



UCAM

UNIVERSIDAD CATÓLICA
DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO
Programa de Doctorado Ciencias del Deporte

Efectos agudos y crónicos de la ingesta de 2S-
Hesperidina sobre marcadores de rendimiento,
metabólicos, bioquímicos y de composición corporal en
ciclistas amateur

Autor:

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Murcia, Diciembre de 2021



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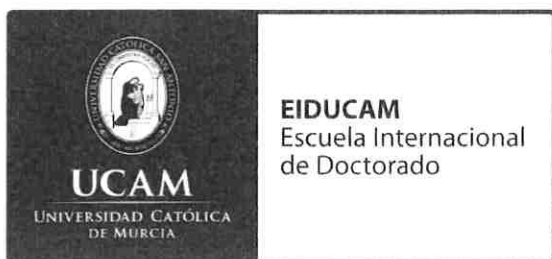
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AUTORIZACIÓN DEL DIRECTOR DE LA TESIS PARA SU PRESENTACIÓN

El Dr. D. Pedro Emilio Alcaraz Ramón y el Dr. D. Cristian Marín Pagán como Directores⁽¹⁾ de la Tesis Doctoral titulada “Efectos de la ingesta de hesperidina a corto y largo plazo en marcadores de rendimiento, estrés oxidativo, inflamatorios, metabólicos y bioquímicos en ciclistas amateur” realizada por D. Francisco Javier Martínez Noguera en el Programa de Doctorado Ciencias del Deporte, **autoriza su presentación a trámite** dado que reúne las condiciones necesarias para su defensa.

Lo que firmo, para dar cumplimiento al Real Decreto 99/2011 de 28 de enero, en Murcia a 30 de septiembre de 2021.

A large, stylized handwritten signature in black ink, appearing to be 'PE Alcaraz'.

A large, stylized handwritten signature in black ink, appearing to be 'C Marín'.

⁽¹⁾ Si la Tesis está dirigida por más de un Director tienen que constar y firmar ambos.

AGRADECIMIENTOS

Tras mucho tiempo, sacrificio y dedicación para finalizar esta Tesis Doctoral, tengo que agradecer a muchas personas el apoyo para poder completar esta fase de mi vida.

En primer lugar, a mis padres Francisco y Antonia que siempre me han apoyado en todas las decisiones que he tomado en mi vida, inculcándome siempre valores de respeto, esfuerzo y sobre todo luchar por los objetivos que me propuesto en la vida. Sin su esfuerzo desde pequeño probablemente no estaría donde estoy hoy, por ello les estoy enormemente agradecido.

A mi hermana Josefina, aunque tenemos nuestros diferentes puntos de vista de ver las cosas siempre la querré como es, ya que, luego tiene su lado cariñoso. Sin olvidarme, de mi tía Lola que es otra integrante más de la familia, siendo una de las mejores personas que conozco, no cambies nunca.

A mi mujer Esperanza, que parte de esta Tesis Doctoral es suya, ya que, ha tenido que duplicarse a nivel familiar para poder llevar la crianza de nuestras 2 preciosas hijas para delante, debido a mi falta de tiempo por el trabajo y realización de este manuscrito. Gracias por todo tu apoyo en todos los proyectos en los que me involucre y por haber sabido entender mi entrega y dedicación a este trabajo, por todo lo que hemos construido a lo largo de tantos años de camino juntos, te recompensaré.

Y como no, esta Tesis va dedicada a mi hija Martina y Estrella, ya que son lo mejor que me ha pasado en esta vida y han sido el motor para finalizar este documento, que tanto tiempo les ha robado de mi presencia. Mis princesas son mi orgullo y mi razón de ser.

A mi director; a Pedro E. Alcaraz por confiar en mí para este proyecto, en la que considero que es para mí la Tesis Doctoral que quería realizar, que todo el mundo no puede decir eso. Esto me ha permitido aprender de un gran profesional e investigador de las ciencias del deporte, con una gran capacidad de trabajo y una gran persona. Y gracias también por contar conmigo para todo tipo de proyecto en los que has creído que podía encajar, dándome la oportunidad de poder desarrollarme a nivel profesional.

A mi otro director, Cristian Marín Pagan por contar conmigo también para realizar esta Tesis Doctoral tan importante para mí, y en la cual has estado codo con codo junto a mí echando mil horas al día para poder sacar las mediciones como de fiesta, donde hemos pasado muy buenos ratos. Agradecerte también todo el esfuerzo realizado y la predisposición a resolver mis dudas y permitirme aprender de ti dentro de tu área de conocimiento que tanto me gusta. Espero seguir colaborando con los 2 y continuar creciendo.

A todos los componentes del laboratorio del CIARD, al que yo considero que tiene a los mejores profesionales y personas del mundo, como son Linda, Tomás, Kostas, Antonio, Davide, Juan Carlos, Andrea y Sajith, pero también a Jorge, Elena y Laura que, aunque no estén actualmente en el laboratorio, también forman parte de este trabajo, ya que han participado de forma directa o indirecta, mediante sus conocimientos o los momentos vividos fuera del laboratorio, que han sido muchos. Gracias por todo, esta Tesis es parte vuestra.

A la Universidad Católica de Murcia, por su apoyo institucional, que es el espacio donde se ha podido materializar esta Tesis Doctoral, junto a personas de una calidad humana enorme.

Y, por último, a la empresa HealthTech BioActives (HTBA), especialmente a dos grandes profesionales y personas como son Jesús Cano y Juan Ignacio Cacho, por su apoyo y colaboración en este proyecto, los cuales acudieron a nuestro laboratorio para que les ayudáramos a evaluar la eficacia de la 2S-hesperidina, que es la protagonista principal de esta Tesis Doctoral. Sin vosotros, no se hubiera empezado a recorrer este largo camino, muchas gracias por vuestra visita.

Mil gracias a todos.

CITA

*“La ciencia se compone de errores, que a su vez son los
pasos hacia la verdad”*

Jules Verne

Efectos de la ingesta de hesperidina a corto y largo plazo en marcadores de rendimiento, estrés oxidativo, inflamatorios, metabólicos y bioquímicos en ciclistas amateur

RESUMEN

Introducción

La utilización de suplementos por parte de los deportistas de todos los niveles y disciplinas para mejorar el rendimiento, es una práctica habitual que se lleva realizando durante muchos años. Ya que, como es conocido y sobre todo su impacto en el alto rendimiento, la nutrición es un factor clave para la mejora de las adaptaciones al entrenamiento. Que apoyado por una óptima suplementación puede tener un efecto sinérgico junto a la nutrición, aumentando las adaptaciones al entrenamiento y disminuyendo el riesgo de patologías asociadas a la práctica deportiva.

Dentro de los suplementos deportivos, están emergiendo en los últimos años los antioxidantes, los cuales provienen principalmente de extractos de fruta y verduras, donde las moléculas predominantes son los polifenoles. Estas moléculas han mostrado diversos efectos, desde una capacidad antioxidante e inflamatoria, reguladora del metabolismo de los lípidos y glucosa, anticáncer, anti COVID-19, hasta la estimulación de la síntesis del óxido nítrico, previniendo enfermedades de diferente etiología. Pero los polifenoles están compuestos por varios subgrupos, donde podemos encontrar los flavonoides que son moléculas que se encuentran principalmente en frutas cítricas, donde uno de las principales moléculas bioactivas es la hesperidina.

Los efectos mostrados por la administración de hesperidina son de amplio espectro, ya que se ha observado un efecto antiinflamatorio, antioxidante, hipoglucemiante, antidislipidémico, entre otros, tanto en humanos como en animales. Pero su efecto en el rendimiento deportivo ha sido poco estudiado, más en animales que en humanos. Debido a la escasa evidencia de la ingesta de hesperidina sobre el rendimiento deportivo en humanos, creemos necesario evaluar de forma profunda si la suplementación con 2S-hesperidina, que es una nueva fórmula (diferente a las utilizadas en otros estudios), es capaz de mejorar el rendimiento deportivo y que mecanismos son los responsables de este, en caso de que lo hubiera.

Objetivos

El objetivo principal de esta tesis fue evaluar tanto la ingesta aguda como crónica (8 semanas) de 2S-hesperidina en marcadores de rendimiento, estrés oxidativo, inflamatorios, metabólicos y bioquímicos en ciclistas amateur, los objetivos específicos fueron.

- Determinar las diferencias en el sistema antioxidante endógeno y en la composición corporal entre ciclistas profesionales y amateur.

- Evaluar la ingesta aguda de 2S-hesperidina (500 mg en una sola toma) en marcadores del sistema antioxidante endógeno, de la potencia anaeróbica y metabólicos a intensidades del umbral ventilatorio 1 en ciclista amateur.
- Evaluar la ingesta de 2S-hesperidina durante 8 semanas en marcadores de rendimiento durante un test incremental, y en marcadores metabólicos, del sistema antioxidante endógeno, inflamatorios, gasometría y del estado ácido-base durante un test rectangular en ciclistas amateur.

Resultados

Como resultados de esta tesis se han publicado 6 artículos y 1 bajo revisión. En el estudio 1 se encontraron valores más altos en la catalasa (CAT), el glutatión oxidado (GSSG) y la relación GSSG/GSH y valores más bajos en la superóxido dismutasa (SOD) en ciclistas profesionales (PRO) en comparación con amateur (AMA) ($p < 0.05$). Además, se encontró una correlación inversa entre la potencia producida en los umbrales ventilatorios 1 y 2 (VT1 y VT2) y la GSSG/GSH ($r = 0.657$ y $r = 0.635$; $p < 0.05$, respectivamente) en PRO.

En el estudio 2, se encontraron valores significativamente más bajos en la densidad mineral ósea, el contenido mineral óseo y la masa libre de grasa en PRO en comparación con AMA ($p < 0.05$). Además, se produjo una potencia significativamente mayor en VT1 y VT2 y en el VO_{2MAX} en PRO en comparación con AMA ($p < 0.05$).

En el estudio 3 de efectos agudos, se encontraron diferencias significativas en la potencia media (2.27%, $p = 0.023$), la velocidad máxima (3.23%, $p = 0.043$) y la energía total (Σ 4 sprint) (2.64%, $p = 0.028$) entre el grupo que consumió 2S-hesperidina y placebo en los mejores datos de la prueba de sprint repetida. También se observaron pequeños cambios en la actividad de CAT, SOD, la ratio GSSG/GSH, así como en los productos de lipoperoxidación (sustancias reactivas al ácido tiobarbitúrico; TBARS), en distintos puntos de la prueba rectangular, aunque no fueron significativos.

En el estudio 4, después de 8 semanas de suplementación, hubo un aumento en la potencia generada en el umbral funcional estimado (3.2%; $p = 0.05$) y la potencia máxima (2.7%; $p = 0.05$) en un test incremental en el grupo que ingirió 2S-hesperidina en comparación con el placebo. En el test rectangular, hubo una disminución del VO_2 (L/min) (8.3%; $p = 0.01$) y del VO_{2R} (consumo de oxígeno relativo al peso) (mL/kg/min) (8.9%; $p = 0.01$) en VT2 en el placebo. Sin embargo, no hubo diferencias entre los grupos. En el test de Wingate, hubo un aumento significativo ($p = 0.05$) de la potencia máxima y relativa en ambos grupos, pero sin diferencias entre ellos.

En el estudio 5, Tras la ingesta de 2S-hesperidina (8 semanas), encontramos en el test rectangular un aumento de SOD después de la fase de ejercicio hasta el agotamiento ($p = 0.045$) y de la fase de recuperación aguda ($p = 0.004$), una disminución del área bajo la curva de GSSG ($p = 0.016$), y una disminución de la proteína quimioatrayente de

monocitos 1 después de la fase de recuperación aguda ($p = 0.004$), después de la intervención.

En el estudio 6, en comparación con el placebo, el análisis DXA mostró una disminución del porcentaje de grasa corporal (%BF) (-10.4%; $p = 0.035$) y de la masa grasa de las extremidades inferiores (-10.5%; $p = 0.029$) a favor del grupo 2S-hesperidina. Tras la evaluación de los datos antropométricos, se observó una disminución del %BF (-3.7%; $p = 0.006$), de la grasa corporal total (-3.0%; $p = 0.047$), del Σ de 8 pliegues cutáneos (-6.1%; $p = 0.008$) en el grupo de 2S-hesperidina, pero no en el de placebo. Además, hubo un aumento del porcentaje de masa muscular (1.0%; $p = <0.001$) y de la masa muscular total (1.7%; $p = 0.011$) tras la ingesta de 2S-hesperidina sin cambios en el placebo.

En el estudio 7, se encontró un aumento del metabolismo del CO_2 en FatMax1, VT1, FatMax2 y EPOC y una disminución del Lac en FatMax1, VT1, VT2, FatMax2 y EPOC después de 8 semanas de suplementación con 2S-hesperidina. Además, en el grupo experimental, el estado ácido-base mejoró en FatMax1, VT1, FatMax2 y EPOC en el test rectangular y previno una disminución de la $p\text{O}_2$ en VT2, en ciclistas amateur.

Conclusiones

En base a los resultados encontrados, podemos establecer que existen diferencias en marcadores del sistema antioxidante endógeno y del estado óseo entre PRO y AMA. Además, la ingesta aguda de 2S-hesperidina mejoró el rendimiento anaeróbico, pero no el placebo, lo que sugiere los beneficios potenciales del uso de 2S-hesperidina en deportes con un alto componente anaeróbico. La ingesta crónica de 2S-hesperidina mejora la potencia generada en el umbral funcional estimado y la potencia máxima tras un test incremental en ciclistas amateur. Y en un test rectangular, la ingesta crónica de 2S-hesperidina aumento de la capacidad antioxidante endógena ($\uparrow\text{SOD}$) tras el esfuerzo máximo y disminución del estrés oxidativo ($\downarrow\text{AUC-GSSG}$), disminuyendo la inflamación ($\downarrow\text{MCP1}$) tras la fase de fase de recuperación aguda. Además, la ingesta crónica de 2S-hesperidina disminuyó la masa grasa en ciclistas amateurs y mostró un efecto promotor del desarrollo muscular. Por último, la ingesta crónica de 2S-hesperidina disminuyó el Lac en intensidades bajas-moderadas-submáximas, y aumento el balance ácido-base a intensidades bajas-moderadas durante y tras un test rectangular en ciclistas amateur.

Palabras clave: Fisiología, nutrición deportiva, rendimiento, antioxidantes, metabolismo, polifenoles y flavonoides.

Effects of short- and long-term hesperidin intake on markers of performance, oxidative stress, inflammatory, metabolic and biochemical markers in amateur cyclists.

ASBTRACT

Introduction

The use of supplements by athletes of all levels and disciplines to improve performance has been a common practice for many years. It is well known that, nutrition is a key factor in improving training adaptations, especially for high performance. Optimal supplementation can have a synergistic effect together with nutrition, increasing adaptations to training and reducing the risk of pathologies associated with sports practice.

Within sports supplements, antioxidants have been emerging in recent years, mainly from fruit and vegetable extracts, where the predominant molecules are polyphenols. These molecules have shown diverse effects, ranging from antioxidant and inflammatory capacity, regulating lipid and glucose metabolism, anti-cancer, anti-COVID-19, to stimulation of nitric oxide synthesis, preventing diseases of different etiologies. But polyphenols are made up of various subgroups, including flavonoids, which are molecules found mainly in citrus fruits, where one of the main bioactive molecules is hesperidin.

The effects of hesperidin administration are broad-spectrum, with anti-inflammatory, antioxidant, hypoglycaemic, anti-dyslipidaemic and other effects observed in both humans and animals. However, its effect on sports performance has been little studied, more so in animals than in humans. Due to the limited evidence of hesperidin intake on sports performance in humans, we believe it is necessary to evaluate in depth whether supplementation with 2S-hesperidin, which is a new formula (different from those used in other studies), is capable of improving sports performance and what mechanisms, if any, are responsible for this.

Objectives

The main objective of this thesis was to evaluate both acute and chronic (8 weeks) ingestion of 2S-hesperidin on markers of performance, oxidative stress, inflammatory, metabolic and biochemical markers in amateur cyclists, the specific objectives were:

- To determine the differences in the endogenous antioxidant system and body composition between professional and amateur cyclists.
- To evaluate the acute intake of 2S-hesperidine (500 mg in a single dose) on markers of the endogenous antioxidant system, anaerobic and metabolic power at ventilatory threshold 1 intensities in amateur cyclists.

- To evaluate the intake of 2S-hesperidin for 8 weeks on markers of performance during an incremental test, and on metabolic, endogenous antioxidant system, inflammatory, blood gas and acid-base status markers during a rectangular test in amateur cyclists.

Results

This thesis presents 6 published articles and 1 under review. In study 1, higher values for catalase (CAT), oxidized glutathione (GSSG) and GSSG/GSH ratio and lower values for superoxide dismutase (SOD) were found in professional (PRO) compared to amateur (AMA) cyclists ($p < 0.05$). In addition, an inverse correlation was found between power produced at ventilatory thresholds 1 and 2 (VT1 and VT2) and GSSG/GSH ($r = 0.657$ and $r = 0.635$; $p < 0.05$, respectively) in PRO.

In study 2, significantly lower values for bone mineral density, bone mineral content and fat-free mass were found in PRO compared to AMA ($p < 0.05$). In addition, there was significantly higher power in VT1 and VT2 and VO_{2MAX} in PRO compared to AMA ($p < 0.05$).

In study 3 of acute effects, significant differences were found in mean power (2.27%, $p = 0.023$), peak velocity (3.23%, $p = 0.043$) and total energy (Σ 4 sprint) (2.64%, $p = 0.028$) between the group consuming 2S-hesperidin and placebo in the best repeated sprint test data. Small changes in CAT activity, SOD, GSSG/GSH ratio, as well as lipoperoxidation products (thiobarbituric acid reactive substances), were also observed at different points in the rectangular test but were not significant.

In study 4, after eight weeks of supplementation, there was an increase in power generated at the estimated functional threshold (3.2%; $p = 0.05$) and peak power (2.7%; $p = 0.05$) in an incremental test in the 2S-hesperidin ingestion group compared to placebo. In the rectangular test, there was a decrease in VO_2 (L/min) (8.3%; $p = 0.01$) and VO_2R (oxygen consumption relative to weight) (mL/kg/min) (8.9%; $p = 0.01$) at VT2 in the placebo. However, there were no differences between groups. In the Wingate test, there was a significant ($p = 0.05$) increase in maximal and relative power in both groups, but no difference between them.

In study 5, following 2S-hesperidin intake (8 weeks), we found in the rectangular test an increase in SOD after the exercise to exhaustion phase ($p = 0.045$) and the acute recovery phase ($p = 0.004$), a decrease in the area under the GSSG curve ($p = 0.016$) and a decrease in monocyte chemoattractant protein 1 after the acute recovery phase ($p = 0.004$), following the intervention.

In study 6, compared to placebo, DXA analysis showed a decrease in percent body fat (%BF) (-10.4%; $p = 0.035$) and lower extremity fat mass (-10.5%; $p = 0.029$) in favour of the 2S-hesperidin group. After evaluation of anthropometric data, a decrease in %BF (-3.7%; $p = 0.006$), total body fat (-3.0%; $p = 0.047$), Σ 8 skinfold (-6.1%; $p = 0.008$) was observed in the 2S-hesperidin group, but not in the placebo group. In addition, there

was an increase in percentage muscle mass (1.0%; $p = <0.001$) and total muscle mass (1.7%; $p = 0.011$) after 2S-hesperidin intake with no change in placebo.

In study 7, an increase in CO₂ metabolism was found in FatMax1, VT1, FatMax2 and EPOC and a decrease in Lac in FatMax1, VT1, VT2, FatMax2 and EPOC after 8 weeks of 2S-hesperidin supplementation. Furthermore, in the experimental group, acid-base status improved in FatMax1, VT1, FatMax2 and EPOC in the rectangular test and prevented a decrease in pO₂ in VT2, in amateur cyclists.

Conclusions

Based on the results found in this thesis, we can establish that there are differences in markers of the endogenous antioxidant system and bone status between PRO and AMA. In addition, acute intake of 2S-hesperidin improved anaerobic performance, but not placebo, suggesting the potential benefits of 2S-hesperidin use in sports with a high anaerobic component. Chronic ingestion of 2S-hesperidin improves power output at the estimated functional threshold and maximal power output after an incremental test in amateur cyclists. And in a rectangular test, chronic 2S-hesperidin intake increased endogenous antioxidant capacity (↑SOD) after maximal exertion and decreased oxidative stress (↓AUC-GSSG), decreasing inflammation (↓MCP1) after the acute recovery phase. In addition, chronic intake of 2S-hesperidin decreased fat mass in amateur cyclists and showed a muscle development-promoting effect. Finally, chronic ingestion of 2S-hesperidin decreased Lac at low-moderate-submaximal intensities, and increased acid-base balance at low-moderate intensities during and after a rectangular test in amateur cyclists.

Keywords: Physiology, sports nutrition, performance, metabolism, antioxidants, polyphenols y flavonoids.

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SIGLAS

AaDpO₂ - Gradiente Alveolar-arterial

ABE - Exceso actual de bases

ACS2 - Acil-CoA sintetasa 2

AGCC - Ácidos grasos de cadena corta

AIS - Australian Institute of Sports

AMPK - Proteína quinasa activada por adenosín monofosfato

ARN_m - Ácido ribonucleico mensajero

ADP - Adenosín difosfato

AMP - Adenosín monofosfato

ATP - Adenosín trifosfato

AUC - Área bajo la curva

BF - Masa grasa

BF% - Masa grasa

CAT - Catalasa

C-FABP - proteína de unión a los ácidos grasos cutánea

CK - Creatina quinasa

C_{máx} - Concentraciones plasmáticas máximas

CMS - Células mononucleares de la sangre

CO - Monóxido de carbono

CO₂ - Dióxido de carbono

COHb - Carboxihemoglobina

CQ₁₀ - Coenzima Q₁₀

CRP - Proteína C reactiva

DHEA - Dehidroepiandrosterona

DMAA - Metilhexanamina

DMBA - 1,3-dimetilbutilamina

EIAH - Hipoxemia arterial inducida por el ejercicio

EMG - Electromiografía

eNOS - Óxido nítrico sintasa endotelial

EPOC - Exceso de consumo de oxígeno después del ejercicio

FAT6 - Desaturasa de ácidos grasos 6

FAT7 - Desaturasa de ácidos grasos 7

FC - Frecuencia cardiaca

FECO₂ - Fracción de CO₂ espirada

FEO₂ - Fracción de O₂ espirada

FFA - Ácidos grasos libres

FatMax - Intensidad a la que se produce la máxima oxidación de las grasas

FFM - Masa libre de grasa

FR - Radical libre

FT - Fibras rápidas

Ga - Glutamato

GE - Gross mechanical efficiency

Glu - Glucosa

GLUT4 - Transportador de glucosa específico del músculo esquelético

Gm - Glutamina

GPX - Glutación peroxidasa

GSH - Glutación reducido

GSSG - Glutación oxidado

Hb - Hemoglobina

HCO₃⁻ - Anión bicarbonato

H₂CO₃ - Ácido carbónico

HCl - Ácido clorhídrico

Hct - Hematocrito

HDL - Lipoproteínas de alta densidad

H-FABP - proteína de unión a los ácidos grasos del

HMG-CoA - 3-hidroxi-3-metil-glutamil coenzima A

HO1 - Hemoxigenasa 1

HOCl - Ácido hipocloroso

H₃PO₄ - Ácido fosfórico

H₂SO₄ - Ácido sulfúrico

ICAM1 - Molécula de adhesión intercelular 1

IFN γ - Interferón gamma

IL1ra - Receptor de la interleucina 1

IL2-R - Receptor de interleucina 2 soluble

IL - Interleucina

IL1 β - Interleucina 1 β

IMTG - Triglicéridos intramusculares

ITT - Contrarreloj individual

KAT1 - 3-cetoacil-CoA tiasa 1

Kj - Kilojulios

Lac - Lactato

LDL - Lipoproteínas de baja densidad

LOO - Radical peroxilo de los ácidos grasos

LPS - Lipopolisacárido

MCP1 - Proteína quimioatrayente de monocitos 1

MCT Transportador de monocarboxilatos

MDT15 - Subunidad mediadora 15

MET - Equivalentes metabólicos

MetHb - Methemoglobina

MLSS - Máximo estado estable de lactato

MTB - Ciclismo de montaña

NADH - Dinucleótido de nicotinamida-adenina

NF- κ B - Factor nuclear Kappa B

NK - Células natural killer

NO - Óxido nítrico

NOX2 - Nicotinamida-adenina-dinucleótido-fosfato oxidasas 2

NOX4 - Nicotinamida-adenina-dinucleótido-fosfato oxidasas 4

NRF1/2 - Factor respiratorio nuclear 1 y 2

O₂ - Oxígeno

O₂Hb - Oxihemoglobina

ODC - Curva de disociación del oxígeno

ONOO \cdot - Peroxinitrito

p50 - Presión parcial de oxígeno al 50% de saturación de oxígeno

PAO₂ - Presión alveolar de oxígeno

PaO₂ - Presión arterial de oxígeno

PAPP γ - Receptor activado por proliferadores peroxisomales γ

PAS - Presión arterial sistólica

PC - Fosfocreatina

PCO₂ - Presión parcial de dióxido de carbono

PCR - Proteína C reactiva

PCT - Proteínas de choque térmico

PDH - Piruvato deshidrogenasa

PDK - Piruvato deshidrogenasa quinasa

PETCO₂ - Presión parcial de dióxido de carbono exhalado o “end-tidal”

PFK - Fosfofructoquinasa

PGC-1 α - Coactivador 1 α del receptor activado gamma del proliferador de peroxisomas

PGE2 - Prostaglandinas 2

PHA - Fitohemaglutinina

P_i - Fosfatos

PIM1 α - Proteína inhibidora de macrófagos 1 α

PIM1 β - Proteína inhibidora de macrófagos 1 β

PLA2 - Fosfolipasa A2

P_{MAX} - Potencia máxima de salida

PO₂ - Presión parcial de oxígeno en sangre

POD2 - Acetil-CoA carboxilasa 2

PPM - Pulsaciones por minuto

RBI - Resistencia básica I

RBII - Resistencia básica II

RBIII - Resistencia básica III

RBP4 - Proteína de unión al retinol 4

RCD - Resistencia corta duración

RER - Ratio de intercambio gaseoso

RHb - Desoxihemoglobina

RLD - Resistencia de larga duración

RLDI - Resistencia de larga duración I

RLDII - Resistencia de larga duración II

RLDIII - Resistencia de larga duración III

RLDIV - Resistencia de larga duración IV

RMD - Resistencia mediana duración

RNS - Especies reactivas de nitrógeno

RO - Radical alcoxilo

RONs - Especies reactivas de oxígeno y nitrógeno

RSS - Especies reactivas del azufre

SARMS - Moduladores selectivos de los receptores de andrógenos

SBC – Bicarbonato estándar

SBE – Exceso de bases estándar

SCD - Estearoil-CoA desaturasa

Shunt - Relative physiological Shunt

SIRT1 - Sirtuina 1 desacetilasa dependiente de nicotinamida adenina dinucleótido

SNC - Sistema nervioso central

SO₂ - Saturación de oxígeno

SOD - Superóxido dismutasa

SREBP1 - Factor de transcripción de unión a elementos reguladores de esteroides 1

SSE - Síndrome de sobreentrenamiento

ST - Fibras lentas

sVCAM1 molécula de adhesión celular vascular soluble 1

TBARS - Sustancias reactivas del ácido tiobarbitúrico

TCA - Ciclo de los ácidos tricarbónicos

tCO₂ – Concentración total de dióxido de carbono en sangre

tO₂ – Concentración total de oxígeno en sangre

T_{máx} – Tiempo para alcanzar la concentración plasmática máxima

TNF α - Factor de necrosis tumoral α

TNF α -R - Receptor de TNF α

UL1 - Primer umbral láctico

UL2 - Segundo umbral láctico

VE - Ventilación

VE/VCO₂ - Equivalente ventilatorio para el CO₂

VE/VO₂ - Equivalente ventilatorio para el O₂

VLDL - Lipoproteínas de muy baja densidad

VO₂ - Consumo de oxígeno

VO_{2MAX} - Consumo máximo de oxígeno

VO_{2R} - Consumo de oxígeno relativo al peso

VT1 - Umbral ventilatorio 1

VT2 - Umbral ventilatorio 2

W - Potencia

XO - Xantina oxidasa

GLOSARIO

En este manuscrito van a aparecer diferentes términos que son frecuentes en la literatura sobre el ciclismo. Para una mayor comprensión y contextualización de los términos, se presentan sus definiciones en este apartado.

El término **anaeróbico** se considera un concepto metabólico sin oxígeno que representa las concentraciones y la actividad de las enzimas. En el presente manuscrito, el término anaeróbico se refiere a los procesos metabólicos que no dependen del oxígeno, independientemente de su disponibilidad

El **consumo máximo de oxígeno** (VO_{2MAX}) representa el oxígeno máximo utilizado, y está limitado por el suministro de oxígeno y sujeta a las limitaciones de la capacidad cardiovascular central y periférica y a la demanda de oxígeno de los tejidos (1).

El **umbral de lactato 1** (UL1) se define como la intensidad del ejercicio que provoca un aumento de 1 mmol/L en la concentración de lactato en sangre por encima del valor medio en reposo (2).

El **umbral de lactato 2** (UL2) es la intensidad de ejercicio más alta en la que la concentración de Lac en sangre permanece estable y se establece con un valor fijo de 4 mmol/L (3). Además, suele coincidir con el estado estable de lactato máximo (MLSS). El MLSS se alcanza cuando la concentración de Lac en sangre varía <1 mmol/L durante los últimos 20 minutos de ejercicio de intensidad constante, lo que refleja un equilibrio entre la producción y la eliminación de Lac (4). Otro nombre común para el UL2 es el inicio de la acumulación de Lac en sangre (5).

El **umbral ventilatorio 1** (VT1) es el punto en el que se produce un aumento no lineal del volumen espiratorio (VE) y de la producción de dióxido de carbono (VCO_2), en combinación con un descenso de la fracción de CO_2 espirada ($FECO_2$) y una elevación de la fracción de O_2 espirada (FEO_2). En la práctica, el VT se identifica como un punto de hiperventilación con respecto al consumo de oxígeno (VO_2) y se refleja en un aumento sistemático del equivalente ventilatorio para el oxígeno (VE/VO_2) sin un aumento concomitante del equivalente ventilatorio para el dióxido de carbono (VE/VCO_2) (6). El VT1 se corresponde estrechamente con el LT1.

El **umbral ventilatorio 2** (VT2), es el punto de compensación respiratoria, que se identifica como un aumento tanto del VE/VO_2 como del VE/VCO_2 y una disminución

de la presión parcial de dióxido de carbono exhalado o “end-tidal” (PETCO₂).⁽⁷⁾ El VT₂ se corresponde muy estrechamente con el LT₂.

La **eficiencia** es una medida del trabajo efectivo y se expresa más comúnmente como el porcentaje de energía total gastada que produce trabajo externo ⁽⁸⁾.

La **eficiencia mecánica** es el porcentaje de la energía química total gastada que contribuye al trabajo externo, el resto se pierde en calor. Dentro de esta definición, la eficiencia mecánica es igual al trabajo mecánico real realizado dividido por el aporte de energía $\times 100$ ⁽¹⁾.

La **eficiencia bruta** es la relación entre el trabajo realizado durante el ciclismo y la energía total gastada y se expresa en forma de porcentaje ⁽⁹⁾. Es importante recordar que la medición de la eficiencia bruta se limita a las intensidades que provocan una relación de intercambio respiratorio de <1.00 .

La **economía** es una medida del VO₂ por unidad de potencia producida. Es la cantidad de oxígeno por litro por unidad de energía transferida al cicloergómetro ⁽¹⁾.

El **torque** que puede ser entendido como el análogo de rotación a la fuerza lineal (fuerza de giro), y se calcula multiplicando la fuerza perpendicular por la distancia desde el pivote (o eje de rotación).

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CAPÍTULO I.
INTRODUCCIÓN

CAPÍTULO I. INTRODUCCIÓN

El ciclismo es un deporte de alta exigencia física y psicológica, ya que, tanto en los entrenamientos como en la competición se combinan periodos de mucho volumen (capacidad aeróbica) con momentos de muy alta intensidad (capacidad anaeróbica), que normalmente es donde se decide al ganador de las competiciones (1). Son numerosos los factores que contribuyen al máximo rendimiento deportivo en ciclismo. A nivel general, una sólida base de acondicionamiento físico y experiencia deportiva, además de un programa de entrenamiento y nutrición, éste último basado predominantemente en una alimentación variada, suficiente y equilibrada, es esencial (10). Sin embargo, los factores intrínsecos de los ciclistas asociados al rendimiento deportivo están vinculados a factores fisiológicos y mecánicos.

En relación a los factores fisiológicos, se ha visto que valores elevados del consumo de oxígeno máximo $\text{VO}_{2\text{MAX}}$ ($\sim 74 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) y un umbral ventilatorio 2 (VT2) y de lactato 2 (UL2) aproximadamente en el 90% del $\text{VO}_{2\text{MAX}}$ (1), son requisitos esenciales para tener éxito en el ciclismo de carretera. Estos valores son los que normalmente tienen los ciclistas profesionales, con valores inferiores en ciclistas amateur (11). Por otro lado, se ha evidenciado que la acidosis metabólica es otro factor determinante en el rendimiento de los ciclistas, que si aparece de forma temprana puede anticipar la fatiga (1). Debido a una alta tasa de recambio de adenosín trifosfato (ATP) en el músculo esquelético durante el ejercicio de alta intensidad, aumenta la producción de iones de hidrógeno (H^+), lo que conduce a la acidosis muscular que se asocia con la pérdida de rendimiento (12). Además, el patrón respiratorio en ciclistas ha mostrado una afectación en el rendimiento, de tal manera, que adaptaciones en este patrón pueden mejorar la eficiencia y el coste metabólico de la respiración en los ciclistas profesionales (13).

Otro factor no menos importante en el rendimiento de los ciclistas es la composición corporal. Donde el aspecto más relevante a tener en cuenta es el peso y la masa grasa, encontrándose ciclistas profesionales con un bajo peso (70 kg) y porcentaje de masa grasa (BF%) (8-10%) (14, 15), comparado con los ciclistas amateur (75 kg y 11%, respectivamente) (16, 17). La composición corporal es un factor que cuando se relativiza a la potencia aumenta su importancia, sobre todo en corredores escaladores que realizan esfuerzos en el VT2 durante 30 min hasta 1 h en subidas con grandes porcentajes de desnivel (15).

En relación a los factores mecánicos, uno de los factores determinantes el en rendimiento de ciclistas es la potencia máxima (P_{MAX}), donde en el nivel profesional se han encontrados P_{MAX} por encima de los 500W y una potencia relativa al peso entre 6-

7.5 W·kg⁻¹ en un test incremental (18). Al igual que en el VO_{2MAX}, los niveles de P_{MAX} y potencia relativa al peso en ciclistas amateur son notablemente inferiores (300-450 W y 5.0-6.0 W·kg⁻¹) a los ciclistas profesionales (11). La potencia anaeróbica, también es otro factor a tener en cuenta, ya que es esencial para ciertos momentos de competición, sobre todo al inicio de las competiciones para coger una escapada, esprintar en las metas volantes o en meta (19, 20). En este sentido, también existen grandes diferencias entre ciclistas profesionales (1279 W P_{MAX}) y amateur (843 W P_{MAX}) en esfuerzos de 30 s con intensidad máxima (21, 22).

Además, la eficiencia también es un factor importante en el rendimiento del ciclismo, ya que describe la relación entre el trabajo mecánico realizado y la energía metabólica necesaria para realizar dicho trabajo, es denominada como gross efficiency (GE) (23). Los ciclistas profesionales han mostrado una mayor GE que ciclistas elite y amateurs (24). Está descrito que los ciclistas con un alto GE son capaces de generar una mayor potencia durante una contrarreloj individual (ITT) de 1 hora con el mismo coste energético que los ciclistas con un GE más bajo (23). El último factor que influye en el rendimiento del ciclista es la biomecánica. Los cambios en diferentes variables pueden afectar a las necesidades energéticas del ciclismo. Estas variables incluyen: cambios en la posición, configuración y orientación del cuerpo; cambios en la distancia entre el asiento y el pedal; y la interacción de la carga de trabajo, la potencia producida y el ritmo de pedaleo (25). Los cambios en estas variables alteran los ángulos de las articulaciones, las longitudes de los músculos y las longitudes de los brazos, afectando así a las relaciones tensión-longitud, fuerza-velocidad-potencia de los músculos multiarticulares y a la eficacia de la producción de fuerza (25).

Tras los factores fisiológicos y mecánicos, la nutrición se podría considerar otro pilar fundamental para que el ciclista pueda desarrollar su máximo potencial. En los últimos años se ha avanzado mucho en el campo de la nutrición deportiva, investigando nuevas estrategias alimentarias y suplementos, con el fin de aumentar el rendimiento deportivo en deportistas de resistencia, en concreto en los ciclistas (26). Dentro de los suplementos deportivos (proteínas, hidratos de carbono, creatina, aminoácidos, etc.), los que están siendo estudiados de forma amplia son los que tienen un perfil antioxidante, como son los polifenoles (efecto antioxidante y antiinflamatorio), para intentar averiguar si son capaces de mejorar el rendimiento (27). Son un grupo amplio de moléculas y provienen principalmente de frutas y verduras, estos se clasifican según su estructura en diferentes grupos: flavonoides, estilbenos, ácidos fenólicos, lignanos y otros (28). En relación a los flavonoides, son los polifenoles más comunes en la dieta humana y, en consecuencia, los más estudiados. Se han descrito más de 4.000 variedades y se dividen en seis subclases: flavonoles, flavonas, flavanonas, flavanoles, antocianinas e isoflavonas (29)

Dentro de las flavononas, que son flavonoides y se encuentran principalmente en frutas cítricas (naranjas, limones, etc.), podemos encontrar la hesperidina, que va a ser la protagonista de esta Tesis Doctoral. En particular, esta molécula ha mostrado un potente efecto antioxidante y antiinflamatorio tanto en modelos animales como en humanos (30). Y aumento de la síntesis de óxido nítrico (NO) y mejora de la disfunción endotelial en pacientes con síndrome metabólico (31), entre otros efectos. Aunque la hesperidina ha sido muy estudiada en modelos patológicos, existe escasa evidencia en relación al rendimiento deportivo.

Anteriormente a esta tesis doctoral, solo hay un estudio en humanos que evaluó los efectos crónicos de la suplementación de 4 semanas con 2S-hesperidina (500 mg/d) en ciclistas entrenados mediante una prueba de 10 min en un cicloergómetro. Se observó un aumento significativo de la potencia media producida en el grupo que consumió 2S-hesperidina (14.9 W = 5.0%) sin cambios significativos en el placebo (3.8 W = 1.3%), pero con diferencias significativas entre grupos (32). Además, resultados similares fueron encontrados al suplementar con 2S-hesperidina durante 4 semanas a ratas, ya que, estas mejoraron el rendimiento (33, 34).

En base a la escasa evidencia encontrada en relación a la ingesta de hesperidina y el rendimiento deportivo en humanos y la novedosa fórmula de 2S-hesperidina. Siendo la 2R-hesperidina, el isómero utilizado principalmente en los estudios y la forma comúnmente comercial, sin embargo, la nueva fórmula de 2S-hesperidina (utilizada en nuestro estudio; isómero predominante de forma natural en la fruta) ha sido poco investigada. Y teniendo en cuenta los hallazgos encontrados por Biesemman et al. (35), que demostró como la hesperidina puede ser capaz de aumentar el ATP intracelular en un 33% y la capacidad de reserva mitocondrial en un 25%, además de mejorar el estado antioxidante. Además, del conocimiento del mecanismo de la hesperidina in vitro, así como en las pruebas presentadas anteriormente en humanos, la hesperidina es un buen candidato para mejorar el rendimiento.

Debido a estos motivos pensamos que era necesario realizar 2 ensayos clínicos clarificar si la ingesta de 2S-hesperidina puede mejorar el rendimiento en ciclistas amateur. Para tal fin, primero realizó un estudio sobre los efectos agudos de la ingesta de 2S-hesperidina, para comprobar si una única dosis (500 mg) era suficiente para encontrar una mejora del rendimiento o algún cambio en el sistema antioxidante endógeno que justificara la posible mejora del rendimiento en ciclistas amateur. Y posteriormente, se evaluó los efectos crónicos de la ingesta de 2S-hesperidina (ingesta de 8 semanas de 500 mg/d) sobre el rendimiento y marcadores bioquímicos, metabólicos y antioxidantes de ciclistas amateur. ¿Será la 2S-hesperidina una nueva ayuda ergogénica en un futuro próximo?

CAPÍTULO II.
ANTECEDENTES

CAPÍTULO II: ANTECEDENTES

2.1. EL CICLISMO

El ciclismo es uno de los deportes con el mayor número de practicantes en hombres como en mujeres. Esto ha sido evidenciado a nivel nacional, por un incremento en las licencias federativas desde 31,787 en el año 2000 alcanzando las 75,638 licencias en el 2021. -Además, el ciclismo ha tenido una expansión tanto como actividad recreativa/fitness y como a nivel competitivo (36). Pruebas como el duatlón y triatlón, el ciclismo de montaña, las carreras regionales, y una afición creciente en nuevas capas sociales hace que este deporte se está convirtiendo en uno de los más populares. Siendo el ciclismo en carretera olímpico desde Atenas en 1896 y el mountain bike (MTB) desde Atlanta 1996 (36).

Existen varias modalidades de ciclismo, pero el ciclismo de carretera y el MTB son las más practicadas, aunque también existen el de pista, enduro y ciclocross. En el ciclismo de carretera, el formato de las carreras puede variar desde competiciones de un solo día (como criteriums, contrarrelojes, carreras en circuito y salida y llega en distinto sitio) hasta carreras por etapas de 3 semanas, además, el terreno puede variar de predominantemente plano a extremadamente montañoso (37). Por el contrario, las carreras de MTB se celebran en su mayoría en un solo día y los competidores completan varias vueltas de un circuito sobre un terreno diverso, diferente a la carretera, que consiste en caminos de tierra y grava, senderos estrechos y campos abiertos. Las carreras de MTB suelen incluir descensos técnicos y una proporción significativa de subidas (37). Además, las competiciones de ciclismo de carretera y de MTB requieren que el ciclista posea la capacidad de generar una potencia relativamente alta de corta duración durante la salida, en las subidas pronunciadas y en la llegada a meta (1).

A nivel general en el ciclismo, se pueden diferenciar 2 factores determinantes en el rendimiento de un ciclista, que son: factores fisiológicos y factores mecánicos

Además, dentro de los factores fisiológicos se pueden diferenciar claramente los siguientes: 1) Consumo máximo de oxígeno (VO_{2MAX}); 2) Potencia máxima (P_{MAX}); 3) potencia anaeróbica; 4) eficiencia 5) umbrales ventilatorios; 6) umbrales de lactato (Lac); 7) balance ácido-base; 8) eficiencia ventilatoria; 9) composición corporal; 10) la fatiga y 11) el sobreentrenamiento. Y por otro lado, tenemos los factores biomecánico.

2.1.1. Factores fisiológicos que influyen en el rendimiento de ciclistas de carretera

2.1.1.1. Consumo máximo de oxígeno (VO_{2MAX})

El VO_{2MAX} representa el oxígeno máximo utilizado por el organismo durante el ejercicio, y está limitado por el suministro de oxígeno y sujeto a las limitaciones de la capacidad cardiovascular central y periférica y a la demanda de oxígeno de los tejidos (38). El VO_{2MAX} está fijado por los límites metabólicos y de transporte de oxígeno o por una combinación de ambos. Aunque no hay que descartar que sea una cualidad importante para el rendimiento ciclista de resistencia, el VO_{2MAX} por sí solo no es un buen predictor del rendimiento de resistencia cuando se comparan atletas con una capacidad de resistencia similar (39). Ya que el Lac puede jugar un rol muy importante. Sin embargo, los mecanismos responsables que justifican que el Lac se posiciona como un mejor predictor del rendimiento de resistencia que el VO_{2MAX} siguen siendo examinados (39). Se ha presentado el argumento de que el VO_{2MAX} está limitado por el suministro de oxígeno a las mitocondrias musculares (40, 41). Pero los niveles de Lac están relacionados con la capacidad de transportar Lac e iones de hidrógeno o protones (H^+) fuera de las fibras musculares, y con la capacidad del músculo esquelético para captar Lac. Es probable que los factores centrales limiten el VO_{2MAX} , mientras que la respuesta del Lac al ejercicio está relacionada principalmente con factores periféricos de la musculatura entrenada. Estos factores incluyen el porcentaje de fibras tipo I (ST), las actividades de las enzimas oxidativas y la capacidad respiratoria (5, 42-44).

En este sentido parece que el VO_{2MAX} tiene un valor predictivo limitado para el rendimiento en grupos homogéneos de atletas de alto rendimiento, pero sí que puede usarse como valor de referencia que puede categorizar al deportista (45). Actualmente, la medición del VO_{2MAX} sigue recomendándose para evaluar y seleccionar a los ciclistas de élite y como requisito previo para rendir a un alto nivel (46, 47). Además, hasta cierto punto parece que los valores bajos de VO_{2MAX} pueden ser indicativos de fatiga o sobreentrenamiento (1).

El potencial del ciclista para triunfar en las carreras de carretera podría medirse a partir de los valores fisiológicos de los ciclistas profesionales. Hay pruebas sustanciales que demuestran que los ciclistas profesionales de éxito (Tour de Francia y la Vuelta a España) poseen altos valores de VO_{2MAX} ($\sim 74 \text{ mL}\cdot\text{kg}^{-1} \text{ min}^{-1}$) y un UL2 en el $\sim 90\%$ del VO_{2MAX} (48). No obstante, Hawley y Noakes encontraron que los ciclistas amateur tenían un VO_{2MAX} de $55 \text{ mL}\cdot\text{kg}^{-1} \text{ min}^{-1}$, con una alta heterogeneidad, con un UL2 inferior a los ciclistas profesionales (49). Por otro lado, Lucia et al. (50) informó que los principales contrarrelojistas del Giro de Italia, el Tour de Francia o la Vuelta a España (vueltas por etapas) eran capaces de tolerar altas cargas de trabajo constantes submáximas cercanas a su VT2 o al $\sim 90\%$ del VO_{2MAX} durante ~ 60 minutos.

Cuando se pretende mejorar el VO_{2MAX} de ciclistas, existen dos estrategias principales: el entrenamiento y la nutrición. Aunque es conocido que el VO_{2MAX} está determinado genéticamente en gran medida, puede ser mejorado por el entrenamiento. Por ejemplo, se ha reportado mejoras con un entrenamiento con intervalos cortos de alta intensidad (3 series de 13 repeticiones de 30 s) comparado a intervalos largos de baja intensidad (4 series de 1 repetición de 300 s). Tras 10 semanas de entrenamiento con intervalos cortos aumentó significativamente el VO_{2MAX} (8.6%) pero no el entrenamiento de intervalos largos de baja intensidad (2.6%) (51). Por otro lado, la intervención a nivel nutricional mediante ayudas ergogénicas también ha documentado mejoras en el VO_{2MAX} de ciclistas. En este sentido, se ha visto que la ingesta crónica de fosfato sódico en ciclistas entrenados mejoró los niveles de VO_{2MAX} (6.4%) frente al placebo (52). Además, la suplementación con 1000 mg de quercetina durante 7 días mejoró también el VO_{2MAX} (3.9%) de ciclistas entrenados, comparado a placebo (53).

Tal y como hemos comentado con anterioridad, el VO_{2MAX} parece no ser determinante en el rendimiento de ciclistas del mismo nivel. Otros factores que se han estudiado en profundidad son los umbrales ventilatorios, por su relación con el rendimiento.

2.1.1.2. Potencia máxima (P_{MAX})

La potencia máxima producida en el VO_{2MAX} (P_{MAX}) durante una prueba incremental hasta el agotamiento, es una expresión mecánica de la capacidad aeróbica máxima (54). Como factores limitantes de la P_{MAX} son similares a los encontrados en el VO_{2MAX} , siendo los factores metabólicos y de transporte de oxígeno o por una combinación de ambos, los que en mayor medida afectan a esta cualidad del ciclista (39).

Existen pruebas sustanciales que demuestran que la P_{MAX} obtenida durante un test incremental máximo en bici puede utilizarse como predictor del rendimiento de un ciclista (49, 55). Los valores medios de la potencia máxima alcanzada en ciclistas profesionales durante una prueba incremental varían en función del protocolo utilizado. Los valores más bajos, de 400 a 450 W (6.0 a 6.5 $W \cdot kg^{-1}$), se registran durante pruebas con incrementos de 4 min, mientras que las potencias de 450 a 500 W (6.5 a 7.5 $W \cdot kg^{-1}$) pueden obtenerse durante protocolos más cortos (es decir, incrementos de 1 min de 25 W) (18). Sin embargo, en ciclistas amateur se ha registrado niveles de P_{MAX} de 383 W (5.3 $W \cdot kg^{-1}$) con un protocolo con incrementos de 35 W cada 3 min (56).

Por otro lado, Hawley y Noakes (49) encontraron una correlación significativa ($r = -0.910$; $p < 0.001$) entre la P_{MAX} durante un test incremental maximal y una prueba de ciclismo de 20 km. Estos investigadores llegaron a la conclusión de que la P_{MAX} puede

ser un buen parámetro para evaluar el rendimiento ciclista y puede utilizarse como un predictor del rendimiento en ciclismo (49, 55). Además, una relación potencia/peso de $>5.5 \text{ W}\cdot\text{kg}^{-1}$ se considera un requisito necesario para los ciclistas que compiten al más alto nivel (57). Sin embargo, este criterio debe utilizarse con precaución, ya que el protocolo utilizado durante la prueba puede afectar al resultado de la potencia.

En relación al entrenamiento, se ha visto que la organización de tres modelos diferentes de entrenamiento de alta intensidad en un orden específico de mesociclo periodizado o en una distribución mixta durante un período de entrenamiento de 12 semanas produce las mismas mejoras en la P_{MAX} en ciclistas entrenados (58). Actualmente, no existen estudios que demuestren una mejora en de la P_{MAX} , tras la ingesta de suplementos deportivos. Sin embargo, sí se han encontrado mejoras en el $\text{VO}_{2\text{MAX}}$, que es una zona de ejercicio cercana a la P_{MAX} en un test incremental en ciclistas, tras la ingesta de suplementos, tal como se mencionó en el apartado relacionado al $\text{VO}_{2\text{MAX}}$. Esta falta de evidencia en relación a la ingesta de suplementos y la P_{MAX} , posiblemente sea debida a que la gran mayoría de investigaciones se han centrado en estudiar el $\text{VO}_{2\text{MAX}}$, como variable de rendimiento.

Además, de la P_{MAX} que es una expresión mecánica de la máxima producción energética, es un factor determinante en el rendimiento de los ciclistas, pero también lo es la potencia anaeróbica, que veremos a continuación.

2.1.1.3. Potencia anaeróbica

La prueba más utilizada para describir la potencia y la capacidad anaeróbicas es el test de Wingate (sprint de 30 s) (59). Este tipo de esfuerzo requiere de la degradación de las fuentes anaeróbicas como la fosofocreatina (CP) y de la glucólisis que termina en la producción de Lac (59). Al inicio del esprint, tanto el sistema de los fosfágenos como el glucolítico se activan por completo (60-62). La glucólisis acelerada, la degradación de la CP y el metabolismo oxidativo proporcionan aproximadamente el 50-55%, el 23-29% y el 16-25%, respectivamente, del ATP requerido por el músculo que se está ejercitando durante un sprint de 30 segundos (19, 20, 63, 64). Además, la resíntesis de ATP hasta el ~80-100% del valor en reposo requiere 2-4 minutos de recuperación (65, 66). Para los ciclistas que compiten, estas dinámicas metabólicas sirven de base para diseñar la estrategia del esprint, ya que, iniciar el sprint hacia la meta demasiado pronto provocará una reducción gradual de la velocidad y, con toda seguridad, la pérdida del primer puesto. Esta teoría está respaldada por el hecho de que la degradación de la CP comienza al inicio del ejercicio intenso y alcanza una tasa máxima en 10 segundos, y luego deja de contribuir al suministro de energía a medida que el almacén de CP se agota en algún momento entre 10 y 30 segundos (60-62, 64). Es evidente que la potencia anaeróbica tiene unas limitaciones. En este sentido, se ha sugerido como factores

limitantes de la potencia anaeróbica en ciclistas, el volumen magro de las piernas, la arquitectura muscular, la activación neuromuscular y componentes metabólicos (tipo de fibras musculares y contenido de enzimas) (afectando a la potencia pico) (67).

A la hora de analizar los datos de potencia anaeróbica (esprint de 30 s; test de Wingate) en ciclistas profesionales, hay que tener en cuenta la homogeneidad de sus diferentes categorías de ciclistas que componen este grupo. Por ejemplo, los ciclistas especialistas en esprint son capaces de producir 1279 W de P_{MAX} , una potencia relativa al peso de $19 \text{ W}\cdot\text{kg}^{-1}$ y una cadencia máxima de 216 rpm. Pero los corredores profesionales especialistas en montaña y en el llano tienen valores inferiores de P_{MAX} , potencia relativa al peso y cadencia máxima (1034 y 1187 W; 15.5 y $16.7 \text{ W}\cdot\text{kg}^{-1}$; 188 y 198 rpm; respectivamente) (68). Los altos niveles de potencia tanto absoluta como relativa en el test de esprint de 30 s en los esprinters profesionales son debidas principalmente a factores genéticos y al entrenamiento específico del sprint que puede provocar modificaciones metabólicas, como un aumento significativo de las actividades enzimáticas que catalizan las reacciones anaeróbicas (22). Sin embargo, se han encontrado valores inferiores en el esprint de 30 s en ciclistas amateur, específicamente, valores de P_{MAX} 843 W y una potencia relativa al peso de $11.2 \text{ W}\cdot\text{kg}^{-1}$ (21). Como es evidente, estos datos indican que la potencia anaeróbica en ciclistas profesionales es superior a la de los ciclistas amateur, lo que le confiere una ventaja a la hora de realizar esfuerzos de corta duración y alta intensidad, como son los inicios de las competiciones para coger la escapada, esprints intermedios y finales.

A la hora de mejorar la potencia anaeróbica mediante el entrenamiento, se ha visto que 12 semanas de entrenamiento de fuerza en ciclistas entrenados (se añadió un entrenamiento de fuerza a su entrenamiento normal) mejoró los valores de P_{MAX} (6%) en un test de Wingate frente a un grupo control (que siguió su entrenamiento cotidiano) (69). Con respecto a la nutrición deportiva, se ha visto que la ingesta aguda de zumo de remolacha (70 mL) mejora la producción de potencia máxima (6%) y en la potencia media durante los primeros 15 s (6.7%) en un test de Wingate (esprint 30 s) en ciclistas entrenados (70).

Como ya hemos visto, la potencia anaeróbica es importante para tener éxito en el ciclismo, pero la eficiencia también es otro factor determinante en el rendimiento de los ciclistas.

2.1.1.4. Eficiencia

La eficiencia en el ciclismo describe la relación entre la potencia mecánica de producida y la potencia metabólica utilizada, y es determinante en el rendimiento de la resistencia (71). Por lo tanto, la eficiencia bruta (Gross mechanical efficiency: GE), el

índice más usado para el cálculo de la eficiencia ciclista puede expresarse de la siguiente manera (71):

$$GE (\%) = (\text{mechanical power output } (J \cdot s^{-1}) / \text{metabolic power input } (J \cdot s^{-1})) \cdot 100$$

Aunque la GE no es una medida precisa de la eficiencia muscular (72), es un buen indicador de la eficiencia de todo el cuerpo y, por tanto, podría ser relevante desde un punto de vista práctico (73). Además, las mediciones de GE realizadas durante las pruebas de laboratorio han demostrado ser fiables (8).

Los determinantes fisiológicos y metabólicos de esta variable aún no se conocen del todo (8), hay varios factores que pueden influir en la GE, como la cadencia de pedaleo (74), la dieta (75), el sobreentrenamiento (76), la genética (77) o la distribución del tipo de fibra (78). Información reciente indica que en los ciclistas profesionales la tasa de aumento del VO_2 , provocada por el ejercicio incremental, disminuye con cargas de trabajo moderadas y altas hasta la máxima potencia alcanzable (79). Además, la eficiencia mecánica parece aumentar con el incremento de la intensidad del ejercicio. Estos hallazgos revelan que los ciclistas profesionales de carretera adquieren una alta eficiencia ciclista que les permite sostener cargas de trabajo extremadamente altas durante largos periodos de tiempo. En este sentido, los corredores profesionales muestran una considerable resistencia a la fatiga de las unidades motoras reclutadas a altas intensidades submáximas (18, 80). No obstante, si es conocido que los corredores profesionales suelen recorrer aproximadamente unos 35.000 km al año y competir unos 90 días al año y que este alto volumen de entrenamiento influye en conseguir una mayor eficiencia en el ciclista (79).

En ciclistas profesionales se encontró una GE del 24.5 % a una intensidad al 80 % VO_{2MAX} (79). En los ciclistas amateur se han encontrado diferente eficiencia (GE) en distintas intensidades, 16.5 % al 30 % VO_{2MAX} , 19 % al 40 % VO_{2MAX} , 20 % al 50 % VO_{2MAX} , 20.5 % al 60 % VO_{2MAX} , 21 % al 70 % VO_{2MAX} y 21.5 % al 80 % VO_{2MAX} (81).

La eficiencia del ciclista profesional durante el ejercicio intenso parece estar relacionada positivamente con el porcentaje de fibras ST en el músculo vasto lateral (82). Una mayor proporción de fibras de tipo I (ST) en el músculo se asocia con un menor coste de oxígeno en el ejercicio submáximo y, por tanto, con una mayor eficiencia bruta (78). Esta eficiencia es un reflejo del aumento del metabolismo aeróbico y de los incrementos relacionados en la producción de potencia muscular (72).

Por otro lado, el entrenamiento puede ser un factor con el que se puede mejorar la eficiencia en el ciclismo. En este sentido, se ha visto que ciclistas entrenados que realizan un entrenamiento de fuerza máxima (3 veces por semana; medias sentadillas, 4 series de 4 repeticiones, más su entreno normal) durante 8 semanas mejoran la eficiencia (4.7%) (test submaximal de 3h 70% VO_{2MAX}) y aumenta el tiempo hasta el

agotamiento en la potencia aeróbica máxima (en un test incremental).(83). A nivel nutricional, también se ha visto que tanto la ingesta de hidratos de carbono (durante 2.5 h de ciclismo al 75% del VO_{2MAX}) (84) como de quercetina (polifenol) (1000 mg para 3 semanas, medido en 3 h al 57% de la P_{MAX} en cicloergómetro) (85), son capaces de mejorar la eficiencia en ciclistas. Por lo tanto, este sería otro factor a tener en cuenta, para mejorar el rendimiento en ciclistas en intensidades submáximas. En este sentido, para comprender mejor los mecanismos metabólicos tanto en ejercicios de baja, moderada y alta intensidad, en las siguientes apartados vamos a profundizar en los umbrales ventilatorio y de lactato.

2.1.1.5. Umbrales ventilatorios

A la hora de determinar los umbrales ventilatorios nos encontramos con 2 puntos claramente diferenciados, que son el VT1 y VT2. El VT1 es el punto en el que se produce un aumento no lineal del volumen espiratorio (VE) y de la producción de dióxido de carbono (VCO_2), en combinación con un descenso de la fracción de CO_2 espirada ($FECO_2$) y una elevación de la fracción de O_2 espirada (FEO_2) (6). En la práctica, el VT1 se identifica como un punto de hiperventilación con respecto al consumo de oxígeno (VO_2) y se refleja en un aumento sistemático del equivalente ventilatorio para el oxígeno (VE/VO_2) sin un aumento concomitante del equivalente ventilatorio para el dióxido de carbono (VE/VCO_2) (6). Por otro lado, el VT2 es el punto de compensación respiratoria, que se identifica como un aumento tanto del VE/VO_2 como del VE/VCO_2 y una disminución de la presión parcial de dióxido de carbono exhalado o "end-tidal"(al final de la espiración) ($PETCO_2$) (7).

Como factores limitantes en el VT1 y VT2, podemos encontrar la cantidad de fibra ST, y la capilarización y la capacidad oxidativa a nivel muscular (24, 86). Además, en el VT2 habría que añadir como factores limitantes la capacidad de tamponamiento y la capacidad de reclutamiento de fibras (siendo mayor en ciclistas de élite), reduciendo así la producción de potencia relativa y el estrés en una fibra determinada (24, 86).

Cuando hablamos de VT1, estamos indicando una intensidad por debajo del VT2, denominada comúnmente umbral aeróbico, con una alta utilización de las grasas como sustrato energético. Por ejemplo, en ciclistas profesionales se ha encontrado el VT1 en el 65% del VO_{2MAX} (24), pero en ciclistas amateur se encuentra por debajo del 60% del VO_{2MAX} (87). Esta diferencia en el VT1, se debe principalmente a que los ciclistas profesionales tienen un alto porcentaje de fibras ST (24, 78), El ejercicio prolongado realizado a una carga de trabajo submáxima, como el que realizan los atletas de resistencia, depende en gran medida de los tipos de fibras ST, y éstas experimentan un aumento como resultado de la adaptación fisiológica muscular al entrenamiento de resistencia (24).

En relación al VT2, se ha visto que es un importante factor de rendimiento incluso en eventos de resistencia extrema como las carreras profesionales de ciclismo (24). Por lo tanto, tener un VT2 elevado es una ventaja para los ciclistas, ya que las etapas de montaña con subidas que duran entre 30 y 60 min exigen que el ciclista trabaje cerca del VT2 o cerca del inicio de la acumulación del Lac en sangre (13, 15). De hecho, se ha demostrado que, durante algunas semanas de la temporada de competición, los ciclistas profesionales pueden completar varias horas de ejercicio a una intensidad superior a la VT2 (88).

A la hora de diferenciar a los ciclistas por su nivel en referencia al VT2, Lucia et al. (24) observó que los ciclistas profesionales alcanzan el VT2 en el 87% del VO_{2MAX} , datos similares a los encontrados por el mismo autor en un estudio posterior en otro grupo de ciclistas profesionales (88.2% VO_{2MAX}) (89). Sin embargo, en ciclistas amateur, los niveles a los que se encuentra el VT2 son inferiores (78% del VO_{2MAX}) a los de los ciclistas profesionales (90). Hay que mencionar, que el VT2 representa una intensidad de trabajo en la que la acumulación de Lac en sangre aumenta considerablemente y se produce una hiperventilación para amortiguar la acidosis (es decir, la compensación ventilatoria) (91). Por lo tanto, el VT2 representa la tasa metabólica más alta a la que el organismo es capaz de mantener una acidosis metabólica elevada pero estable.

Tradicionalmente, los umbrales ventilatorios se han utilizado durante mucho tiempo junto con el VO_{2MAX} para evaluar los cambios en la condición física de los ciclistas producidos por el entrenamiento (92). Hace más de tres décadas, Wasserman y sus colegas fueron pioneros en la aplicación de métodos alternativos para estimar el umbral de Lac mediante análisis de intercambio de gases (91, 93). Además, hay que tener en cuenta, que la medición del Lac en sangre requiere técnicas invasivas como la toma de muestras de sangre capilar, venosa o arterial, y puede tener una resolución limitada para detectar con precisión los cambios metabólicos temporales durante el ejercicio incremental. Esto puede ser una desventaja frente a la medición de los umbrales ventilatorios. A la vista de estos resultados y aunque la relación mecánica entre la rápida acumulación de Lac, su acidosis asociada y los umbrales ventilatorios sigue siendo controvertida y variable (94-96), estos umbrales fisiológicos siguen siendo utilizados como norma para la clínica/prescripción de ejercicio y en entornos de investigación para la asignación, normalización y ajuste de ritmos de trabajo.

A la hora de intentar mejorar el VT1 y VT2 en ciclistas, se ha visto que 8 semanas de un entrenamiento polarizado tiene mayores mejoras en el VT1 y VT2 (\uparrow del VO_2 L/min en un 11.9 y 13.3 %, respectivamente) que un entrenamiento por bloques (\uparrow 7.8 y 10.1 %, respectivamente) (97). En relación a la suplementación deportiva, se ha visto que la ingesta de 1.2 g de omega-3 (con \uparrow ácido docohexánico) mejora la potencia media generada en VT2 sin cambios en el VO_2 en ciclista amateur (98). Sin embargo,

también ha sido documentado que la ingesta 28 días L-arginina ($2 \times 6 \text{ g}\cdot\text{d}^{-1}$) y de 14 días de *Cordyceps sinensis* ($1000 \text{ mg}\cdot\text{d}^{-1}$) no fueron capaces de mejorar la potencia generada en VT1, VT2 y $\text{VO}_{2\text{MAX}}$ en ciclistas entrenados (99, 100).

Hay que tener en cuenta, que los umbrales ventilatorios (VT1 y VT2) son puntos fisiológicos a tener en cuenta por los entrenadores, ya que estos les permiten evaluar los cambios mediados por el entrenamiento y tomar las oportunas decisiones. Además, también existen otros 2 umbrales asociados a las concentraciones de Lac, que son el umbral de Lac 1 (UL1) que está relacionado al VT1 y el umbral de Lac 2 (UL2) que está relacionado al VT2. Su relación está vinculada en el punto en el que aparecen en un test incremental, pero son medidos de forma diferente, ya que, el VT1 y VT2 se analizan mediante los equivalentes ventilatorios, y el UL1 y UL2 son analizados mediante las concentraciones de lactato en sangre.

2.1.1.6. Umbrales de lactato

El UL1 se define como la intensidad del ejercicio que provoca un aumento de 1 mmol/L en la concentración de Lac en sangre por encima del valor medio en reposo (2). Y el UL2 es la intensidad del ejercicio que provoca una concentración de Lac de 4 mmol/L (5). Los factores limitantes para el UL1 y el UL2 son similares a los del VT1 y VT2, por su relación en cuanto al punto donde se encuentran en un test incremental (24, 86).

Encontramos que los ciclistas profesionales tienen el UL2 en el $\sim 90\%$ del $\text{VO}_{2\text{MAX}}$ (48), por el contrario, los valores encontrados en ciclistas amateur (bien entrenados) están sobre el 76% $\text{VO}_{2\text{MAX}}$ (101). Además, se ha demostrado que la potencia generada en el UL2 es un predictor válido ($r = 0,88$) del potencial de un ciclista (14). Sin embargo, hay evidencias científicas que indican que las condiciones de estado estable a intensidades de ejercicio que provocan valores de Lac en sangre difieren del valor fijo de 4 mmol/L correspondiente al UL2 (102, 103). Por lo que, es recomendable testar el máximo estado estable en cada deportista. Además, se ha determinado de que el UL2 es la intensidad de trabajo en estado estacionario más alta posible que puede mantenerse durante un tiempo prolongado (~ 60 min) y, por lo tanto, es un excelente índice de resistencia (5, 45, 102). Adicionalmente, Coyle et al. (14) demostró que el VO_2 en el UL2 es un fuerte predictor ($r = 0.960$) del rendimiento entre ciclistas entrenados con una potencia aeróbica máxima similar.

La medición del Lac en sangre se utiliza ampliamente en la medicina deportiva, aunque existe un debate sobre cómo afecta el Lac a la fatiga en los atletas de resistencia (104). Sin embargo, la concentración de Lac en la sangre en relación con la intensidad del ejercicio es un marcador relevante del rendimiento de resistencia (105-107). Esto puede visualizarse en una curva de Lac en sangre utilizando un test de esfuerzo

incremental, a medida que la carga de trabajo del atleta aumenta con el tiempo, se miden las concentraciones de Lac en sangre en intervalos definidos. Durante el ejercicio de alta intensidad se forma Lac junto con H^+ en los músculos (107), seguido de una mayor eliminación de Lac del plasma (106, 108). Cuando la eliminación se satura, el Lac en sangre empieza a aumentar cuando la producción supera el aclaramiento. Este aumento (exponencial) del Lac en sangre durante la curva del Lac es importante, ya que la intensidad del ejercicio correspondiente se asocia con el rendimiento de la resistencia, existiendo una correlación con la transición del entrenamiento aeróbico al anaeróbico.

Atendiendo a los factores que pueden modificar los umbrales de lactato, se ha mostrado que 6 semanas de entrenamiento polarizado mejoran el rendimiento (W) en UL1 (9%) comparado a un entrenamiento en el umbral (2%), sin cambios significativos en UL2 en ciclistas entrenados (109). Con respecto a la ingesta de polifenoles, se ha visto que el consumo de $400 \text{ g}\cdot\text{d}^{-1}$ de pulpa de açai (alto en polifenoles) durante 15 días disminuye la producción de Lac y aumenta la potencia generada en el umbral anaeróbico (zona próxima al UL2), sin cambios significativos en el grupo placebo (110).

Anteriormente, hemos visto la evaluación de las concentraciones de la Lac, es una herramienta útil para el control metabólico del ciclista, en el siguiente apartado vamos a ver su relación con el rendimiento.

2.1.1.7. Relación entre el rendimiento y los niveles de lactato en sangre

La concentración de Lac en sangre a varias intensidades de ejercicio de ciclismo es altamente predictiva del rendimiento de resistencia, lo que hace que su medición sea valiosa para evaluar el rendimiento futuro (4, 111, 112). Especialmente es significativa en el máximo estado estable de lactato (MLSS), que es la intensidad de ejercicio más alta a la que la concentración de Lac en sangre permanece estable, que refleja un equilibrio entre la producción y la eliminación de Lac (4). Se ha observado que los ciclistas entrenados alcanzan la MLSS a una intensidad equivalente al 90% de su velocidad media simulada de contrarreloj de 5 km (113). En este sentido, Harnish et al. (114) demostraron que el MLSS puede estimarse de forma no invasiva, con un error del 2%, utilizando una contrarreloj de ciclismo de 5 km y 40 km.

Por otro lado, Bentley et al. (115) encontraron una correlación positiva entre la potencia generada en UL2 y el rendimiento en una contrarreloj de 20 min ($r = 0.67$) y 90 min ($r = 0.91$) en ciclistas amateur. Además, la P_{MAX} resultó ser especialmente útil por su valor predictivo del rendimiento ciclista en una hora. La importancia de este hallazgo radica en que el descubrimiento de la P_{MAX} no requiere la medición del VO_2 o del Lac. Sin embargo, si la medición del Lac está disponible, es una medida valiosa para la prescripción de la intensidad del entrenamiento (116).

Por lo tanto, el control de la producción de lactato puede ser de utilidad para conocer el impacto a nivel metabólico en el organismo de los ciclistas. Pero el siguiente apartado nos ayudará a entender mejor los mecanismos de regulación del Lac.

2.1.1.8. Acidosis metabólica

La mayoría de los textos de fisiología y bioquímica demuestran claramente la importancia de la reacción del Lac para mantener el redox citosólico (reacción de oxidación-reducción) y permitir que la glucólisis continúe durante el ejercicio intenso. Además, Donovan y Brooks (117) pudieron demostrar la importancia del Lac como sustrato neoglucogénico durante el ejercicio. El hallazgo más sorprendente fue la observación de que los individuos bien entrenados eran más capaces de mantener los niveles de glucosa en sangre a través de la gluconeogénesis del Lac que los individuos no entrenados (117). Recordemos que la gluconeogénesis es la síntesis de glucosa a partir de precursores que no son carbohidratos, como el glicerol, los cuerpos cetónicos o los aminoácidos. Aunque muchos han llegado a aceptar estas facetas positivas del metabolismo del Lac, algunos individuos siguen creyendo que estos beneficios se producen a costa de aumentar la acidosis cuya génesis es el ácido láctico (1).

Una revisión de Robergs et al. (107) identificó claramente como los intermediarios de la glucólisis se protonan en un pH fisiológico donde el ácido láctico existiría como Lac y no como ácido láctico, siendo el Lac de sodio la principal forma de ácido láctico en los sistemas fisiológicos. Sin embargo, este autor sugiere una mayor dependencia del recambio de ATP no mitocondrial como fuente potencial de producción de protones que se asocia con una mayor creación de Lac y el desarrollo de acidosis (107). Además, cuando se supera la capacidad de amortiguación de la célula, se desarrolla la acidosis (107). Claramente, existen mecanismos plausibles que explican que el ácido láctico no es la fuente principal de protones durante el ejercicio. En este sentido, Robergs et al. (107) señalan la evidencia de que el Lac sirve para disminuir las concentraciones de iones hidrógeno en lugar de aumentarlas. La conversión de piruvato en Lac no sólo oxida la forma reducida del dinucleótido de nicotinamida-adenina (NADH), sino que también utiliza un H^+ de la solución para unirse al carbono, sirviendo así para disminuir la concentración de H^+ en lugar de contribuir a su aumento. En una investigación reciente en ciclistas profesionales, Santalla et al. (118) demostraron que, a pesar de un pH indicativo de acidosis, no había ningún deterioro aparente inducido por la acidosis en la función del músculo esquelético en los deportistas. Estos resultados también sugieren, de forma indirecta, que la eficiencia de la contracción muscular no se vio alterada de forma significativa y añade más apoyo a que la producción de Lac retrasa la acidosis en lugar de contribuir a ella. Debería ser evidente que la producción de Lac no debería considerarse una faceta negativa del aumento de la intensidad del ejercicio. Todos los aspectos de la producción de Lac son

beneficiosos. La producción de Lac sirve para mantener el redox citosólico, producir nueva glucosa, consumir H^+ del citosol, así como permitir el transporte de H^+ desde la célula (1).

Aunque una mayor capacidad de producir y eliminar el Lac de la célula ayuda a retrasar la aparición de la acidosis, la producción de Lac retrasa, no empeora, la acidosis (107). Para el ciclista de competición, esto significa que para un determinado VO_2 durante el ciclismo intenso, una alta producción de Lac es beneficiosa. Su producción es aún más beneficiosa si va acompañada de una alta capacidad de transporte de Lac y protones desde la célula. Se sabe que ambos factores aumentan con el entrenamiento de resistencia y sprint (119).

Pese a todo, aunque la producción de Lac no es la causa de la acidosis, sigue siendo un importante indicador indirecto de un ajuste en el metabolismo que causa la acidosis. La elevada producción de Lac puede deberse a la contribución del metabolismo anaeróbico del glucógeno y la glucosa (120) o a la sobreproducción aeróbica de piruvato y su posterior conversión en Lac (121).

El reclutamiento del tipo de fibra muscular también tiene implicaciones en la aparición de la acidosis metabólica. Por ejemplo, cadencias de pedaleo elevadas con reducción de la fuerza en el pedal, reducen la fuerza utilizada por golpe de pedal, lo que implica una reducción de la fatiga muscular en las fibras tipo II (FT) (122). Sin embargo, cuando se aumenta la cadencia de pedaleo sin una reducción de la fuerza al pedal o se emplea una marcha más dura, las fibras FT se reclutan progresivamente (122). Las fibras musculares FT tienen una menor densidad mitocondrial que las fibras ST y, por lo tanto, son más dependientes de la glucólisis y del recambio de ATP citosólico (107). Combinadas, estas dos características metabólicas dan lugar a una mayor tasa de liberación de protones del catabolismo, lo que se traduce en una importante producción de protones cuando la intensidad del ciclismo requiere un mayor reclutamiento de fibras musculares FT. En consecuencia, las fibras FT contribuirán sustancialmente a la acidosis porque tienen menos masa mitocondrial para facilitar la regeneración de ATP y la captación de protones (107).

2.1.1.9. Eficiencia ventilatoria

El dióxido de carbono (CO_2) se produce en el metabolismo celular y se expulsa a la atmósfera mediante la ventilación (VE), pero durante este proceso el CO_2 juega un papel fundamental en la regulación del pH corporal, el tono vascular y en el control de la ventilación (123). La relación entre la tasa de salida de CO_2 (VCO_2) y la VE en diferentes circunstancias se ha descrito ampliamente como una medida de la eficiencia respiratoria a una tasa metabólica determinada (124). Durante el esfuerzo incremental,

la pendiente de la relación lineal entre el VE y la VCO_2 (pendiente VE/VCO_2) es el método más utilizado para evaluar la eficiencia ventilatoria (123).

Los músculos respiratorios pueden alcanzar el límite de su capacidad durante un ejercicio de alta intensidad o larga duración. Los datos de las investigaciones sugieren que los músculos inspiratorios pueden ser un potencial factor de limitación del rendimiento en el ejercicio (125). Esto puede ser debido, a que un entorno ácido elevado ($\downarrow pH$) de los músculos respiratorios o la competencia por el flujo sanguíneo entre los músculos locomotores y los respiratorios pueden contribuir a la fatiga de los músculos inspiratorios tras un ejercicio de alta intensidad (126). Romer et al. (125) observaron que una disminución de la tasa de flujo inspiratorio máximo después de una contrarreloj ciclista de 20 km y 40 km. Además, el trabajo respiratorio durante el ejercicio intenso compromete el flujo sanguíneo debido a la redistribución del flujo sanguíneo entre la pared torácica y los músculos locomotores (127). También se ha observado que la fatiga muscular inspiratoria ralentiza la tasa de relajación necesaria para la recuperación muscular (128).

El patrón de respiración del ciclista parece tener una cierta influencia en el rendimiento. Los ciclistas profesionales muestran un patrón respiratorio único a altas cargas de trabajo, caracterizado por la ausencia de un cambio taquipnéico, es decir, continúan aumentando la VE mediante el incremento del volumen tidal frente a la frecuencia respiratoria (13). Se sugiere que esta adaptación de la respiración puede mejorar la eficiencia y el coste metabólico de la respiración y explicar en parte la cinética del VO_2 mostrada por los ciclistas profesionales (13).

Parece ser que el entrenamiento de resistencia tradicional no proporciona el estímulo de entrenamiento necesario para fortalecer los músculos inspiratorios, por lo que puede ser conveniente un protocolo de entrenamiento específico de dichos músculos (125). En este sentido, el entrenamiento de los músculos inspiratorios que incorpora 30 esfuerzos inspiratorios dinámicos dos veces al día durante 6 semanas contra una carga de umbral de presión equivalente al ~50% de la presión bucal inspiratoria máxima, ha demostrado ser un estímulo de entrenamiento eficaz. Sin embargo, las evidencias en este tema no son muy robustas, ya que, también se ha visto que las mejoras en la función muscular respiratoria no parecen ser transferibles al VO_{2MAX} o a atletas que compiten (129).

Actualmente, a nivel nutricional existe ninguna estrategia ni alimentaria ni ayuda ergogénica que haya demostrado cambios en el patrón respiratorio del ciclista que puedan mejorar el rendimiento.

Hay que tener presente, que aunque todavía falta mucha investigación sobre la relación entre el patrón respiratorio y el rendimiento. El factor respiratorio tiene su

importancia dentro de los factores que afectan al rendimiento del ciclista, posiblemente en menor medida que la composición corporal, que puede ser un factor más decisivo para el éxito en el ciclismo y abordaremos en el próximo punto.

2.1.1.10. Composición corporal

La composición corporal es un factor importante en el ciclismo, ya que el balance entre el peso, la masa grasa y muscular corporal debe determinarse para averiguar cambios en los distintos componentes de la composición corporal y alcanzar un estado óptimo de rendimiento (130). Existen varios métodos para medir la composición corporal, pero los utilizados principalmente en el deporte son la antropometría y la densitometría. Los procedimientos antropométricos nos permiten determinar la masa grasa (BF) y muscular (MM), que son de gran ayuda, ya que podemos conocer la distribución de estos tejidos tanto en miembros superiores como inferiores y evaluar sus cambios, además de cómo afectan al rendimiento de cada deportista (131). Los factores más importantes que pueden modificar la composición corporal son la nutrición y el entrenamiento (132).

El ciclismo de carretera es un deporte que requiere actuar en una gran variedad de situaciones (por ejemplo, carreteras llanas o de subida) y situaciones competitivas (por ejemplo, ciclismo individual o con drafting detrás de numerosos ciclistas). A su vez, el rendimiento en el ciclismo en cada uno de los terrenos de competición viene determinado en parte por las características morfológicas individuales (masa corporal, altura, superficie corporal, índice de masa corporal, área frontal e índice de masa corporal (IMC) (15).

Así pues, las características antropométricas pueden ser muy diferentes dependiendo de cada especialidad en ciclismo de carretera. Los especialistas en contrarreloj o en terreno llano suelen ser más altos y pesados (180-185 cm de altura, 70-75 kg de peso, IMC de ~22) que aquellos que suelen tener mejor rendimiento en las subidas (175-180 cm de altura, con un peso de 60-66 kg y un IMC de 19-20 (15, 89). Las características antropométricas de los mejores ciclistas en la actualidad, capaces de destacar en ambos tipos de terrenos, se acercan a las de los contrarrelojistas (es decir, ~180 cm de altura y ~70 kg de peso) (15). El porcentaje de grasa corporal, en cambio, no es tan elevado. Por otra parte, el porcentaje de masa grasa (BF%) no difiere significativamente entre los distintos tipos de ciclistas: a partir de valores cercanos al 10% (mediante técnicas de pliegues cutáneos) durante los meses de invierno, disminuye gradualmente durante la temporada, para alcanzar valores en torno al 9% durante la primavera y cerca del 8% durante las carreras de 3 semanas (al final de los meses de primavera y verano) (88, 133, 134). Por otro lado, también se ha descrito las características antropométricas de ciclistas amateur, encontrándose un peso y altura

promedio de 71 kg y 175 cm, respectivamente, unido a un IMC de 22.9 y un BF% del 11% (medido con densitometría). Sin embargo, otro estudio presentó valores de peso, altura y BF% de 76 kg, 181 cm y 15%, respectivamente superiores a los del estudio anterior en ciclistas amateur, pero con IMC similar (17). En este estudio también se observó una masa libre de grasa (FFM) de 65.5 kg.

A la hora de mejorar la composición corporal, en concreto reducir la masa grasa y el peso, se ha visto que la combinación de una dieta baja en hidratos de carbono (15% hidratos de carbono en la dieta) y el entrenamiento (8 semanas; 3 x semana 90 min al 80% de la potencia máxima + 20 min contrarreloj) en ciclistas puede mejorar el rendimiento en un test de 20 min (132). Además, se ha observado como el consumo de té verde, (con alto contenido en polifenoles) durante 12 semanas ha reducido la masa grasa (BF) en hombres y mujeres obesos (135).

Cambios en la composición corporal pueden verse afectados por distintos factores, como hemos comentado anteriormente, pero dentro del entrenamiento. Una adecuada manipulación de la carga del entrenamiento puede ser una estrategia eficaz. Concretamente, el volumen del entrenamiento es un factor que puede influir de una manera importante en la composición corporal (1).

2.1.1.11. Volumen de entrenamiento

Los ciclistas profesionales de carretera entrenan en distancias de aproximadamente 30000-35000 km al año (48). Este volumen de rodaje diferencia claramente al ciclista profesional de los ciclistas menos dotados genéticamente, aunque "bien entrenados", que recorren, de media, menos de la mitad de esta distancia (250 frente a 625 km/semana) (11). Las distancias recorridas durante las carreras de carretera de varias etapas oscilan entre 5 km y casi 300 km. La Vuelta a España, una carrera por etapas de 22 días, cubre ~3725 km, de los cuales ~3635 km son de competición en línea y ~89km son etapas de ITT. Del mismo modo, la carrera por etapas del Tour de Francia cubre ~3899 km, de los cuales ~3796 km son de competición en línea y ~103 km de ITT (1). Otro factor que diferencia a los ciclistas profesionales del resto de ciclistas es el número de carreras que disputan, ya que, el número de días de carrera en un grupo de 22 ciclistas profesionales de un equipo europeo fue de 101 ± 6 d/año, con un rango de 88-112 d/año (11). En el caso de los ciclistas amateur (bien entrenados), Jeukendrup et al. establecieron que tenían una frecuencia de entrenamiento semanal de 3-7 días, con una duración media de los entrenos de 60-240 min, experiencia en la práctica de ciclismo de 3-5 años y participaban de 0-20 competiciones al año (11). Hay que decir, que los ciclistas amateurs son los predominantes en el ciclismo a nivel mundial, siendo la muestra de ciclistas profesionales muy inferior.

2.1.1.12. Fatiga

En el ciclismo la fatiga muscular puede definirse como la incapacidad de mantener una potencia requerida o esperada durante un tiempo determinado (136). Se ha demostrado que los sujetos que poseen un alto porcentaje de fibras FT son más sensibles a la fatiga que los que tienen más fibras ST (137). Un ejercicio de esprints repetidos (en ciclismo) provoca la fatiga de los músculos glúteo mayor y vasto lateral, y cuando esto pasa, estos músculos monoarticulares producen menos fuerza y potencia. Se observan dos tipos de fatiga (1) la fatiga periférica, en la que se produce un aumento de la relación actividad electromiográfica (EMG)/fuerza; y (2) la fatiga central, caracterizada por una relación EMG/fuerza constante asociada a una disminución de la fuerza de los músculos agonistas (138). Simultáneamente, se da una menor activación de los músculos antagonistas, lo que permite al ciclista mantener la transferencia eficazmente de fuerza y de potencia al pedal (138).

El ejercicio prolongado de ciclismo deteriora la capacidad de fuerza muscular asociada a los cambios en las propiedades contráctiles y neuronales de los extensores de la rodilla (1). Pero no parece haber ninguna relación entre el patrón de reclutamiento del tipo de fibra y la fatiga neuromuscular y la consiguiente reducción de la fuerza durante el ejercicio de ciclismo, más bien, existe un componente central de la fatiga. Los déficits de activación muscular no son significativamente diferentes entre las cadencias (1). En línea con estas afirmaciones, Lepers et al. (139) descubrieron que a mayor cadencia de pedaleo, la entrada neural central a los músculos *vastus medialis* y *vastus lateralis* no se modificaba. Además, los resultados sugieren que el impulso central se altera menos cuando se utiliza una cadencia de pedaleo alta (69-103 rpm). Es interesante señalar dos conclusiones importantes: 1) el impulso central se altera menos cuando se utiliza una cadencia alta; y 2) la cadencia elegida libremente no minimiza los efectos de la fatiga en las capacidades de fuerza de los extensores la parte posterior de la pierna (1). En la práctica, esto significa que se puede utilizar eficazmente una combinación de diferentes cadencias altas.

Se ha observado que existen diferentes mecanismos que producen la fatiga del sistema nervioso central (SNC) durante el ejercicio prolongado (140). Más concretamente, se han examinado los aumentos de serotonina o un agotamiento de las catecolaminas durante el ejercicio como posibles contribuyentes a la aparición temprana de la fatiga (141, 142). Sin embargo, Piacentini et al. (143) demostraron que un inhibidor de la recaptación noradrenérgica no influía en el rendimiento de una contrarreloj individual (ITT) de 90 min. Además, la ingestión de 5-hidroxitriptófano (5 mg/kg), un precursor de la serotonina, no tuvo ningún efecto sobre el rendimiento ciclista prolongado al 65% del VO_{2MAX} seguido de un esfuerzo incremental hasta la

fatiga (144). Por lo tanto, parece que el componente central de la fatiga no es fácil de relacionar con el aumento o la disminución de los neurotransmisores.

Existe poca investigación sobre la prevención de la fatiga, especialmente durante las carreras por etapas. A este respecto, algunos investigadores han planteado la hipótesis de que existe un sistema subconsciente aprendido de anticipación/regulación, conocido como "teleanticipación", que se origina en el SNC (145). Este mecanismo de retroalimentación subconsciente sirve para disminuir la salida eferente del córtex motor. Antes del comienzo de una competición determinada, se supone que el SNC del ciclista conoce su nivel de forma física, su capacidad de resistencia y sus limitaciones, tal y como las ha obtenido en competiciones similares anteriores (146). Se conoce la carga total de ejercicio y el tiempo que el cuerpo del ciclista puede tolerar el nivel metabólico dado (146). Teniendo esta información se produce, a nivel subconsciente, el límite de carga de ejercicio para evitar la fatiga prematura antes de la conclusión del evento. Esto se lleva a cabo mediante un "programador central" que establece los límites superiores tolerables para las cargas competitivas totales (146). Se postula que la limitación se logra a través de una disminución de la salida eferente de la corteza motora. Este mecanismo teórico puede ser similar a la hipótesis del "controlador central", en la que se teoriza que un controlador central y neural limita el gasto cardíaco regulando la masa de muscular que puede activarse cuando se acercan los límites metabólicos (147). En este sentido, Lucia et al. (148) observaron que durante el Tour de Francia y la Vuelta a España, los ciclistas nunca alcanzaban sus límites máximos de ejercicio durante dos días consecutivos.

2.1.1.13. Sobreentrenamiento

Existe un fino equilibrio entre la carga de entrenamiento y la recuperación. Demasiado entrenamiento y muy poca recuperación pueden alterar este equilibrio y el resultado es un fenómeno que se denomina "sobrecarga" (149). Los ciclistas que abandonan las carreras por etapas debido a la insuficiente recuperación metabólica causada por la disminución de los niveles de glucógeno y de fosfatos son ejemplos de sobrecarga. Cuando se corrige con prontitud, la recuperación de la sobrecarga suele producirse en unos 14 días (150).

Si el entrenamiento y la recuperación no se reequilibran, el sobreesfuerzo conduce al síndrome de sobreentrenamiento (SSE). Un síntoma común de este síndrome es la reducción del rendimiento ciclista y la consiguiente fatiga, que provoca síntomas como irritabilidad, problemas de sueño y falta de motivación. Los mecanismos implicados incluyen importantes alteraciones en el eje hipotálamo-hipófisis-tiroides, así como una función neuromuscular alterada. Entre las alteraciones,

también se encuentra el aumento de la FC en reposo y en ejercicio, así como la modulación de la variabilidad de la FC (aumento de la actividad simpática) (151).

La FC es un indicador sensible del estado de entrenamiento de los ciclistas, en reposo está controlada por el sistema nervioso simpático y parasimpático autónomo. Cuando la actividad simpática aumenta, la FC aumenta y la variabilidad de la FC a corto plazo disminuye (152). Sin embargo, cuando la actividad parasimpática aumenta, la FC disminuye y la variabilidad de la FC a corto plazo aumenta (152). Se ha informado de que la variabilidad de la FC a corto plazo refleja la actividad del sistema nervioso simpático y parasimpático y el equilibrio entre estas dos divisiones. Dado que el equilibrio y la actividad de estas divisiones del sistema nervioso autónomo cambian con el entrenamiento y el SSE, la variabilidad de la FC puede utilizarse para indicar los efectos del entrenamiento (152). Por ejemplo, la FC disminuye y la variabilidad de la FC aumenta con el efecto positivo del entrenamiento. En el SSE y en el estado de SSE simpático, la FC aumenta y la variabilidad de la FC disminuye. En el estado de SSE parasimpático o en el agotamiento, tanto la FC como la variabilidad de la FC disminuyen (152).

Si se utiliza la FC durante el sueño para interpretar el estado de entrenamiento o el SSE, hay que tener en cuenta que la FC máxima intrínseca diaria durante el sueño varía en ~8 ppm (153). Lucia et al. (148) demostraron que en las carreras ciclistas profesionales de 3 semanas, el mejor marcador posible que sugiere el SSE y la fatiga es una disminución de la FC máxima. Este fenómeno, a su vez, reflejaría un cierto estado de desregulación del sistema simpático-adrenal. De hecho, se ha informado de un cierto estado de "agotamiento hormonal" al final de las carreras de 3 semanas (148, 154).

Además, el ciclista sobreentrenado suele mostrar incompetencia en el rendimiento, fatiga prolongada o incapacidad para entrenar a los niveles esperados (1). También puede observarse dolor y sensibilidad muscular, dolor muscular persistente que aumenta con cada sesión de entrenamiento y elevación de la creatina quinasa sérica. La sobrecarga puede ser tanto psicológica como física, siendo frecuente la alteración del estado de ánimo, la reducción de la capacidad de rendimiento máximo y la incompetencia competitiva durante semanas y meses (1).

El SSE se ha relacionado con los niveles bajos de glutamina (154). Se ha sugerido que la presencia de una menor concentración de glutamina podría ser un efecto negativo del estrés del ejercicio. La glutamina es el aminoácido más abundante en el cuerpo, donde el músculo esquelético proporciona la mayor parte de la glutamina plasmática requerida por el tracto gastrointestinal, las células del sistema inmunitario y el riñón durante la acidosis (1). Sin embargo, dos son los factores que parecen ser los responsables de la disminución de la concentración de glutamina con el paso del

tiempo a) el aumento de los niveles de glucocorticoides puede disminuir las reservas musculares de glutamina; y b) la disminución de la ingesta nutricional de proteínas puede afectar a las reservas, ya que se ha sugerido un aumento de la ingesta de proteínas para los atletas de resistencia, especialmente durante el entrenamiento intenso (1).

Por otro lado, se ha observado que la concentración de glutamato en los atletas sobreentrenados presenta otra perspectiva del SSE. Parece que las concentraciones elevadas de glutamato se asocian a periodos de entrenamiento de muy alta intensidad en los que se observan altas concentraciones de Lac en sangre y, coincidentemente, altas concentraciones de iones hidrógeno. El estado normal de reposo o de bajo volumen de entrenamiento está representado por una concentración de glutamina de $585 \pm 54 \mu\text{mol/L}$ y por una concentración de glutamato de $101 \pm 16 \mu\text{mol/L}$. Una concentración de glutamina de $585 \mu\text{mol/L}$ o inferior parece indicar una "capacidad de trabajo desarrollada" o tolerancia al volumen de ejercicio, en atletas que han desarrollado una base aeróbica durante años de entrenamiento (155).

Los cambios en las concentraciones de glutamina y glutamato parecen seguir un patrón de disminución de la glutamina y de aumento de la concentración de glutamato con el aumento de la carga de entrenamiento. En consecuencia, se ha propuesto que el estado de entrenamiento puede representarse mediante la relación entre la glutamina y el glutamato (Gm/Ga). La relación Gm/Ga en reposo o al inicio de la temporada de entrenamiento sería $>5.88 \mu\text{mol/L}$, pudiendo ser un valor máximo de $7.66 \mu\text{mol/L}$ (155). Una concentración media de glutamina de $522 \pm 53 \mu\text{mol/L}$, de glutamato de $128 \pm 19 \mu\text{mol/L}$ y una relación Gm/Ga de $4.15 \pm 0.57 \mu\text{mol/L}$ se han propuesto como los valores extremos para los atletas que no han cumplido las condiciones de SSE y están gestionando la carga de entrenamiento impuesta (155). Una relación Gm/Ga de $3.58 \mu\text{mol/L}$ es un indicador de SSE. Este nivel puede utilizarse como guía para sugerir que el sobreesfuerzo, que lleva al SSE, puede manifestarse de forma temprana (155). En este caso, es necesario realizar pruebas en serie de Gm y Ga comenzando antes de la temporada de entrenamiento.

Además, se ha visto que los atletas que tienen una menor tolerancia al volumen o a la intensidad de la carga de entrenamiento pueden ser identificados por relaciones en Gm/Ga relativamente más bajas de aproximadamente una desviación estándar ($5.04 \mu\text{mol/L}$) del valor medio de entrenamiento en reposo o de principios de temporada (155). Asimismo, una reducción del Lac máximo comparado a un mantenimiento de este en atletas adaptados a un programa de entrenamiento duro, sugiere un SSE. A este respecto, Snyder et al. (156) sugieren que uno de los criterios más sensibles y fáciles de medir el exceso de entrenamiento es la relación entre el Lac sanguíneo y la percepción del esfuerzo percibido (RPE) (Lac : RPE). Cuando la concentración de Lac en sangre

(medida en mmol/L) se reduce y el RPE (utilizando una escala de 10) permanece inalterado, la relación de ambos (multiplicada por 100) se reducirá a probablemente <100 (aquí la concentración de Lac en sangre es menor que el RPE). Tal ratio sugiere un sobreesfuerzo/sobreentrenamiento.

Los ciclistas que experimentan "piernas pesadas" mientras ruedan están expresando un síntoma común de SSE. Los grandes volúmenes de entrenamiento, la inflamación sistémica y los niveles elevados de citoquinas proinflamatorias, directa y/o indirectamente, inducen a la anorexia, lo que resulta en la reducción de la ingesta calórica (1). Además, la lesión local de la membrana muscular y la reducción de la disponibilidad de los transportadores de glucosa GLUT-4 en la membrana de las células musculares, atenúa el movimiento de la glucosa hacia la célula para la resíntesis del glucógeno. Ambos factores pueden contribuir a la reducción de la síntesis de glucógeno muscular en la SSE. Además, la reducción del glucógeno muscular podría explicar a su vez las "piernas pesadas". Asimismo, la reducción de los niveles de Lac en sangre durante el ejercicio submáximo y máximo podría ser consecuencia de la reducción del glucógeno muscular (1).

El tratamiento de la SSE incluye la determinación y eliminación de aquellos factores, tanto en la vida diaria como en el entrenamiento, que conducen a un SSE evidente. El sueño regular y el descanso, son también factores muy importante (1). Debe garantizarse una cantidad y calidad adecuada a nivel nutricional, independientemente de la pérdida de apetito relacionada con el SSE.

2.1.2. Factores biomecánicos que influyen en el rendimiento de ciclistas de carretera

2.1.2.1. Biomecánica

La biomecánica es una materia que estudia el movimiento del cuerpo humano en los diferentes deportes. en el ciclismo comprende el estudio de la posición en la bicicleta del ciclista que puede influir en el rendimiento y las lesiones (157). Diferentes tecnologías son utilizadas para evaluar la biomecánica de los ciclistas, entre ellas se encuentran sensores de fuerza, dispositivos de captura de movimiento y el registro electromiográfico que mide las fuerzas ejercidas en el pedal, el sillín y el manillar, así como los pares articulares creados por la actividad muscular (157).

Aunque estas técnicas permiten obtener un análisis biomecánico detallado de los movimientos del ciclismo, el rendimiento en este deporte sigue siendo difícil de explicar en su totalidad. La mayor parte de las dificultades para comprender la relación entre la biomecánica y el rendimiento se deben a las limitaciones impuestas por la bicicleta, la fisiología humana y el sistema músculo-esquelético (157).

Una bicicleta puede considerarse como un dispositivo que convierte el trabajo producido en el pedal por el ciclista en energía cinética. En cada instante, la potencia del cigüeñal representa la potencia desarrollada por los dos pedales que se calcula para cada pedal utilizando el producto escalar (producto punto) entre la fuerza vectorial aplicada en el pedal (N) y la velocidad del pedal (m/s) (157).

La eficacia del patrón de aplicación de la fuerza se evalúa mediante el análisis de las fuerzas del pedal (158). Una medida clásica es la relación entre la fuerza ortogonal y la fuerza total aplicada sobre el pedal, denominada eficacia mecánica. Los ciclistas profesionales tienen una mejor técnica de pedaleo (es decir, una mayor proporción de impulso positivo) que los ciclistas de élite y de club cuando pedaleaban a una potencia ≥ 200 W (158).

Además, se ha observado que al cambiar la posición del asiento (por ejemplo, el ángulo del tubo del asiento, la altura del asiento), el ángulo del tronco o la longitud de la biela, la configuración del segmento cambia, haciendo que los músculos de las extremidades inferiores trabajen en diferentes porciones de la curva de fuerza-longitud (159). Se sabe que la posición afecta al rendimiento del ciclismo, lo que sugiere que las características de fuerza-longitud también influyen significativamente en el rendimiento del pedaleo (159). Por ejemplo, una posición vertical del tronco parece ser una ventaja en términos de par máximo de biela y eficiencia global, aunque su ventaja disminuye con el aumento de la velocidad en el campo, debido a la resistencia aerodinámica (160). La posición óptima también puede determinarse como aquella que minimiza una determinada función del coste energético basada en el VO_2 , el momento articular o las medidas de EMG (161, 162).

Además, se ha demostrado la influencia de algunos factores biomecánicos como la aerodinámica en el rendimiento ciclista (163). En este sentido, teniendo en cuenta que el componente de resistencia al aire durante el ciclismo es proporcional a la velocidad³ (v^3); por consiguiente, es el principal factor de coste energético a altas velocidades. Esta resistencia aerodinámica representa más del 90% de la resistencia total que encuentra el ciclista a velocidades superiores a 30 km/h (164). A velocidades superiores a 50 km/h, la resistencia aerodinámica es la variable que más determina el rendimiento (164, 165).

Sin embargo, cuando el ciclista dobla los codos y se agacha con el torso casi paralelo al suelo, la resistencia al viento se reduce en un 20%. En la posición de descenso, las manos en el centro de la parte superior del manillar, la barbilla apoyada en las manos y las bielas paralela al suelo, la resistencia al viento se reduce en un 28% aproximadamente (166). La resistencia al viento es responsable de la mayor parte del coste metabólico del ciclismo (80 a 90%) (167). Kyle y Burke (168) sugieren 4 métodos eficaces para disminuir la resistencia al viento del ciclismo: (a) reducir el área frontal

del ciclista y la bicicleta; (b) mejorar el flujo de aire alrededor de la zapatilla eliminando las correas y las pinzas de los dedos; (c) reducir la turbulencia del aire causada por la bicicleta, con tubos aerodinámicos que reaccionan mejor con el viento cruzado que los tubos estándar. Las ruedas más pequeñas, menos radios, neumáticos más estrechos, bujes estrechos llantas aerodinámicas, ruedas cubiertas y radios aerodinámicos reducen la resistencia aerodinámica; y (d) la racionalización de los accesorios, por ejemplo, una botella de agua con ineficientemente aerodinámica añade una mayor resistencia a la bicicleta a 48 km/h). Además, la ropa también puede producir un incremento de la resistencia, por ejemplo, un traje completo de una pieza de material "Lycra Spandex" con una aerocapucha reducirá la resistencia global al viento en un 11%. Otro ejemplo, es el uso o no del casco. Cubrir el pelo largo con un casco aerodinámico reduce la resistencia en un 7% y puede mejorar hasta 1 min en una ITT de 40 km (169). Se ha estimado que el ciclista Lemond pudo reducir su ITT ganadora del Tour de Francia de 1989 en al menos 10 segundos llevando un casco aerodinámico. Por otro lado, se ha mostrado que un casco con contornos suaves, en lugar de cavidades y bordes afilados, puede reducir la resistencia al aire del 10 al 15% (170).

Por último, y en relación al efecto del entrenamiento sobre algunas variables mecánicas, se ha observado que 25 semanas de entrenamiento combinado de fuerza y resistencia condujo a una aparición más temprana del pico de torque durante el golpe de pedal, mientras que el entrenamiento de resistencia solamente no lo hizo (51). Esto podría ser debido a que el pico torque aparece antes en la producción de potencia submáxima estandarizada permitiendo un mayor flujo sanguíneo en los músculos de trabajo. Al acortar el marco de tiempo con una producción de fuerza relativamente alta durante cada golpe de pedal, aumentando así la duración de la fase de relajación favorable al flujo sanguíneo (51). Actualmente, no existe evidencia de que la ingesta de un suplemento o pauta nutricional mejore alguno de los factores que componen la biomecánica.

Tras finalizar con los factores fisiológicos y mecánicos que influyen en el rendimiento de ciclistas, ahora se va a abordar otro punto importante, en el que se explicaran los diferentes tipos de resistencia, así como su relación con los diferentes factores fisiológicos.

2.2. LA RESISTENCIA

Actualmente, el concepto de resistencia hace referencia a esfuerzos con duraciones amplias que van desde los 20 segundos hasta 6 horas o más. Pero la mayoría de definiciones establecen un concepto en común y este es la capacidad psicofísica del deportista para resistir a la fatiga (171-174). Por lo tanto, el principal factor que limita el rendimiento de un deportista en modalidades de resistencia es la

fatiga. Pero hay que tener en cuenta, que la resistencia depende de muchos factores tales como la velocidad, la fuerza muscular, las capacidades técnicas de ejecución de un movimiento eficiente, la capacidad para utilizar económicamente los potenciales funcionales, el estado psicológico cuando se ejecuta el trabajo y otros factores más (175). Independientemente del tipo de deporte practicado, tiene que existir una fortaleza psicológica, interpretada como una motivación para soportar el dolor y la incomodidad, ya que, aquellos deportistas capaces de dominar este aspecto pueden llegar a tener una ventaja sobre sus rivales (175).

Desde un punto de vista fisiológico, la resistencia se determina por la relación entre la magnitud de las reservas energéticas accesibles para su utilización y la velocidad de consumo de la energía durante la práctica deportiva (176), lo que deriva la siguiente ecuación:

$$\text{Resistencia} = \text{reservas energéticas (J)} / \text{velocidad de consumo energético (J/min)}$$

Pero también se puede definir como un proceso complejo de adaptación morfofuncional producido a nivel celular en los músculos esqueléticos que intervienen en la actividad física desarrollada. Por lo tanto, consideramos la resistencia como la capacidad para soportar la fatiga frente a esfuerzos prolongados y/o recuperarse más rápidamente después de los esfuerzos (175).

2.2.1. Tipos de resistencia

La resistencia se clasifica según diferentes criterios como pueden ser, el volumen de la musculatura implicada (resistencia general o local), la especificidad de la modalidad deportiva (resistencia base o general y resistencia específica), en función de la obtención de energía muscular (resistencia aeróbica y anaeróbica), según la duración del esfuerzo (resistencia de corta, media y larga duración) y en base a las capacidades físicas (resistencia a la fuerza, a la fuerza explosiva y a la velocidad) (Tabla 1) (175). Debido a la gran diversidad de tipos de resistencia y atendiendo a un punto de vista práctico y metodológico, vamos a diversificar los tipos de resistencia diferenciando 2 formas fundamentales de ésta que se dan en cualquier modalidad deportiva: la resistencia básica y la específica, basándonos en el adaptado de Zintl et al. (171).

Tabla 1. Formas y tipos de resistencia desde un punto de vista metodológico. Adaptado de Zintl et al. (171).

Formas	Características	Tipos
RESISTENCIA BÁSICA	Carácter básico para desarrollar otras capacidades	<p>a) Resistencia de base I</p> <p>Resistencia básica independiente de la modalidad deportiva (ejercicios generales)</p> <p>b) Resistencia de base II</p> <p>Resistencia básica relacionada con la modalidad deportiva (ejercicios específicos)</p> <p>c) Resistencia de base III acíclica</p> <p>Resistencia en deportes colectivos/combate con cambios acíclicos de la carga</p>
RESISTENCIA ESPECÍFICA	Enfocada en la estructura de carga específica de cada modalidad, relación óptima entre intensidad y duración de la carga	<p>d) Resistencia de corta duración (35 s – 2 min)</p> <p>e) Resistencia de mediana duración (2 – 10 min) <i>resistencia de velocidad y fuerza</i></p> <p>f) Resistencia de larga duración I (10 – 35 min)</p> <p>g) Resistencia de larga duración II (35 – 90 min)</p> <p>h) Resistencia de larga duración III (90 – 6 h)</p> <p>i) Resistencia de larga duración IV (> 6h)</p>

Cuando hacemos referencia a la resistencia aeróbica nos remitimos a la capacidad de resistencia a la fatiga durante las actividades en las que la resíntesis de ATP se genera, principalmente, mediante el metabolismo aeróbico. Sin embargo, hay que mencionar, que las causas de la fatiga durante el ejercicio prolongado pueden ser debido a varios factores, los de mayor importancia son: la acumulación de productos de desecho del metabolismo oxidativo intenso y prolongado que puede limitar el rendimiento como es la acumulación protones de hidrógeno (acidosis metabólica) y el calor (177).

Dentro del ámbito deportivo, la planificación del entrenamiento para desarrollar la resistencia aeróbica tendrá como objetivo generar adaptaciones para retrasar la aparición de la fatiga durante la competición, aumenta la capacidad de soportar cargas de trabajo de los entrenamientos, disminuir el tiempo de recuperación entre esfuerzos y estabilizar la técnica deportiva. Además, una mejora de la resistencia aeróbica puede retrasar la aparición de la fatiga en ejercicio repetido de alta intensidad, por restablecimiento de forma rápida de las concentraciones de fosfocreatina (PC) y ATP durante el descanso, obteniendo como resultado mayor rendimiento en periodos de ejercicio subsiguientes al inicial (178). Esto va en línea a lo encontrado en un estudio donde se ha utilizado la resonancia magnética como instrumento de medición, encontrando una relación entre el nivel de resistencia aeróbica y la recuperación de los niveles de PC tras esfuerzos de moderada y alta intensidad (179). En la Tabla 2, aparecen de forma específica las características de los distintos tipos de resistencia que existen, según el adaptado de Navarro (175).

Tabla 2. Resumen de las características de los tipos específicos de resistencia dinámica en función del tiempo de esfuerzo, intensidad de carga y vías energéticas utilizadas. Adaptado de Navarro (174).

	RCD	RMD	RLD			
			I	II	III	IV
Duración de la carga (min)	35 s-2 min	2-10 min	10-35 min	35-90 min	90 min - 6h	>6h
Intensidad de la carga	Máxima	Máxima	Submáxima	Submáxima	Mediana	Ligera
PPM	185-200	190-210	180-190	175-190	150-180	120-170
%VO₂MAX	100	100-95	90-95	80-95	60-90	50-60
Lactato (mmol/l)	10-18	12-20	10-14	6-8	4-5	>3
Consumo energético						
Kcal	60	45	28	25	20	18
Kj/min	250	190	120	105	80	75
Kj total	380-460	445-1680	1680-3150	3150-9660	9660-27000	>27000
Vía energética	Predomino/anaeróbico	Aeróbica/anaeróbica	Predominio aeróbico has totalmente aeróbica			

Anaeróbica/aeróbica	65:35 45:55	50:50 20:80	15:85	5:95	2:98	1:99
Aláctico (%)	15-30	0-5	0	0	0	0
Láctico (%)	50	40-55	20-30	5-10	<5	<1
Aeróbica (HC) (%)	20-35	40-60	60-70	70-75	60-50	<40
Aeróbica (grasas) (%)	0	0	10	20	40-50	>60
Degradación del glucógeno muscular (%)	10	20	40	60	80	95
Lipólisis, ácidos grasos (mmol/l)	0.50	0.50	0.80	1.0	2.0	2.5
Glicólisis, lactato (mmol/l)	18	20	14	8	4	2
Proteólisis, alanina (mmol/l)	500	500	400	350	250	200
Urea (mmol/l)	0	1	1-2	2-3	3-6	4-8
Cortisol (µmol/l)	400	400	350	300	400	500
Sustrato energético	Glucógeno y fosfatos	Glucógeno	Glucógeno	Glucógeno	Grasas +	Grasas,

Sustrato energético principal α	Glucógeno y fosfatos α	Glucógeno muscular α	Glucógeno muscular y hepático α	Glucógeno muscular y hepático α y grasas α	Grasas α y glucógeno α	Grasas, proteínas y glucógeno α
<p>RCD. = resistencia corta duración; RMD. = resistencia media duración; RLD. = resistencia larga duración; PPM. = pulsaciones por minuto; %VO_{2MAX} = porcentaje del consumo máximo de oxígeno.α</p>						

2.3. EFECTOS DEL EJERCICIO DE RESISTENCIA EN EL ESTRÉS OXIDATIVO

2.3.1. Concepto de estrés oxidativo

En 1985 se definió por primera vez el término estrés oxidativo como "una alteración del equilibrio del estado oxidante/antioxidante a favor del primero" (180). Aunque esta definición se ha utilizado ampliamente durante más de dos décadas, es probable que dicha definición de estrés oxidativo sea modificada en el futuro, debido a la complejidad asociada a la evaluación del equilibrio redox celular. De hecho, se ha argumentado que el término estrés oxidativo desafía una simple definición de pro-oxidante frente a antioxidante y que la descripción de un "estrés oxidativo" sólo es útil si se conocen los detalles moleculares del desequilibrio (181). En un esfuerzo por refinar el significado del estrés oxidativo, Dean Jones (181) ha propuesto que este término se redefina como una alteración de la señalización y el control redox. Independientemente de que esta nueva definición obtenga una aceptación generalizada, cabe anticipar que la descripción del estrés oxidativo sufrirá futuras modificaciones a medida que avance el campo de la biología redox.

Independientemente de cómo se defina el estrés oxidativo, un entorno pro-oxidante persistente en las células puede modificar las moléculas sensibles al redox. Un enfoque común para evaluar el estrés oxidativo en los sistemas biológicos implica la medición del aumento o la disminución de una molécula sensible al redox que responde al estrés oxidativo. En general, los marcadores fiables del estrés oxidativo poseen las siguientes cualidades: 1) son químicamente únicos y detectables, 2) aumentan o disminuyen durante periodos de estrés oxidativo, 3) poseen vidas medias relativamente largas y 4) no se ven afectados por otros procesos celulares (por ejemplo, el ciclo celular, el metabolismo energético, etc.) (182).

Durante los periodos de estrés oxidativo, los pro-oxidantes sobrepasan las defensas antioxidantes de las células y dañan los diferentes componentes celulares. Así, el estrés oxidativo en los sistemas biológicos suele caracterizarse por la siguiente secuencia: 1) aumento de la formación de radicales y otros oxidantes, 2) disminución de los antioxidantes de pequeño peso molecular y/o solubles en lípidos, 3) alteración del equilibrio redox celular, y 4) daño oxidativo a los componentes celulares (es decir, lípidos, proteínas y/o ADN). Por lo tanto, los biomarcadores del estrés oxidativo suelen pertenecer a una de las 4 categorías. La primera categoría de biomarcadores implica la detección de oxidantes. Desgraciadamente, la medición directa de la producción de radicales en células vivas es difícil porque los radicales son altamente reactivos y tienen una vida media corta. Por ello, para medir la producción de oxidantes en las células se suelen utilizar moléculas exógenas, como sondas fluorescentes o trampas de espín.

Cuando se añade a un sistema biológico, la sonda o trampa de espín se convierte en un producto radical modificado único con una vida media relativamente larga que puede cuantificarse como una medida de la producción de oxidantes (138, 163). No obstante, dado que un aumento de la producción de oxidantes no define necesariamente una condición pro-oxidante, las medidas de aumento de la producción de oxidantes por sí solas no son marcadores definitivos del estrés oxidativo. Además, una desventaja potencial del uso de sondas o trampas de espín es que estas moléculas pueden perturbar el sistema biológico investigado (136). Otro inconveniente de este enfoque es que muchas trampas de espín y sondas fluorescentes son tóxicas para las células (136).

2.3.2. Fuentes de oxidantes en los músculos en ejercicio

Desde el descubrimiento del efecto oxidativo inducido por el ejercicio físico, algunos investigadores han estudiado las posibles fuentes de producción de especies reactivas de oxígeno (ROS) en varios tejidos. Aunque los oxidantes podrían producirse en una variedad de tejidos durante el ejercicio, se ha establecido que el músculo esquelético es la fuente dominante de producción de ROS durante el mismo (183). Las posibles fuentes de producción de ROS inducidas por el ejercicio a nivel muscular han sido ampliamente investigadas e incluyen las siguientes: mitocondrias, fosfolipasa A2 (PLA2), y nicotinamida-adenina-dinucleótido-fosfato oxidasas 2 y 4 (NOX2 y NOX4) localizadas en 4 sitios dentro de las fibras musculares: mitocondrias, sarcolema, retículo sarcoplásmico y túbulos T (Figura 1) (183-187).

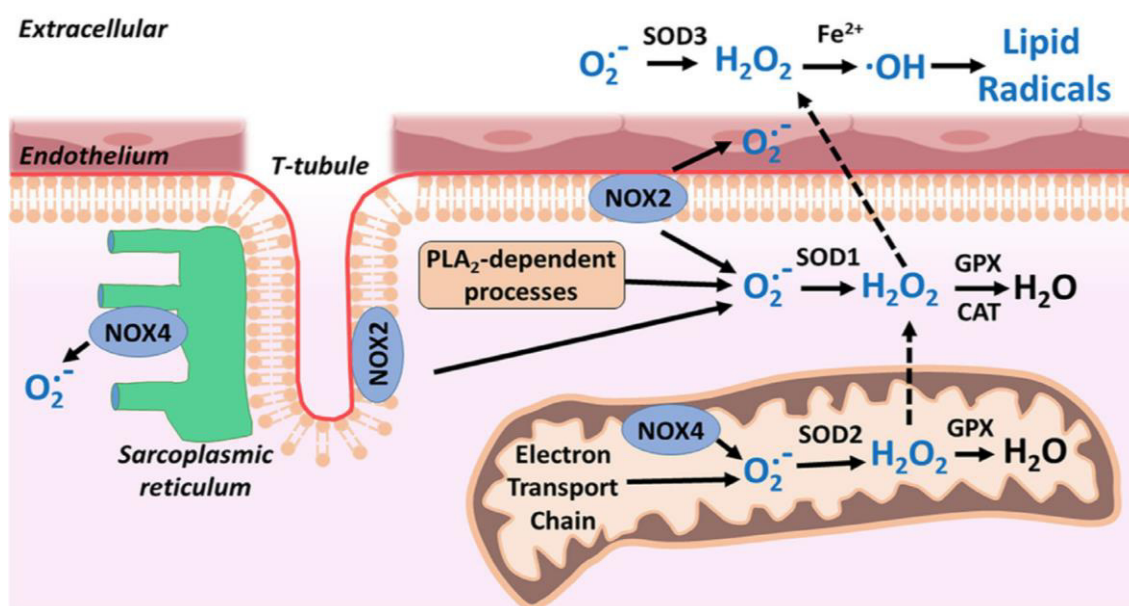


Figura 1. Sitios potenciales de producción de especies reactivas de oxígeno (ROS) en los músculos esqueléticos en contracción. Modificada de Powers et al. (188). CAT = catalasa; GPX

= glutatión peroxidasa; H_2O_2 = peróxido de hidrógeno; NOX = NADPH oxidasa; $O_2^{\bullet-}$ = superóxido; OH^{\bullet} = radical hidroxilo; PLA2 = fosfolipasa A2; SOD = superóxido dismutasa.

Aunque los primeros estudios propusieron que las mitocondrias son la fuente probable de producción de ROS en las fibras musculares durante el ejercicio, esta predicción no parece ser del todo exacta, dado que la producción de ROS en las mitocondrias del músculo esquelético disminuye durante el ejercicio (188). En concreto, se ha estimado que entre el 2% y el 5% del oxígeno molecular consumido en las mitocondrias forma $O_2^{\bullet-}$ (189). Basándose en estos hallazgos, se hipotetizó que el aumento de la fosforilación oxidativa en las mitocondrias dentro de los músculos esqueléticos en contracción daría lugar a un aumento proporcional de la producción de $O_2^{\bullet-}$. Sin embargo, los estudios actuales revelan que las mitocondrias producen menos $O_2^{\bullet-}$ durante la respiración en estado activo en comparación con la respiración en estado basal (190-192). Por lo tanto, las pruebas disponibles indican que las mitocondrias no son el principal lugar de producción de ROS en el músculo esquelético durante el ejercicio.

Esto puede ser debido a que las mitocondrias pueden consumir $O_2^{\bullet-}/H_2O_2$ producido dentro del compartimento de la matriz o el H_2O_2 que se difunde en las mitocondrias desde orígenes citosólicos. Por lo tanto, es posible que las mitocondrias consuman más ROS de las que se generan durante condiciones energéticas similares al ejercicio (193). A pesar de ello, se sabe que los niveles totales de ROS del músculo esquelético aumentan en las fibras musculares durante las contracciones *in vitro*, e *in vivo* durante el ejercicio físico en roedores como en humanos (Figura 2). Las fuentes principales de formación de ROS durante la contracción del músculo esquelético incluyen las NOX, que son enzimas especializadas en generar $O_2^{\bullet-}$ (es decir, sin otra función conocida), junto con una menor contribución de la xantina oxidasa (XO) (193). La XO está especialmente implicada en la producción de ROS durante el ejercicio anaeróbico, y se ha informado de una relación lineal entre la XO y los niveles de ácido láctico (304). Una de las razones es el hecho de que la actividad de la XO depende en gran medida de la disponibilidad de sustrato (437). Durante el ejercicio intenso, debido a la alta tasa de degradación del ATP, se forman hipoxantina y xantina de generación de AMP que sirven como sustratos para la XO, que utiliza el oxígeno molecular para generar ROS (194).

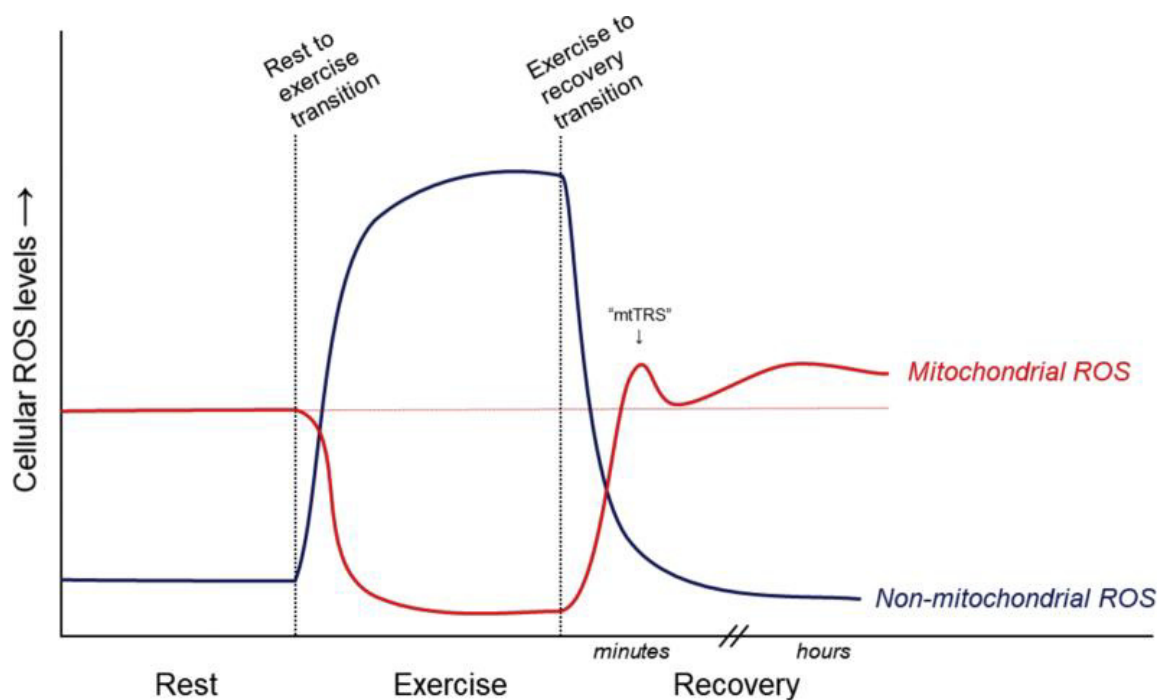


Figura 2. Propuesta de las contribuciones relativas de las fuentes mitocondriales y no mitocondriales de ROS a los niveles celulares generales de ROS en el músculo esquelético durante y en los minutos y horas siguientes a una única sesión de ejercicio de resistencia. Modificada de Mason et al. (193). mtTRS: pico de transición mitocondrial.

La PLA2 es una enzima que escinde los fosfolípidos de la membrana para liberar ácido araquidónico. Este ácido graso cuando está libre es un sustrato para varios sistemas enzimáticos generadores de ROS, incluidas las lipoxigenasas (195). Es importante destacar que la activación de la PLA2 puede activar las NADPH oxidasa (NOX) (196), y el aumento de la actividad de la PLA2 en el músculo esquelético también puede promover la producción de ROS en las mitocondrias (197) y el citosol (198). Hay que tener en cuenta, que en el músculo esquelético existen 2 isoformas de PLA2 tanto dependientes como independientes del calcio y que ambas isoformas son capaces de estimular la generación de ROS en el músculo (197, 198). La hipótesis es que las enzimas independientes del calcio regulan la actividad oxidante citosólica de las células del músculo esquelético en condiciones de reposo (198), mientras que la isoforma de PLA2 dependiente del calcio estimula la generación de ROS mitocondrial durante la actividad contráctil (199). No obstante, se requieren investigaciones adicionales para confirmar este postulado.

El músculo esquelético expresa 2 isoformas de NADPH oxidasa (NOX2 y NOX4) (200). La NOX2 se localiza en el sarcolema y en el túbulo T, mientras que la NOX4 se localiza tanto en el retículo sarcoplásmico como en la mitocondria (201). La NOX4 es

constitutivamente activa y no requiere la asociación con subunidades reguladoras (200). Por el contrario, NOX2 se activa mediante agonistas específicos (por ejemplo, angiotensina II, estrés mecánico/contráctil y citoquinas). Por lo tanto, parece probable que NOX4 contribuya a la tasa basal de producción de ROS en las fibras musculares, mientras que NOX2 es la principal fuente de producción de ROS mediada por la NADPH oxidasa en el músculo en contracción (200). De hecho, varios estudios recientes señalan a la NOX como principal contribuyente a la producción de ROS inducida por la contracción (186, 202, 203). Por ejemplo, los investigadores que utilizaron diversas técnicas experimentales para examinar los sitios subcelulares responsables de la producción de $O_2^{\cdot-}$ en las fibras musculares concluyeron que las mitocondrias no son responsables de la producción de $O_2^{\cdot-}$ inducida por la contracción en las fibras musculares; en cambio, concluyeron que la NOX es una fuente importante de producción de $O_2^{\cdot-}$ tanto en reposo como durante las contracciones (186). Otros autores también han llegado a conclusiones similares (202, 203). No obstante, en la actualidad, concluir que la NOX es la fuente dominante de ROS en el músculo esquelético en contracción es complicado por las complejidades asociadas al estudio de la actividad de la NOX en las células. Está claro que se necesitan metodologías mejoradas y estudios adicionales para aclarar esta cuestión.

2.3.3. Impacto a nivel celular de la producción de oxidantes inducida mediante el ejercicio en las fibras musculares

Existe evidencia que las ROS se producen continuamente en el músculo esquelético tanto en reposo como durante el ejercicio; además, las ROS modulan una serie de procesos fisiológicos, como la regulación del flujo sanguíneo, la producción de fuerza muscular y la adaptación del músculo al entrenamiento (204). Las consecuencias de la producción de ROS inducida por el ejercicio de resistencia en el músculo esquelético es debido a varios factores, pero nosotros nos vamos a centrar en 3: 1) el estrés oxidativo inducido por el ejercicio, 2) el impacto de las ROS en la producción de fuerza muscular y 3) la influencia de las ROS en la adaptación del músculo al entrenamiento.

2.3.3.1. Estrés oxidativo inducido por el ejercicio

Aunque el ejercicio de corta duración (<1 minuto) y de baja intensidad (~30% VO_{2MAX}) no parece promover el estrés oxidativo, está bien establecido que ejercicios agudos de larga duración y de alta intensidad, provocan un aumento de sus biomarcadores de estrés oxidativo (p. ej., aumento de la oxidación de proteínas y de la peroxidación de lípidos) tanto en la sangre como en los músculos esqueléticos activos (205, 206). Sin embargo, el entrenamiento de resistencia a corto plazo (5 días consecutivos) y a largo plazo (12 semanas) ha demostrado aumentar la actividad de las

enzimas antioxidantes en los músculos entrenados y eliminar el estrés oxidativo inducido por la contracción debida a una sesión aguda de ejercicio (207, 208). Por otro lado, un metaanálisis reciente concluye que el daño al ADN se produce en los glóbulos blancos inmediatamente después de un ejercicio agudo de resistencia persiste hasta 24 horas (209). Sin embargo, este daño al ADN inducido por el ejercicio no es detectable varios días después del ejercicio; esto es probablemente atribuible al aumento de los mecanismos de regulación de reparación del ADN inducidos por el ejercicio (209).

2.3.3.2. Efectos de las ROS en la producción de fuerza muscular

Se ha demostrado que el impacto de las ROS en la producción de fuerza muscular es bifásico y depende del nivel de ROS dentro de la fibra muscular. Una vez más, la molécula madre de la cascada de las ROS es el radical superóxido que se convierte en H_2O_2 , y existe la probabilidad que tanto el $O_2^{\bullet-}$ como el H_2O_2 influyan en la función contráctil del músculo (210). En reposo, el $O_2^{\bullet-}$ se produce a bajas tasas en las fibras musculares esqueléticas, mientras que, durante el ejercicio, la tasa de producción de $O_2^{\bullet-}$ en el músculo aumenta notablemente. La cantidad de producción total de $O_2^{\bullet-}$ en la fibra muscular depende tanto de la intensidad y la duración del ejercicio como de la temperatura del músculo que se contrae. En general, el ejercicio aeróbico prolongado de intensidad moderada (65%-75% del VO_{2MAX}) da lugar a una mayor producción de ROS en comparación con el ejercicio de baja intensidad y de corta duración. Además, el aumento de la temperatura muscular da lugar a mayores niveles de ROS durante las contracciones (211).

Reid et al. (210, 212-214) publicaron una serie de estudios que demostraban que las ROS tienen una influencia bifásica en la producción de fuerza del músculo esquelético (Figura. 3). Sus trabajos revelan que, en el músculo no fatigado, se requiere un nivel óptimo de ROS para que las fibras musculares generen el 100% de su producción de fuerza isométrica máxima. Por ejemplo, este estudio revela que la eliminación selectiva de $O_2^{\bullet-}$ o H_2O_2 de la fibra muscular utilizando superóxido dismutasa (SOD) o catalasa (CAT), respectivamente, resulta en una disminución de la producción de fuerza máxima muscular. A la inversa, el aumento de los niveles de ROS en la fibra por encima del punto óptimo da lugar a una disminución de la capacidad de los músculos para generar fuerza (215). El hecho de que la administración del antioxidante N-acetilcisteína retrase la tasa de fatiga muscular durante el ejercicio prolongado proporciona más apoyo al concepto de que los niveles elevados de ROS perjudican la producción de fuerza muscular máxima (216-218).

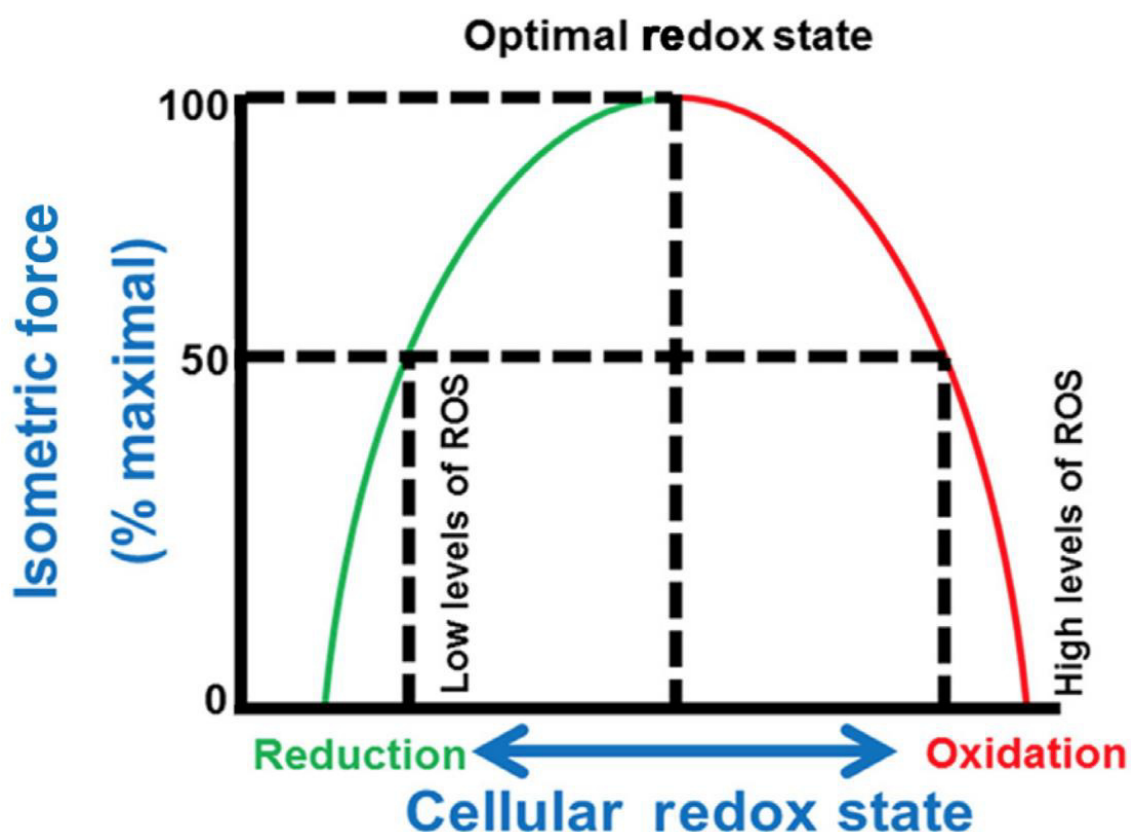


Figura 3. Relación entre el estado redox celular y la producción de fuerza del músculo esquelético. Obsérvese que la producción máxima de fuerza en el músculo esquelético requiere un estado redox óptimo. El alejamiento del estado redox óptimo (es decir, un aumento de la reducción o de la oxidación) da lugar a una disminución de la producción de fuerza isométrica máxima. Modificada de Reid et al. (210). ROS = especies reactivas de oxígeno.

Los mecanismos responsables de que la producción de fuerza muscular puede aumentar o disminuir en respuesta a las alteraciones redox, actualmente no tiene una respuesta definitiva. No obstante, es posible que los cambios en los niveles de calcio libre en el músculo y la sensibilidad miofibrilar al calcio contribuyan al impacto redox en la producción de fuerza muscular. Además, es posible que una disminución de la actividad de la bomba de Na^+/K^+ mediada por las ROS también contribuya a la disminución de la producción de fuerza muscular que se produce durante el ejercicio de resistencia prolongado (204). En relación con el papel que desempeña la sensibilidad al calcio en la producción de fuerza muscular, estudios bien controlados realizados en fibras musculares individuales confirman que los niveles elevados de oxidantes (es decir, H_2O_2) disminuyen la sensibilidad miofibrilar al calcio, lo que da lugar a una disminución de la producción de fuerza muscular a cualquier nivel dado de calcio libre en la fibra (219, 220). Esta observación, explica en parte por qué los

niveles elevados de oxidantes disminuyen la producción de fuerza muscular. En cambio, el impacto de los niveles elevados de oxidantes en los niveles citosólicos de calcio libre durante la contracción muscular está menos claro. Específicamente, aunque está establecido que los canales de liberación de calcio en el retículo sarcoplásmico (es decir, los receptores de rianodina) son sensibles al redox (221, 222), el impacto preciso de la modulación redox en estos canales sigue sin estar bien definido. Por ejemplo, existen pruebas tanto a favor como en contra de la noción de que los niveles elevados de oxidantes interrumpen la liberación de calcio del retículo sarcoplásmico (220, 223). La explicación de esta discrepancia experimental no está clara, pero puede deberse a las diferencias en las condiciones experimentales de numerosos estudios (por ejemplo, temperatura del músculo, niveles de oxidantes). No obstante, en conjunto, las pruebas experimentales indican que los niveles elevados de oxidantes en el músculo esquelético asociados al ejercicio prolongado son capaces de dañar una o más proteínas implicadas en el acoplamiento excitación-contracción, lo que da lugar a una reducción de la producción de fuerza muscular (fatiga) (204).

Por último, es posible que una disminución de la actividad de la bomba de Na^+/K^+ mediada por las ROS también contribuya a la reducción de la capacidad de producción de fuerza muscular que se produce durante el ejercicio de resistencia prolongado (224). Esto es debido a que el ejercicio muscular provoca a nivel intracelular una pérdida de K^+ y un aumento del Na^+ a pesar de la disminución de la actividad de la bomba de Na^+/K^+ (224). Esta disminución del K^+ intracelular y la reducción del gradiente trans-sarcolemal de Na^+ deterioran la excitabilidad de la membrana y, por lo tanto, disminuyen la producción de fuerza muscular (224). Las pruebas experimentales que apoyan el concepto de que la disminución de la actividad de la bomba de Na^+/K^+ mediada por las ROS contribuye a la fatiga muscular derivan de los experimentos en humanos que confirman que la N-acetilcisteína (antioxidante) atenúa la fatiga muscular inducida por el ejercicio, en parte por la mejora de la regulación de los niveles de K^+ intracelular (224).

2.3.3.3. Efectos de las ROS en las adaptaciones musculares al entrenamiento

Como se ha comentado anteriormente, es bien conocido que el aumento de oxidantes inducido por el ejercicio contribuye a la fatiga muscular. Sin embargo, la producción de ROS en el músculo esquelético durante el ejercicio de resistencia prolongado también desempeña un papel importante en las vías de señalización celular implicadas en la adaptación muscular al ejercicio (204). De hecho, tanto los estudios en humanos como en animales demuestran que la prevención de la señalización redox (principalmente se usó vitamina C y E) inducida por el ejercicio

atenúa las adaptaciones (\downarrow biogénesis mitocondrial) inducidas por el entrenamiento en las fibras musculares (225-227).

El músculo esquelético es un tejido muy plástico que experimenta cambios fenotípicos considerables en respuesta al entrenamiento de resistencia. En tan sólo 5-10 días consecutivos de ejercicio de resistencia, se ha comprobado que pueden dar lugar a incrementos sustanciales en la capacidad oxidativa y antioxidante de las fibras musculares esqueléticas (208, 228). En los últimos años, nuestra comprensión de los mecanismos de señalización responsables de las adaptaciones a nivel muscular ha aumentado notablemente. Es importante destacar que muchas de estas vías de señalización celular se inician, o al menos se potencian, mediante señales redox. De hecho, las vías sensibles al redox dan lugar a cambios en la actividad de los factores de transcripción, aumentando o disminuyendo la transcripción de los genes diana (Figura. 4).

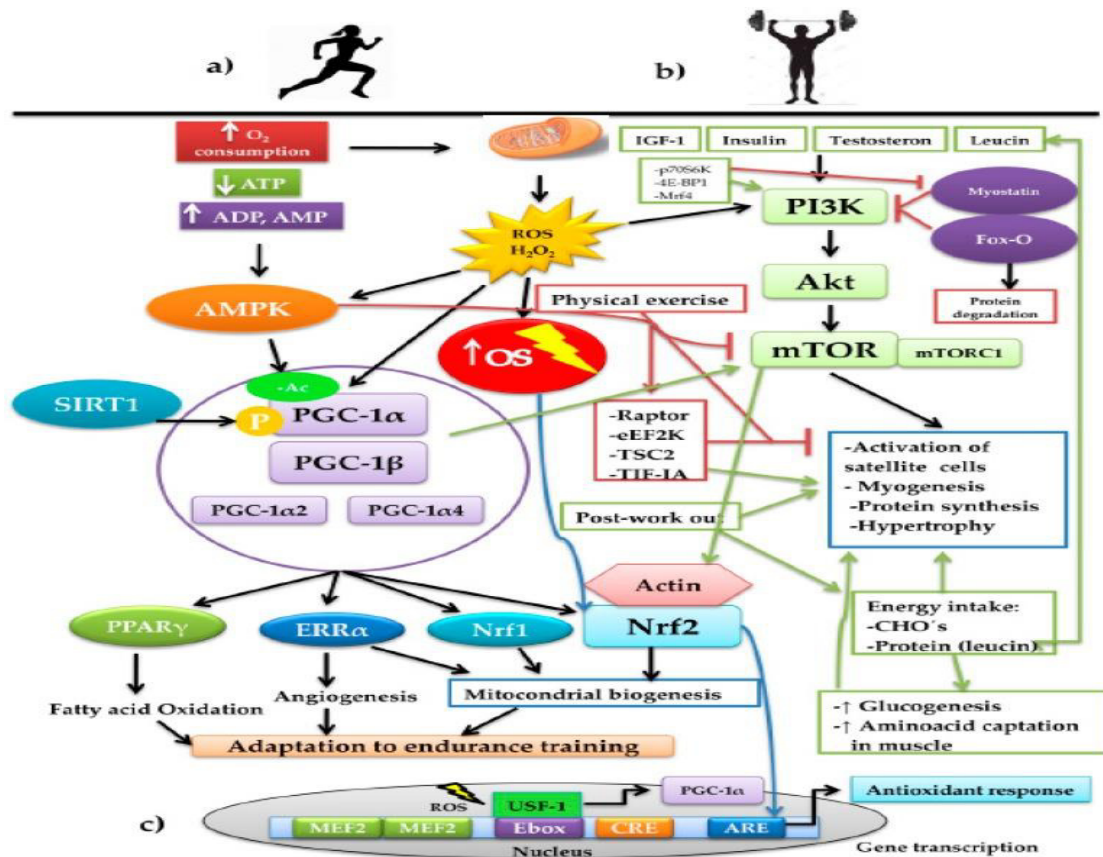


Figura 4. Representación esquemática de la interconexión de las vías de señalización implicadas en el entrenamiento de la resistencia aeróbica y de la fuerza muscular. Modificada de Vargas-Mendoza et al. (229).

Las adaptaciones generadas por los deportes de resistencia aeróbica aumentan el consumo de oxígeno (consumo de O₂). Debido a la demanda energética, se activan las vías de producción de ATP, aumentando la producción mitocondrial de ROS, básicamente H₂O₂, aumentando el SO, con la consiguiente disminución del ATP disponible. El aumento de ADP y AMP activan el sensor energético AMPK que, junto con SIRT1, activa el PGC-1 α . Estos, al interactuar con otros factores de transcripción, como PPAR γ , ERR α , factor respiratorio nuclear 1 y 2 (NRF1 y NRF2), inducen adaptaciones al entrenamiento de duración prolongada mediante el desarrollo de mecanismos, como la oxidación de ácidos grasos, la formación de vasos sanguíneos y la biogénesis mitocondrial (229). (b) El entrenamiento de fuerza más otros factores como el IGF-1, la insulina, la testosterona y la leucina estimulan la vía anabolizante PI3K/Akt/mTOR. Durante el trabajo físico, esta vía es regulada negativamente por la AMPK a través de los mecanismos ejecutados por Raptor, eEF2K, TSC2, y TIF-IA; al finalizar el ejercicio, los mecanismos se revierten, estimulando la síntesis proteica y la fibrogénesis muscular (229). La vía se ve reforzada por la reposición energética post-entrenamiento de carbohidratos y el aminoácido de cadena ramificada, leucina, que ejerce un efecto sobre la activación de las células satélite y la miogénesis. Encontramos inmersos, en el control de la vía, a Fox-O, un potente inductor de la degradación proteica muscular, y a la miostatina, que regula negativamente la vía. Como contrapartida, la miostatina posee los reguladores negativos p70S6K, 4E-BP1 y Mrf4 que, al inhibirla, promueven la vía PI3K/Akt/mTOR. En ambas modalidades de ejercicio converge la interacción entre los factores redox-dependientes, PGC-1 α y NRF2, produciendo las diferentes adaptaciones al entrenamiento (229). (c) Representación de la transcripción nuclear de PGC-1 α y la Ebox de unión por USF-1 mediada por ROS. Por otro lado, la activación de la vía PI3K/Akt/mTOR induce la reorganización de los filamentos de actina, despolimerizando la actina y formando un complejo con NRF2, que permite su posterior translocación al núcleo para su unión a ARE y dirigir la respuesta antioxidante (229)

A este respecto, ahora está claro que la producción de ROS inducida por el ejercicio desempeña un papel importante en la señalización inducida por el ejercicio a través del factor nuclear Kappa B (NF- κ B) y PGC-1 α en las fibras musculares esqueléticas (230, 231). Esto es importante porque tanto el NF- κ B como el PGC-1 α desempeñan un papel necesario en el aumento de los antioxidantes y la biogénesis mitocondrial mediada por el ejercicio. Además de NF- κ B y PGC-1 α , la activación redox de NRF2 desempeña un papel importante en la promoción de la expresión inducida por el ejercicio de muchos componentes clave implicados en el sistema antioxidante endógeno (229). De hecho, NRF2 controla la expresión de componentes clave de los sistemas antioxidantes de glutatión y tioredoxina, así como de las enzimas implicadas en la generación de NADPH (232) (Figura 5). En conjunto, las pruebas disponibles

indican que la producción de ROS inducida por el ejercicio es claramente un requisito para las adaptaciones del músculo esquelético inducidas por el entrenamiento de resistencia.

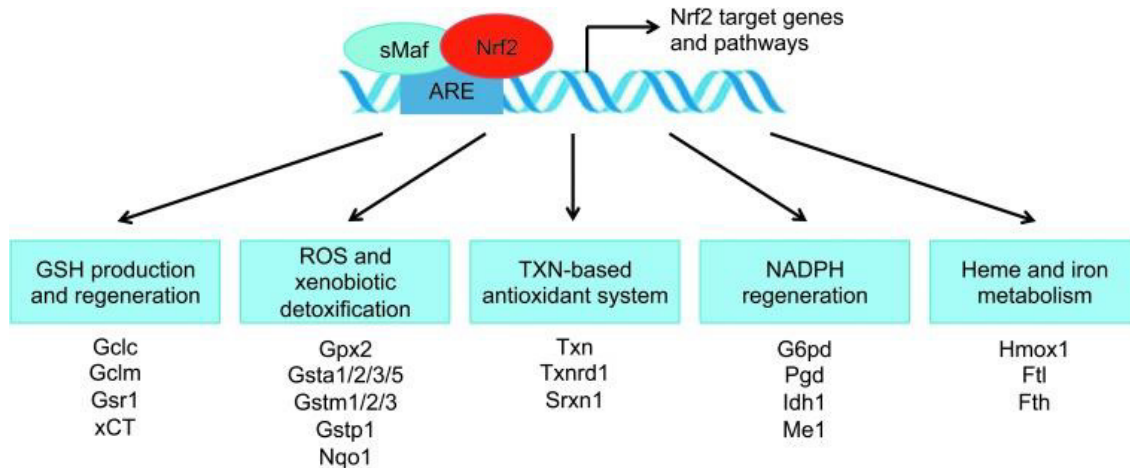


Figura 5. Sistema de defensa citoprotector regulado por NRF2. A través de la regulación coordinada de la producción, utilización y regeneración de GSH y TXN, la regeneración de NADPH, el metabolismo hemo y del hierro, las ROS y la desintoxicación de xenobióticos, NRF2 proporciona el principal sistema de defensa citoprotector de la célula. Modificada de et al. Tonelli (232). GSH, glutatión; HMOX1, hemooxigenasa 1; Idh1, isocitrato deshidrogenasa 1; NADPH, nicotinamida adenina dinucleótido fosfato; Nqo1, NADPH quinona deshidrogenasa 1; Pgd, 6-fosfogluconato deshidrogenasa; ROS, especies reactivas de oxígeno; TXN, tiorredoxina.

Además, existen pruebas de que la señalización de las ROS también está implicada en la hipertrofia inducida por el entrenamiento de resistencia (233). Por ejemplo, en un modelo de cultivo celular, el estrés oxidante inducido por el H_2O_2 puede activar la proteína quinasa B, que promueve la síntesis de proteínas en las células a través de la activación de la diana de rapamicina de los mamíferos (mTOR) (234). La activación de la mTOR estimula la síntesis de proteínas a través del aumento de la traducción del ARNm de las proteínas contráctiles. Además, las pruebas experimentales en un modelo de sobrecarga del músculo plantar revelan que la producción de peroxinitrito ($ONOO^{\bullet-}$), un producto de reacción del NO y el $O_2^{\bullet-}$, desencadena una cascada de señalización que da lugar a la activación directa de mTOR (235). Por lo tanto, parece que la producción de ROS inducida por la contracción muscular son una molécula de señalización clave en la hipertrofia muscular inducida por el entrenamiento de resistencia.

2.3.3.4. Ejercicio y mitohormesis

El hecho de que la producción de ROS inducida por el ejercicio sea perjudicial o beneficiosa para la salud depende probablemente del equilibrio entre los niveles de producción de ROS durante el ejercicio y la competencia de los sistemas antioxidantes celulares para proteger a las células contra un desafío oxidante. En este sentido, varias revisiones recientes han llegado a la conclusión de que el entrenamiento regular con ejercicio no provoca un estrés oxidativo crónico en los músculos activos (236-240). Conceptualmente, esta conclusión está respaldada por la noción de hormesis inducida por el ejercicio. El término hormesis se utiliza en biología para describir una curva bifásica dosis-respuesta en la que un aumento transitorio de los niveles bajos de un factor de estrés (p. ej, radiación, radicales) proporciona un efecto adaptativo beneficioso en las células, mientras que una dosis crónica y/o alta del estresor provoca daños en las células (Figura. 6) (241).

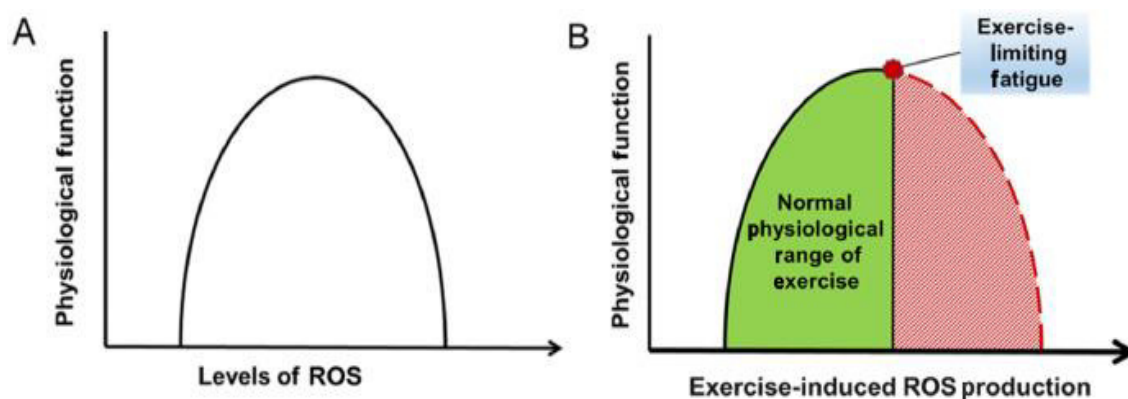


Figura 6. (A) Relación entre los niveles celulares de ROS y la función fisiológica. Esta curva bifásica en forma de campana representa la curva de hormesis de ROS. (B) Relación entre los niveles de especies reactivas de oxígeno en las fibras musculares inducidos por el ejercicio y la función fisiológica. Esta figura predice que el aumento de los niveles de ROS en las fibras musculares inducido por el entrenamiento no alcanza un nivel perjudicial debido a la fatiga inducida por el ejercicio. Modificada de Powers et al. (204). ROS = especies reactivas de oxígeno.

Aunque la investigación de la hormesis tiene una larga historia en biología, la primera descripción de la hormesis inducida por el ejercicio apareció en 2005 (242). Este informe concluyó que los niveles bajos a moderados de producción de ROS inducidos por el ejercicio desempeñan un papel esencial en la adaptación inducida por

el ejercicio del músculo esquelético. Por el contrario, los niveles elevados de producción de ROS provocan daños en el músculo y una disminución de los beneficios fisiológicos asociados a la producción de ROS de baja a moderada. Como se ha comentado anteriormente, se ha establecido que el aumento de la producción de ROS inducido por el ejercicio en el músculo esquelético desempeña un papel necesario en la adaptación del músculo esquelético al entrenamiento. La curva de hormesis en forma de campana predice que los aumentos en la producción de ROS inducidos por el ejercicio promueven beneficios fisiológicos significativos hasta que se alcanza un nivel óptimo de producción de ROS. Sin embargo, si el ejercicio produce un verdadero efecto hormético en el cuerpo, después de alcanzar este cenit de beneficio fisiológico, cualquier aumento adicional en la producción de ROS inducida por el ejercicio daría lugar a daños en los tejidos y a una disminución de las adaptaciones inducidas por el ejercicio (204).

2.4. EFECTOS DEL EJERCICIO DE RESISTENCIA EN EL SISTEMA ENDÓGENO ANTIOXIDANTE

Los FR son moléculas o fragmentos de moléculas con uno o más electrones no apareados. Estos son muy inestables y muy reactivos porque tienden a atrapar un electrón de otras moléculas (oxidación) (243-245). Los FR se producen mediante una transferencia de electrones que requiere un elevado aporte de energía (244) y su tiempo de vida es muy corto (de milisegundos a nanosegundos (Tabla 3) Al reaccionar con otros radicales o moléculas, un FR puede formar nuevos radicales (245). Entre las FR, las ROS se derivan del oxígeno, las especies reactivas de nitrógeno (RNS) derivan del nitrógeno y las especies reactivas de azufre (RSS) derivan del azufre. (Tabla 3). Estas especies pueden formarse por reacciones con las ROS o pueden aumentar la producción de ROS (243).

Tabla 3. Clasificación y principales efectos de los radicales libres

Radical libre	Abreviatura	Vida media	Efectos principales
<i>Especies reactivas de oxígeno</i>	ROS		
Anión superóxido	O ₂ ^{•-}	10 ⁻⁵ s	Oxidación y peroxidación de lípidos
Ozono	O ₃	Estable	

Oxígeno singlete	$^1\text{O}_2$	1 μs	Oxidación de proteínas
Radical hidroxilo	$\text{HO}\cdot$	10^{-9} s	Daño al ADN
Peróxido de hidrógeno	H_2O_2	Estable	
Ácido hipocloroso	HOCl	Estable	
Radical alcoxilo	$\text{RO}\cdot$	10^{-6} s	
Radical peroxilo	$\text{ROO}\cdot$	7 s	
Radical hidroperoxilo	$\text{ROOH}\cdot$		
<i>Especies reactivas del nitrógeno</i>		RNS	
Óxido nítrico	$\text{NO}\cdot$		Peroxidación de lípidos
Dióxido nítrico	$\text{NO}_2\cdot$	1-10 s	Oxidación de proteínas
Peroxinitrito	$\text{ONOO}\cdot^-$	0.05^{-1} s	Daño al ADN
<i>Especies reactivas del sulfuro</i>		RSS	
Radical tiilo	$\text{RS}\cdot$		Oxidación de proteínas Daño al ADN Producción de ROS

Para neutralizar los FR o ROS, en el organismo existen antioxidantes que pueden definirse como unas moléculas que ayudan a reducir la gravedad del estrés oxidativo, ya sea formando un radical menos activo o apagando la reacción en cadena del FR o ROS dañino sobre sustratos como las proteínas, lípidos, hidratos de carbono o el ADN (246). Hay una serie de antioxidantes activos en el organismo que incluyen antioxidantes enzimáticos (endógenos) y no enzimáticos (aportados principalmente por los alimentos), pudiendo todos ellos ser antioxidantes intracelulares o extracelulares (247). (Tabla 4). Las enzimas antioxidantes incluyen la SOD, CAT y glutatión peroxidasa (GPX). Los antioxidantes no enzimáticos incluyen una variedad de amortiguadores de FR como la vitamina A (retinol), vitamina C (ácido ascórbico),

vitamina E (tocoferol), flavonoides, tioles (incluyendo GSH, ubidecarenona (ubiquinona Q₁₀), ácido úrico, bilirrubina, ferritina) y los micronutrientes (hierro, cobre, zinc, selenio, manganeso), que actúan como cofactores enzimáticos (248). La eficacia del sistema antioxidante depende de la ingesta nutricional (vitaminas y micronutrientes) y de la producción endógena de enzimas antioxidantes, que puede modificarse con el ejercicio, el entrenamiento, la nutrición y el envejecimiento (246).

Tabla 4. Localización y acciones de las enzimas antioxidantes

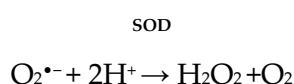
Antioxidantes	Cofactores	Localización Celular	Dianas
SOD-Mn	Manganeso	Mitocondria	Anión superóxido Peroxinitrito
SOD-Zn-Cu	Cobre y Zinc	Citosol y Mitocondria (membrana)	Anión superóxido Peroxinitrito
CAT	Hierro	Peroxisomas, citosol y mitocondrias	Peróxido de hidrógeno
GPX	Selenio	Citosol y mitocondria	Anión superóxido Peroxinitrito

CAT = catalasa; GPX = glutatión peroxidasa; SOD = superóxido dismutasa

2.4.1. Antioxidantes endógenos

2.4.1.1. Superóxido dismutasa

La SOD es la principal defensa contra el radical superóxido y es la primera línea de defensa contra el estrés oxidativo. La SOD representa un grupo de enzimas que catalizan la dismutación del O₂^{•-} y la formación de H₂O₂:

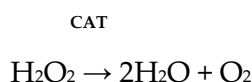


En todas las células, en situación de reposo, la mayor parte del O₂^{•-} producido por las mitocondrias es reducido por la SOD mitocondrial y la otra parte se difunde en el citosol (249). En las células musculares, el 65-85% de la actividad de la SOD se realiza

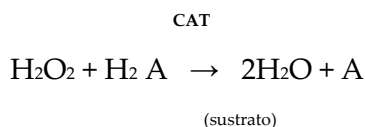
en el citosol (247). Existen diferentes formas de SOD en el organismo (Tabla 4). El manganeso es un cofactor de la forma Mn-SOD, que está presente en las mitocondrias, así como el cobre y el zinc, que son cofactores de la Cu-Zn-SOD, presente en el citosol.

2.4.1.2. Catalasa

La CAT está presente en todas las células y en particular en los peroxisomas, estructuras celulares que utilizan el oxígeno para desintoxicar sustancias nocivas y producir H₂O₂ (250). La CAT convierte el H₂O₂ en agua y oxígeno:

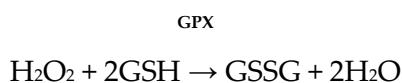


La CAT también puede utilizar el H₂O₂ para desintoxicar algunas sustancias mediante una reacción de peroxidasa. Esta reacción necesita un sustrato como el fenol, el alcohol (etanol; A) o el ácido fórmico (250).



2.4.1.3. Glutación peroxidasa

La GPX presente en el citosol celular y en las mitocondrias tiene la capacidad de transformar el H₂O₂ en agua. Esta reacción utiliza el GSH y lo transforma en glutación oxidado (GSSG):

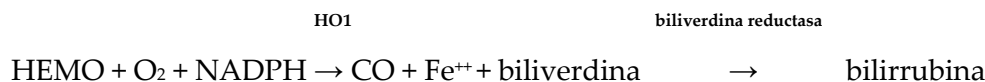


La GPX y la CAT tienen la misma acción sobre el H₂O₂, pero la GPX es más eficiente con una alta concentración de ROS y la CAT tiene una acción importante con una menor concentración de H₂O₂ (159, 250).

2.4.1.4. Hemoxigenasa 1

La hemoxigenasa inducible 1 (HO-1), que regula su expresión con la exposición a oxidantes, irradiación y una serie de agentes que incluyen citoquinas, hormonas, grupo hemo y metales pesados. El importante papel de la HO-1 en la defensa antioxidante surge de la inducción de la síntesis de ferritina, disminuyendo la reserva celular de

hierro libre, y también el aumento de la síntesis de biliverdina y bilirrubina, que se sabe que son potentes antioxidante (251).



2.4.2. Antioxidantes no enzimáticos

2.4.2.1. Vitamina E (tocoferol)

La vitamina E es una vitamina liposoluble compuesta por varias isoformas conocidas como tocoferoles. El α -tocoferol es la forma más activa y abundante (252). La vitamina E (α -tocoferol) se encuentra prácticamente en todas las membranas celulares, pero la mayor reserva de vitamina E unida a la membrana se encuentra en la membrana mitocondrial interna, donde se encuentra el sistema de transporte de electrones. Además, el contenido de vitamina E en el músculo esquelético es ~50% del observado en el tejido hepático, cardíaco y pulmonar (~20-30 nmol/g) (253). La vitamina E interactúa con numerosos antioxidantes como la vitamina C, el GSH, el β -caroteno o el ácido lipoico. Estos antioxidantes tienen la capacidad de regenerar la vitamina E a partir de su forma oxidada (254). Ésta desempeña un papel importante en las membranas celulares porque detiene la peroxidación lipídica, ya que, la estructura molecular de la vitamina E permite la inactivación de las ROS en un entorno lipídico, en particular para los radicales peroxilos ($\text{LOO}\cdot$), que provienen de la oxidación de las lipoproteínas de baja densidad (LDL) en las membranas o en sangre (255, 256). La deficiencia de vitamina E es frecuente en las poblaciones occidentales sanas (256, 257), pudiendo aumentar el estrés oxidativo y la fatiga asociada a la disminución de la capacidad oxidativa y la resistencia. Para evitar esta situación, se ha descrito que los atletas suelen tomar suplementos de vitamina E para prevenir los daños musculares y la fatiga inducidos por el ejercicio (258, 259).

2.4.2.2. Vitamina C (ácido ascórbico)

La vitamina C es una vitamina hidrosoluble y es probablemente el antioxidante más importante en los fluidos extracelulares, pero también es eficaz en el citosol. La vitamina C es más abundante en los tejidos en los que la producción de ROS es más importante (260, 261), siendo definido este fenómeno como una adaptación contra el estrés oxidativo (261). En los fluidos, la vitamina C tiene la capacidad de neutralizar las ROS ($\text{OH}\cdot$, $\text{O}_2\cdot^-$, radical $\text{LOO}\cdot$ de los ácidos grasos y radical alcoxilo ($\text{RO}\cdot$) (260). En el interior de las células, la vitamina C refuerza la acción de la vitamina E y el GSH al regenerar su forma activa después de que hayan reaccionado con las ROS (253, 262, 263). La vitamina C también tiene la capacidad de atrapar los iones de cobre, que

tienen una potente acción oxidante. Por ello, se ha estudiado la administración de suplementos de vitamina C (248). En los atletas se discuten sus efectos preventivos contra el estrés oxidativo (264, 265). Una deficiencia de vitamina C tiene efectos negativos en el rendimiento y la suplementación con vitamina C (especialmente en combinación con otros antioxidantes como la vitamina E) ayuda a mantener un nivel adecuado de vitamina C en los tejidos (258).

2.4.2.3. *Vitamina A (β -caroteno) y vitamina E*

La vitamina A es una vitamina liposoluble presente en muchas sustancias lipídicas. El β -caroteno, presente en las membranas celulares, se convierte en vitamina A cuando el organismo lo necesita. Aunque el mecanismo de su acción in vivo no está claro, se sugiere que el β -caroteno desactiva las ROS (en particular el oxígeno singlete y los radicales lipídicos) y reduce la peroxidación de los lípidos (247, 266). Aunque son menos importantes que la vitamina E dentro del sistema antioxidante, el β -caroteno y la vitamina A actúan en tándem con la vitamina C y la vitamina E para proteger a las células contra las ROS (267). Además, la administración de suplementos de β -caroteno parece tener efectos beneficiosos contra el estrés oxidativo inducido por el ejercicio, aunque sin producir mejora sobre el rendimiento físico (247, 267).

2.4.2.4. *Flavonoides*

Los flavonoides (FIOH) son sustancias fenólicas que se forman en las plantas a partir de los aminoácidos fenilalanina, tirosina y malonato (268, 269). Los estudios in vitro señalan los efectos antioxidantes de los flavonoides que tienen la capacidad de inhibir las enzimas prooxidantes o de formar complejos con iones prooxidantes como Fe^{2+} , Fe^{3+} o Cu^{2+} . Los flavonoides también ejercen una acción de captura directa sobre algunas ROS mediante la donación directa de átomos de hidrógeno. Algunos estudios han confirmado las propiedades antioxidantes in vivo de los flavonoides (270). Además, los flavonoides parecen tener un efecto ahorrador sobre la vitamina E y el β -caroteno (270, 271). Por otro lado, en concreto la quercetina (flavonoide), ha mostrado una mejora del rendimiento en deportes de resistencia (27).

2.4.2.5. *Tioles*

Los tioles son una clase de moléculas caracterizadas por la presencia de residuos de sulfidrilo (-SH) en su sitio activo (272). Los tioles se sintetizan a partir de aminoácidos azufrados: cisteína o la metionina como precursor de la cisteína. Tienen numerosas funciones en los sistemas biológicos, como la síntesis de proteínas, el estado redox, la biogénesis celular y la inmunidad, presentando también un papel importante en el sistema de defensa antioxidante (272). El GSH es el principal tiol presente en un organismo y actúa como un sustrato para la GPX en la inhibición de ROS de la

peroxidasa. El GSH también puede desintoxicar directamente las ROS y mejora la capacidad antioxidante funcional de las vitaminas C y E (273, 274). En presencia de estrés oxidativo, es posible observar una disminución de la relación GSH/GSSG y de la cantidad total de tioles (44, 272, 275, 276). Estos fenómenos parecen estar implicados en la etiología de algunas enfermedades neurodegenerativas como el Parkinson o Alzheimer (277). También se observan en el envejecimiento o tras el ejercicio físico (275, 276). Una baja concentración de GSH en las células puede estar asociada a daños celulares y a una menor eficacia de la inmunidad, lo que puede compensarse con un suplemento de vitamina C y E (278). Estos resultados tienden a demostrar que estos antioxidantes tienen los mismos objetivos y actúan conjuntamente contra los ROS. El ácido lipoico es un tiol que inhibe la peroxidación lipídica y ayuda a reducir las vitaminas C y E de su forma oxidada (254, 279, 280). También puede reducir la cistina (la forma oxidada de la cisteína) en cisteína para promover la tiologénesis (272, 277, 281). La suplementación con ácido lipoico ha mostrado protección contra el daño oxidativo de los lípidos a nivel cardiaco, hepático y muscular en ratas, influyendo favorablemente en las defensas antioxidantes de los tejidos y contrarrestando la peroxidación lipídica en reposo y en respuesta al ejercicio (281). Por tanto, la suplementación con ácido lipoico ayuda a aumentar la protección antioxidante y puede tener algunos efectos terapéuticos (254, 272).

2.4.2.6. Coenzima Q₁₀

La coenzima Q₁₀ (CQ₁₀) es una molécula endógena esencial para la síntesis de ATP y está especialmente presente en la membrana mitocondrial (282, 283). Se sabe que la CQ₁₀ actúa como antioxidante con una acción directa sobre los radicales peroxilo o con una acción indirecta al regenerar las vitaminas C y E (284, 285). La CQ₁₀ también tiene efectos beneficiosos, como la protección contra las enfermedades cardiovasculares, el cáncer y el envejecimiento celular o la apoptosis (282, 283, 286). Sin embargo, la CQ₁₀ actúa como mediadora de la expresión génica y la síntesis de proteínas en el músculo (282). En este caso, la CQ₁₀ actúa como prooxidante dando lugar a O₂^{•-}, que es convertido en H₂O₂ por la SOD, actuando como un segundo mensajero para la expresión genética. La suplementación con CQ₁₀ se ha probado en grupos de deportistas con resultados limitados sobre la reducción del estrés oxidativo y el rendimiento físico (287, 288).

2.4.2.7. Ácido úrico

El ácido úrico es un producto final del metabolismo de las purinas en los seres humanos (275, 289). Se sabe que el ejercicio físico intenso aumenta las concentraciones plasmáticas de ácido úrico (256, 290). El ácido úrico plasmático puede difundirse entonces a los músculos para protegerlos de la oxidación del FR (291). De hecho, el

ácido úrico, en el plasma y en el músculo, es también uno de los antioxidantes más importantes con efectos directos sobre el oxígeno singlete, $\text{LOO}\cdot$, ácido hipocloroso (HOCL), el peroxinitrito ($\text{ONOO}\cdot$) o el ozono (292-295). Algunos estudios demostraron que el ácido úrico representa una gran parte (>50%) de la capacidad antioxidante plasmática. Así, el ácido úrico ayuda a proteger los eritrocitos, las membranas celulares, el ácido hialurónico y el ADN de la oxidación de los FR (296). Otra importante propiedad antioxidante del ácido úrico es la capacidad de formar complejos estables con los iones de hierro. Este proceso inhibe el Fe^{3+} , la oxidación catalizada de la vitamina C y la peroxidación de los lípidos (297, 298). Por lo tanto, el ácido úrico es un protector de la vitamina C, pero también de la vitamina E (263). In vivo, es posible detectar y medir su producto de oxidación inducido por el FR (alantoína) en los fluidos corporales tras episodios de estrés oxidativo, como el ejercicio físico (275, 289, 299, 300).

2.4.2.8. *Proteínas del choque térmico*

Las proteínas de choque térmico (PCT) son una variedad de proteínas de las que se sabe que tienen efectos protectores contra diversos tipos de estrés. Las HSP aumentan con el ejercicio, sobre todo con las variaciones de la temperatura corporal, la inflamación y el estrés oxidativo (301-303). Las PCT se consideran antioxidantes porque protegen a las células y a las proteínas intracelulares contra el daño inducido por las ROS (301, 302). El entrenamiento físico y la administración de suplementos antioxidantes promueven un nivel basal de PCT más bajo, pero la capacidad de tener una rápida liberación de PCT en situaciones de estrés permanece inalterada (301). Dado el potencial de las ROS para dañar las proteínas intracelulares durante los siguientes episodios de contracciones musculares, los datos sugieren que, en condiciones de estrés oxidativo, las vías antioxidantes preexistentes pueden complementarse con la síntesis de HSP (303). Así, las HSP podrían representar un importante mecanismo de protección contra el daño muscular inducido por el ejercicio (301-303).

2.4.2.9. *Ferritina*

El hierro es necesario para el crecimiento y la proliferación normal de las células y puede tener efectos antioxidantes como cofactor de la catalasa. Sin embargo, los iones de hierro pueden tener efectos prooxidantes en la reacción de Fenton o pueden oxidar la vitamina C y reducir la protección antioxidante contra el FR (298, 304). Por lo tanto, el exceso de hierro es potencialmente perjudicial y la ferritina, una de las principales proteínas del metabolismo del hierro, desempeña un papel importante en el mantenimiento del equilibrio del hierro (305). Varios estudios apoyan una función protectora de la ferritina contra el daño mediado por el FR, ya que la ferritina minimiza

la formación de FR al secuestrar el hierro en la sangre o en las células (304, 306, 307). Además, se observa un aumento de la síntesis de ferritina en respuesta al ejercicio físico, el daño celular y la inflamación, que promueven el estrés oxidativo (306-308). En algunos estudios se demostraron vínculos indirectos y directos entre el FR y la expresión genética de la ferritina (304-307).

2.4.2.10. Albumina, ceruloplasmina y bilirrubina

La albúmina, ceruloplasmina y bilirrubina actúan como antioxidantes inespecíficos que rompen la cadena cediendo electrones a los FR (309). La albúmina (una proteína tiol) y la ceruloplasmina están implicadas en el transporte de cobre, por lo que reducen la generación de FR mediante la reacción de Fenton (310, 311). Por otro lado, la bilirrubina, una proteína biliar procedente del metabolismo de la hemoglobina (Hb), aumenta con el estrés oxidativo y tiene efectos antioxidantes en los fluidos corporales (308, 312, 313). Sin embargo, estas proteínas tienen una acción antioxidante limitada porque su acción es indirecta y es efectiva en fluidos corporales como la sangre, lejos de la mayor localización de producción de FR, especialmente durante el ejercicio físico.

2.5. EFECTOS DEL EJERCICIO DE RESISTENCIA SOBRE LAS CITOQUINAS INFLAMATORIAS.

2.5.1. Ejercicio y la respuesta de fase aguda de citoquinas

La respuesta local a una infección o lesión tisular implica la producción de citoquinas que se liberan en el lugar de la inflamación. Estas citoquinas facilitan la afluencia de linfocitos, neutrófilos, monocitos y otras células, y estas células participan en la eliminación del antígeno y la curación del tejido (314). La respuesta inflamatoria local va acompañada de una respuesta sistémica conocida como respuesta de fase aguda. Esta respuesta incluye la producción de un gran número de proteínas de fase aguda derivadas de los hepatocitos, como la proteína C reactiva (PCR), la α 2-macroglobulina y la transferrina. Esto ha sido evidenciado mediante la inyección de TNF- α , IL1 β e IL6 (citoquinas) en animales de laboratorio y en humanos produciendo en la mayoría, si no en todos los casos, se generó una situación similar a la respuesta de fase aguda (315). Por lo tanto, estas citoquinas suelen denominarse "inflamatorias" o "proinflamatorias", aunque puede ser más razonable clasificar la IL-6 como una citoquina "que responde a la inflamación" en lugar de una citoquina proinflamatoria, ya que la IL-6 no induce directamente la inflamación.

El aumento en la IL6 en respuesta al ejercicio ha sido evidenciado en muchos estudios (314, 316), sin embargo, en relación a TNF α , la situación es ambigua, ya que

algunos estudios no han detectado un aumento del TNF α después del ejercicio (316-319), mientras que otros informan de un aumento de las concentraciones plasmáticas de TNF α (320-323). Además, también se ha encontrado un aumento de la concentración de IL6 inmediatamente después de una carrera de maratón, pero no de IL1 β (324). Después de una carrera de maratón, el TNF α y la IL β se multiplican por 2, mientras que las concentraciones de IL6 lo hacen hasta 50 veces, a esto le siguió un marcado aumento de la concentración de IL1ra (325).

Además, estudios recientes demuestran que pueden detectarse varias citoquinas en el plasma durante y después del ejercicio extenuante (322, 323, 325). En particular, con el ejercicio se ha visto una inducción de las citoquinas proinflamatorias TNF α e IL1 β y un aumento drástico de la citoquina que responde a la inflamación, IL6. Esta situación se ve equilibrada con la liberación de inhibidores de citoquinas IL1ra y receptores de TNF α (TNF α -R) y la citoquina antiinflamatoria IL10 (323). Además, las concentraciones de quimiocinas, IL8 y proteína inhibidora de macrófagos 1 α (PIM 1 α) y PIM1 β , se elevan después de un maratón (314). Estos resultados sugieren que los inhibidores de citoquinas y las citoquinas antiinflamatorias restringen la magnitud y la duración de la respuesta inflamatoria al ejercicio. La presencia de múltiples citoquinas (TNF α , IL1 β , IL6, receptores de IL2 e interferón gamma (IFN γ)) en la orina después del ejercicio muestra que es posible la expresión de un amplio espectro de citoquinas en respuesta al ejercicio (326).

Hay varias explicaciones posibles para los resultados variables sobre las citoquinas proinflamatorias que responden a la inflamación en relación con el ejercicio (327). Entre ellas se encuentran: 1) el tipo de actividad física, así como la intensidad y la duración del ejercicio y 2) la especificidad y la sensibilidad de los ensayos es otra posible explicación (314).

Mediante la utilización de una técnica de PCR comparativa para detectar el ácido ribonucleico mensajero (ARNm) de las citoquinas en biopsias de músculo esquelético y células mononucleares de la sangre (CMS) recogidas antes y después de un ejercicio de resistencia (correr >2h) (325). Antes del ejercicio, el ARNm de la IL6 no pudo detectarse en el músculo ni en los CMS, pero si se detectó en las biopsias musculares después del ejercicio, sin embargo, el ARNm de la IL6 no se encontró en las muestras de CMS. Se encontraron mayores cantidades de ARNm de IL1ra en las muestras de CMS después del ejercicio. Este estudio sugiere que el ejercicio induce la producción local de IL6 en el músculo esquelético y posteriormente desencadena la producción de IL1ra a partir de los CMS circulantes (325).

En un estudio donde los sujetos realizaban una carrera de larga duración (89.4 km) mostró que el 81% de los atletas tenían concentraciones de endotoxina en plasma por encima del límite superior de 0.1 ng/mL, incluido el 2% con niveles de plasma superiores a 1 ng/mL, un valor que se considera letal en humanos (328). Curiosamente,

estos autores observaron que los valores más elevados de endotoxina se midieron en los sujetos con peor estado físico que completaron la carrera en más de 8 h. Este grupo también descubrió que no había un aumento de los niveles de LPS después de la carrera de 21.1 km (328). Sin embargo, si se ha registrado un aumento de los niveles plasmáticos de LPS en atletas que participaron en una competición de triatlón (329). La relación entre la enfermedad leve post-ejercicio en 39 ciclistas después de una carrera de 161 km y la endotoxemia, encontró que ésta no era la causa de la enfermedad post-esfuerzo y no estaba relacionada con la rabdomiólisis (330). La disparidad entre los estudios sobre corredores (328) y ciclistas (330) con respecto a la enfermedad asociada a la endotoxemia no es fácil de entender. Sin embargo, es posible que el traumatismo intestinal durante la carrera, pero no durante el ciclismo, pueda comprometer la función de barrera de la pared intestinal y, por tanto, aumentar la carga portal de endotoxinas (314).

Aunque se han publicado informes contradictorios, la mayoría de los autores están de acuerdo en que un factor derivado de los monocitos es responsable de la degradación de las proteínas musculares (314). Las pruebas de los experimentos in vivo indican que la IL1, el TNF α y la IL6 contribuyen al catabolismo de las proteínas musculares, pero los experimentos in vitro no apoyan este concepto (314). Es posible que la IL1 y el TNF α requieran un cofactor o un procesamiento (es decir, una escisión), o que sean intermediarios que inducen un factor inhibidor que actúa en el propio músculo. Se ha comprobado que la IL1 β aumenta en el músculo hasta 5 días después de completar el ejercicio (331).

La presencia de IL1 en el músculo esquelético varios días después del ejercicio sugiere que la IL1 puede estar implicada en el daño muscular prolongado o en la degradación de las proteínas (314). La suplementación con aminoácidos de cadena ramificada (BCAA) reduce la degradación proteica neta sin reducir la IL6, lo que sugiere que la IL6 no está estrechamente relacionada con el catabolismo muscular después de un ejercicio excéntrico prolongado (332).

Se han encontrado niveles elevados de prostaglandinas 2 (PGE2) y dolor muscular de aparición tardía 24 h después del ejercicio excéntrico. El curso temporal de la PGE2 y el dolor muscular indica una posible relación. La fuente de producción de prostaglandinas podría ser el macrófago, que es la célula predominante a las 24 h. Se ha demostrado que la IL1 α , la IL1 β y el TNF α inducen la síntesis de prostaglandinas en las células endoteliales, las células musculares lisas y el músculo esquelético (333). Por lo tanto, la producción de citoquinas inflamatorias en respuesta al ejercicio puede estimular la producción de prostaglandinas. Los elevados niveles de IL6 en plasma que se encuentran inmediatamente después de la finalización de un ejercicio exhaustivo constituyen un apoyo adicional. Así pues, la producción o liberación de IL6 precede a la acumulación de neutrófilos y macrófagos en el músculo, al aumento de PGE2, al

aumento de CK y a la sensación de dolor muscular de aparición retardada (314). Además, existen similitudes en las respuestas de citoquinas observadas en sujetos tras un ejercicio intenso y en pacientes con traumatismos y sepsis. En los modelos experimentales de meningitis y sepsis, las endotoxinas inducen un aumento del TNF α , seguido de un aumento de la IL1 β , y considerablemente más tarde de la IL6 (334) (Figura 7). Sin embargo, en los pacientes traumatizados, el patrón de liberación de citoquinas es diferente, con un aumento de la IL6 pero no del TNF α (335). Trabajos recientes muestran que la respuesta de las citoquinas al ejercicio que daña los músculos es similar a la observada en los pacientes traumatizados (322, 323).

Se ha sugerido un modelo de la respuesta de las citoquinas en relación con el ejercicio (Figura 7). La alteración mecánica de las miofibras inicia la producción local y sistémica de citoquinas. La liberación secuencial de citoquinas se asemeja a la observada en relación con el traumatismo (es decir, alta IL6 y baja TNF α e IL1 β) (322). Aunque se han detectado niveles elevados de IL6, esta IL6 sólo induce un modesto aumento de la PCR. No se ha determinado el curso temporal de la PCR (336).

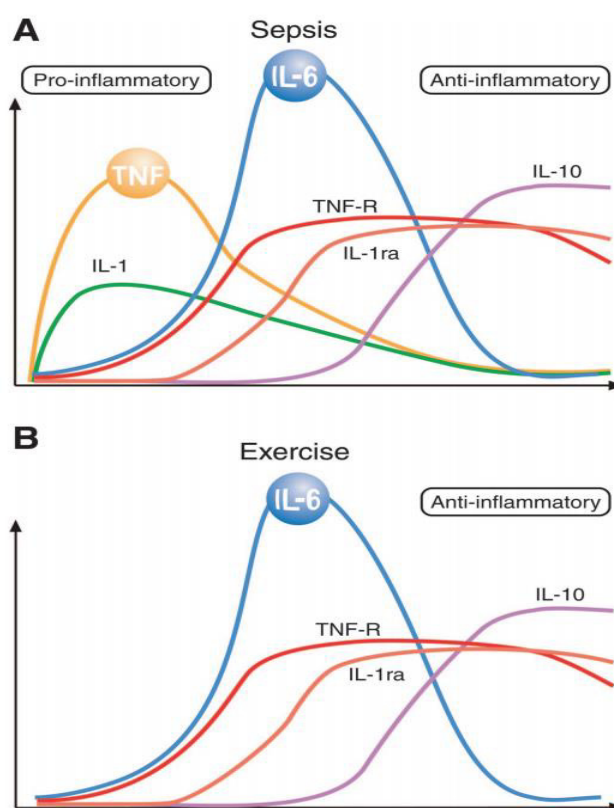


Figura 7. En la sepsis (A), la cascada de citoquinas en las primeras horas consiste en de TNF α , IL1, IL6, IL1ra, TNF-R e IL10. La respuesta de citoquinas al ejercicio (B) no incluye el TNF α y la IL1, pero sí muestra un marcado aumento de la IL6, seguida de la IL1ra, el TNF-R y la IL10.

El aumento de los niveles de PCR no aparecen hasta 8 -12 h después Modificada.de Petersen et al. (337).

Además, muchos de los otros efectos biológicos que se producen con el traumatismo inducido por las citoquinas proinflamatorias, como la disminución del miocardio, la vasodilatación, la agregación de leucocitos y la disfunción de los riñones, el hígado, los pulmones y el cerebro, no se desarrollan en respuesta al ejercicio (314). Sin embargo, el ejercicio no se caracteriza por una respuesta proinflamatoria sistémica plenamente desarrollada. Esta falta de respuesta sistémica puede deberse a una liberación transitoria de citoquinas en respuesta al ejercicio. Alternativamente, esto puede reflejar una adaptación a la respuesta de citoquinas (por ejemplo, una mayor capacidad para inducir citoquinas inhibitoras naturales eficaces y receptores de citoquinas) (314).

2.5.2. Activación del sistema inmune como respuesta al ejercicio

El ejercicio induce numerosos cambios en el sistema inmunitario, pero es controvertido si estos cambios reflejan la activación del sistema inmunitario o la alteración de la composición de las subpoblaciones de linfocitos que influyen en los ensayos funcionales in vitro (338, 339). Ciertamente, muchos de los cambios inducidos por el ejercicio que se han descrito pueden atribuirse a cambios en la composición de los CMS. Por ejemplo, la disminución de la respuesta de los linfocitos a la fitohemaglutinina (PHA) se debe a la disminución de la fracción de las células CD4+, y el aumento de la producción in vitro de IL1 a partir de las CMS estimuladas con endotoxina se debe al aumento del porcentaje de monocitos (314).

Sin embargo, el aumento de los niveles de citoquinas en el plasma después del ejercicio intenso probablemente no se deba simplemente a una redistribución de los monocitos (314). Se registraron concentraciones plasmáticas aumentadas de receptor de interleucina 2 soluble (IL2-R), CD8+, molécula de adhesión intercelular 1 (ICAM1), CD23 and TNF α -R durante o después de un ejercicio de larga duración (340). Estos resultados sugieren que existe cierta activación del sistema inmunitario durante el ejercicio intenso de larga duración. Sin embargo, en otro estudio, los niveles del factor estimulante de colonias de granulocitos y macrófagos permanecieron inalterados tras el ejercicio concéntrico (341).

El ejercicio físico intenso prolongado se ha asociado con un aumento inicial y una disminución retardada de los complejos inmunitarios circulantes (320, 342). Sin embargo, los cambios fueron estadísticamente significativos, pero cuantitativamente pequeños, y los valores sólo fueron ocasionalmente superiores al límite superior y encontramos otros estudios que no reportan estos cambios (343, 344). La falta de

aumento de las concentraciones de inmunocomplejos y también la falta de aparición de productos de división del complemento C3 (C3c y C3d) indican que la activación del complemento inducida por inmunocomplejos no se produce durante el ejercicio concéntrico (344). Además, los niveles del receptor del complemento tipo 1 (CR1, CD35; asociados con procesos inmunitarios e inflamatorios) en los eritrocitos no cambian en relación con el ejercicio concéntrico, ni en sujetos sanos ni en pacientes con artritis reumatoide (344). Una evolución similar de los cambios en la mieloperoxidasa (marcador específico de la activación de los polimorfonucleares) y el C5a (induce quimiotaxis) y la relación altamente significativa entre estas 2 variables prestan cierto apoyo a la hipótesis de que la activación del complemento contribuye a la neutrocitosis post-ejercicio en el ejercicio excéntrico (345).

La disparidad entre los estudios (314) puede explicarse por el hecho de que sólo el ejercicio de larga duración o el que implica un componente excéntrico provoca la activación de la cascada del complemento. En apoyo de esta hipótesis, correr cuesta abajo, pero no caminar cuesta arriba, indujo un aumento de los niveles plasmáticos de mieloperoxidasa y elastasa (346).

2.5.3. Efectos del ejercicio crónico sobre el sistema inmunitario

En contraste con el gran número de estudios sobre la respuesta inmunitaria al ejercicio agudo, se sabe mucho menos sobre el efecto del acondicionamiento físico o el entrenamiento en la función inmunitaria. Esto se debe en gran medida a las dificultades para separar los efectos del acondicionamiento físico del ejercicio físico real, así como la complejidad de realizar estudios longitudinales. Así, los cambios inducidos por el ejercicio físico intenso pueden durar al menos 24 h, e incluso el ejercicio agudo moderado induce cambios inmunológicos significativos durante varias horas. Dado que no es fácil persuadir a los atletas para que se abstengan de su programa de entrenamiento normal, incluso durante un solo día, puede ser difícil obtener resultados sobre los verdaderos "niveles en reposo". La influencia del ejercicio crónico se ha estudiado tanto en modelos animales como humanos, estos últimos incluyendo tanto estudios longitudinales como transversales (314).

Un indicador del ejercicio crónico como factor de estilo de vida es comparar los niveles en reposo de cualquier parámetro inmunológico en controles no entrenados y en atletas condicionados. En 2 estudios, realizados con ciclistas masculinos de competición, se controlaron los efectos del ejercicio agudo exigiendo a los sujetos que no hicieran ejercicio 20 h antes de la toma de muestras de sangre. Todos los sujetos habían practicado deporte durante una media de 4 años, con un volumen de entrenamiento medio de 20,000 km/año. La media de la actividad de las células natural killer (NK) fue del 38.1% en el grupo entrenado en comparación con el 30.3% en el

grupo no entrenado, y la media del % de células NK CD16+ fue del 17% en el grupo entrenado frente al 11% en el grupo no entrenado (347). En otro estudio, se examinaron 15 ciclistas y 10 controles durante un período de entrenamiento de alta o baja intensidad encontrando que, la actividad de las células NK era significativamente elevada en el grupo entrenado, tanto durante el periodo de entrenamiento de baja intensidad (39.2 frente al 30.9%) como durante el periodo de entrenamiento de alta intensidad (55.2 frente al 33.6%) (348). Durante el entrenamiento de baja intensidad, el aumento de la actividad de las células NK en los sujetos entrenados se debió a un mayor porcentaje de células NK. Durante el entrenamiento de alta intensidad, el aumento de la función de las células NK no se debió a un simple aumento numérico: tanto los sujetos entrenados como los no entrenados tenían un número comparable de células NK circulantes. Los mecanismos de esta mayor actividad podrían ser secundarios a las diferencias en la activación de las células NK. Los resultados sugieren que las células NK se activaron en los sujetos entrenados durante el entrenamiento de alta intensidad y que esto puede conducir a un ajuste del número de células CD16+ (NK) en circulación por algún mecanismo desconocido. En estos estudios (347, 348), otras subpoblaciones de linfocitos y las respuestas proliferativas de los linfocitos no diferían entre los sujetos entrenados y los no entrenados.

Otra investigación, comparó 22 corredores de maratón que habían completado al menos 7 maratones, fueron comparados con un grupo de 18 controles sedentarios (349). A pesar de las grandes diferencias entre los grupos en cuanto a VO_{2MAX} , porcentaje de grasa corporal y actividad física, sólo la actividad de las células NK entre las variables del sistema inmunitario medidas resultó ser significativamente diferente entre los grupos (mayor entre los maratonistas). En línea con los datos anteriores, la actividad de las células NK y las respuestas proliferativas estimuladas por PHA fueron significativamente elevadas en un grupo de mujeres de edad avanzada altamente condicionadas en comparación con un grupo inactivo (350).

Las respuestas proliferativas de los linfocitos se han descrito como disminuidas (351), elevadas (350, 352) o sin cambios (347-349, 353, 354) cuando se comparan atletas y no atletas. La función de los neutrófilos se suprime (355, 356) o no se ve influida de forma significativa por el entrenamiento (357, 358). La función de los neutrófilos no se modificó en los atletas durante un período de entrenamiento bajo, pero disminuyó durante los períodos de entrenamiento de alta intensidad (352, 359).

En estudios longitudinales no hay un efecto claro del ejercicio físico sobre el sistema inmune, ya que, por ejemplo, la actividad de las células NK no se ve influida (350). Sin embargo, la actividad de las células NK en las mujeres de edad avanzada que realizaron 16 semanas de ejercicio en cinta rodante aumentó (360). En otro estudio, 15

semanas de caminata mejoraron la actividad de las células NK en mujeres moderadamente obesas y previamente inactivas (361).

2.6. EFECTOS DEL EJERCICIO DE RESISTENCIA SOBRE LA GASOMETRÍA ARTERIAL

2.6.1. Diferencias en los valores de gasometría arterial dependiendo del lugar de extracción

Las mediciones de gases en sangre arterial se utilizan comúnmente en la evaluación de diversas poblaciones clínicas, como los pacientes que sufren trastornos respiratorios. Estas mediciones se utilizan habitualmente para evaluar y diagnosticar la hipoxemia, la hipercapnia y la hipocapnia y los cambios en el estado ácido-base. Las mediciones de gases en sangre arterial también pueden ayudar a identificar el intercambio gaseoso en atletas de resistencia en muy buena forma física que se someten a un ejercicio intenso (362). En este sentido, la eficacia del intercambio gaseoso pulmonar se evalúa mejor mediante la canulación de la arterial radial; sin embargo, esta técnica puede ser incómoda y puede presentar algunos riesgos (363).

Se han propuesto métodos alternativos no invasivos, como la oximetría de pulso, que se utiliza para evaluar saturación arterial de oxihemoglobina, aunque los valores obtenidos se correlacionan mal con la presión arterial de oxígeno (PaO_2) (362). Para medidas de oximetría de pulso, los estudios actuales informan de un sesgo absoluto medio (precisión) de entre el 0 y el 0.3% con un sensor de oximetría frente, y del 20.5 al 2.5% cuando el sensor se coloca en el dedo índice (364, 365). La precisión de la oximetría de pulso comparada con la medición arterial mediante cooximetría es de alrededor del 62.5% (frente) o del 63.0 al 7.3% (dedo) (364, 365). Además, tanto los valores de sesgo de precisión de la pulsioximetría empeoran a niveles de saturación inferiores al 85% (365).

Por otro lado, la toma de muestras de sangre capilar arterializada en lugar de arterial puede ser un sustituto fiable del muestreo arterial. Si se puede conseguir una vasodilatación adecuada mediante la aplicación de una sustancia vasodilatadora tópica en la piel y/o calentando la zona (con agua caliente, toalla o calefactor), la presión parcial de oxígeno en sangre (PO_2) debe converger, y la muestra capilar arterializada debe reflejar fielmente la sangre arterial. Esta técnica no se practica comúnmente hoy en día debido a la variabilidad de los resultados, ya que, algunos estudios muestran una concordancia con la PaO_2 y la saturación arterial de oxihemoglobina en comparación con las muestras arterializadas del lóbulo de la oreja en reposo en adultos (366-368). Sin embargo, otros estudios no muestran dicha concordancia (369-371).

2.6.2. Parámetros analizados en la gasometría arterial

Los analizadores de gases sanguíneos informan de una amplia gama de resultados (Tabla 5), pero los únicos parámetros que se miden directamente son el pH, PO₂ y PCO₂. Otros parámetros, como la saturación de oxígeno de la hemoglobina (HbO₂%), la concentración real y estándar de bicarbonato y el exceso de bases se calculan mediante nomogramas derivados de la experimentación directa. Algunos analizadores de gases en sangre incorporan un cooxímetro (capaz de medir directamente la Hb total, la oxihemoglobina (O₂Hb), carboxihemoglobina (COHb) y metahemoglobina (MetHb)) y bioquímica (por ejemplo, Na⁺, K⁺, Ca²⁺, Cl⁻, lactato, glucosa) (372).

Tabla 5. Parámetros que suelen indicar los analizadores de gases en sangre (372, 373).

Mediciones directas	
<i>pH</i>	<p>Se mide con un electrodo de pH.</p> <p>$pH = -\log_{10} H^+$ - el logaritmo negativo de H⁺.</p> <p>El pH disminuye a medida que aumenta la H⁺.</p> <p>El pH 7.0 corresponde a H⁺ 100 nmol/L.</p> <p>Un cambio de una unidad de pH representa un cambio de diez veces en H⁺.</p>
<i>PaO₂</i>	<p>Se mide con un electrodo polarográfico (Clark).</p> <p>La hemoglobina es el principal portador de O₂, cada molécula une 4 moléculas de O₂. Una pequeña cantidad de O₂ se disuelve en la sangre, pero es esta fracción la que se difunde a través de los capilares para suministrar los tejidos.</p> <p>La pulsioximetría mide la saturación de la hemoglobina con O₂.</p> <p>La PaO₂ es la presión parcial del oxígeno no disuelto, que está en equilibrio con el oxígeno disuelto.</p>
<i>PaCO₂</i>	<p>Se mide con un electrodo de pH modificado.</p> <p>El CO₂ se transporta como HCO₃⁻ (99%), unido a la hemoglobina y disuelto en el plasma. Al igual que en la medición de la PaO₂ sólo se mide la fracción no disuelta.</p>

Mediciones derivadas	
<i>HCO₃⁻ actual</i>	<p>La cantidad real de bicarbonato contenida en una muestra.</p> <p>El plasma se equilibra con pCO₂ de 5.3 kPa (40 mmHg) a 20°C y el HCO₃⁻ calculado a partir de el volumen de CO₂ evolucionado cuando se añade ácido (HCl) a la muestra.</p>
<i>HCO₃⁻ estándar</i>	<p>Es la concentración de bicarbonato cuando la muestra se equilibra con pCO₂ 5.3 kPa (40 mmHg) a 37°C, y con la hemoglobina totalmente saturada de O₂.</p> <p>Se eliminan los efectos de la acidosis/alcalosis respiratoria.</p> <p>El rango normal es de 24-33 mmol/L.</p>
<i>Exceso de bases real</i>	<p>La cantidad de ácido fuerte (o base) necesaria para valorar un litro de sangre hasta un pH de 7.4 a pCO₂ 5.3 kPa (40 mmHg) y 37°C.</p> <p>Se refiere al componente metabólico del trastorno ácido-base, ya que no se tiene en cuenta cualquier cambio de pH debido a la pCO₂.</p>
<i>Exceso de bases estándar</i>	<p>El exceso de base estándar es una expresión del exceso de base in vivo. Se considera como un modelo del líquido extracelular y se calcula utilizando en la fórmula un tercio de la concentración total de hemoglobina. Este parámetro es independiente de la pCO₂ de la muestra y se utiliza como reflejo de los cambios en los componentes no respiratorios del estado ácido-base.</p>
<i>Anion Gap</i>	<p>Anion Gap = ((Na⁺) + (K⁺)) - ((Cl⁻) + (HCO₃⁻))</p> <p>Los valores de una persona normal están entre 12-20mmol/L:</p> <ul style="list-style-type: none"> • ≥20mmol/L, hay un 67% de probabilidad de acidosis metabólica. • ≥30mmol/L, hay un 100% de probabilidad de acidosis metabólica.

PCO₂ = presión parcial de dióxido de carbono, PO₂ = presión parcial de oxígeno, HCO₃⁻ = bicarbonato y HCl = ácido clorhídrico.

2.6.3. Factores que afectan al consumo de oxígeno

Existen grandes diferencias en el VO_2 de los distintos tejidos y en diferentes niveles de actividad (Tabla 6). En la salud, la temperatura central se controla a 36-37.5°C, pero cambios en la temperatura central pueden afectar a la tasa metabólica en la enfermedad. La mayoría de las reacciones químicas se aceleran unas 2.5 veces por cada 10°C de aumento de la temperatura. Este "efecto Q10" contribuye a el aumento del consumo de oxígeno en la fiebre hasta que, a temperaturas muy elevadas, disminuye porque las proteínas se desnaturalizan y el metabolismo se ve afectado. El efecto directo de la temperatura se complica por el coste metabólico de la termorregulación, y el VO_2 también aumenta a medida que desciende la temperatura central cuando se inician los escalofríos (374)

Tabla 6. Consumo de oxígeno (VO_2) en diferentes tejidos (375).

Órganos	Consumo de oxígeno (mL/min por 100 g de tejido)
Cerebro	3
Riñón	5
Piel	0.2
Músculo esquelético (reposo)	1
Músculo esquelético (ejercicio de alta intensidad)	50
Corazón (reposo)	8
Corazón en ejercicio máximo	70

Otros factores que pueden aumentar la tasa metabólica son el aumento de concentraciones circulantes de tiroxina, adrenalina y fármacos como la isoprenalina. El dolor y la ansiedad aumentan el VO_2 por una combinación de inquietud y aumento de la concentración de catecolaminas en el plasma. El trabajo respiratorio es suele ser responsable de <5% del VO_2 en reposo, pero puede aumentar hasta el 30% en personas con enfermedades respiratorias, en cuyo caso el VO_2 se reduce considerablemente con la ventilación mecánica (376).

La PO_2 alveolar (PAO_2) está determinada por la ventilación alveolar y la PO_2 del gas inspirado humedecido, que es de 19.9 kPa (149 mmHg, a 37°C saturado de agua) a

nivel del mar. En la salud, la sangre capilar pulmonar se equilibra con el gas alveolar de manera que la P_{aO_2} es ligeramente inferior porque se produce una derivación de derecha a izquierda desde la circulación bronquial y las venas de Tebas. El gradiente de PO_2 (PAO₂-PaO₂) es normalmente <2 kPa en adultos jóvenes (375).

El oxígeno se transporta en la sangre en solución y se combina con la Hb en el glóbulo rojo. La solubilidad del oxígeno es baja (0.225 mL de O₂ por litro de sangre por kPa o 0.03 mL/L/mmHg); a una PaO₂ normal de 13 kPa (97 mmHg), sólo hay 3 mL de oxígeno disuelto por litro en comparación con los 200 mL/L unidos a la Hb. El porcentaje de los sitios de la Hb que se combinan con el oxígeno (saturación de oxígeno (SO₂)) viene determinado por la PO₂ a la que está expuesta la Hb, tal y como se describe en la curva de disociación de la O₂Hb (Figura 8). La afinidad de los sitios de la Hb por el oxígeno también se ve afectada por otros factores de la sangre. Por ejemplo, el aumento de la presión parcial de dióxido de carbono (PCO₂), el aumento de la temperatura y la reducción del pH (como ocurre en los tejidos metabólicamente de los tejidos metabólicos), reducen la afinidad de los grupos hemáticos por el oxígeno, favoreciendo su liberación de la Hb. Esto se refleja en un desplazamiento hacia la derecha de la curva de disociación de la O₂Hb (Figura 8). Los efectos de la anemia y la intoxicación por monóxido de carbono (CO) en la curva de disociación se muestran en la Figura 8 y sus efectos en el suministro de oxígeno (375).

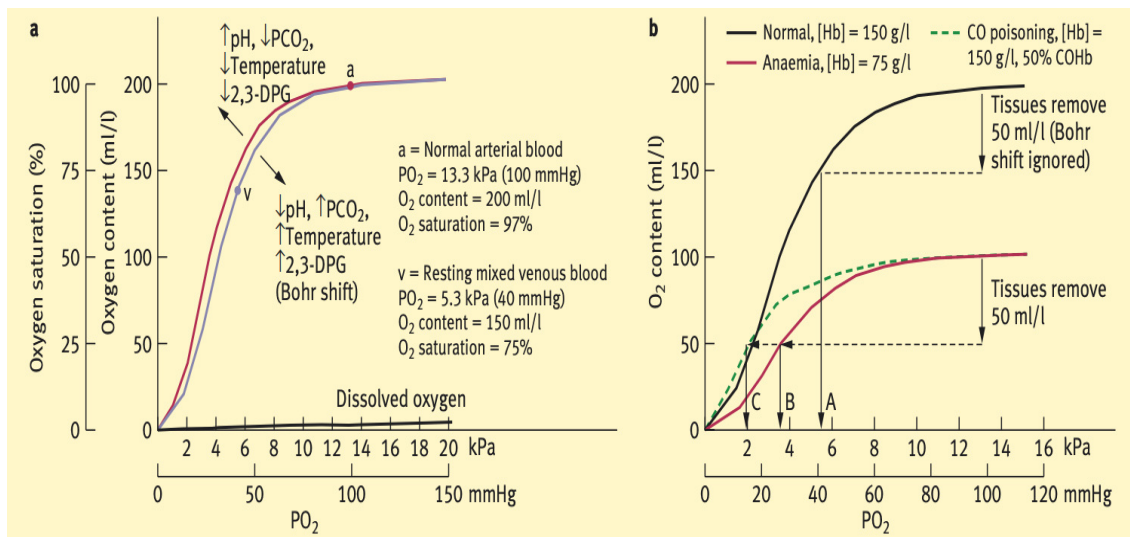


Figura 8. a) Curva de disociación de la oxihemoglobina y b) Curva de disociación de la oxihemoglobina en la anemia y la intoxicación por monóxido de carbono. Modificada de Ward et al. (375).

La Hb está casi totalmente saturada (>97%) de oxígeno a una PaO₂ normal de unos 13 kPa (97 mmHg). Cada gramo de Hb puede combinarse con 1,36 mL de oxígeno, de modo que con una concentración normal de Hb de 150 g/L, el contenido arterial de oxígeno es de unos 200 mL/L ($(150 \times 1.36 \times 0.97) + 3$). La curva de disociación se estabiliza por encima de 8 kPa (60 mmHg, SO₂ = 91%). Por consiguiente, en la salud a nivel del mar, el contenido de oxígeno se ve poco afectado por una pequeña caída de la PaO₂ (por ejemplo, una hipoventilación leve) o un aumento de la PaO₂ (por ejemplo, una hiperventilación o respiración de oxígeno). Por el contrario, en un paciente hipóxico con una PO₂ de <8 kPa (60 mmHg), el contenido de O₂ arterial puede verse notablemente afectado por pequeños aumentos o disminuciones de la PaO₂ arterial, por ejemplo, por pequeños cambios en la ventilación o la concentración de oxígeno inspirado (375).

El oxígeno de la Hb se libera para reponer el oxígeno disuelto en sangre mientras se difunde a los tejidos. Cuanto mayor sea la Hb, mayor será el almacén de oxígeno y más lenta será la caída de la PO₂ a lo largo del capilar. La PO₂ cae rápidamente al principio y luego más lentamente, de modo que la PO₂ capilar media está más cerca de la PO₂ venosa que de la arterial. La PO₂ y la SO₂ venosas varían de un tejido a otro, en función del VO₂ del tejido en relación con su flujo sanguíneo. A pesar del alto VO₂ en el riñón, la SO₂ venosa renal es elevada (90%) debido al gran flujo sanguíneo renal. Sin embargo, el flujo sanguíneo coronario es mucho menos generoso en relación con el VO₂ del miocardio, por lo que la SO₂ venosa coronaria es mucho menor (40%) (375).

2.6.4. Hipoxemia arterial

Existen cinco causas por las que la PaO₂ puede bajar y estas se muestran en la Figura 9. Se puede esperar que el aumento de la concentración de oxígeno inspirado aumenta significativamente la PaO₂ y el contenido de oxígeno en la mayoría de estas condiciones (1, 2, 3 y 5) (Figura 9). Es menos eficaz en una derivación pura derecha-izquierda (Shunt) (4) porque la sangre derivada no está expuesta al aumento de oxígeno y el contenido de O₂ de la sangre que pasa por regiones alveolares que funcionan normalmente puede mejorar poco. De estas cinco causas de hipoxemia arterial, sólo la hipoventilación (2) se acompaña inevitablemente de un aumento de la PaCO₂, que no mejorará al respirar mezclas de gases ricos en oxígeno. La hipoxemia estimula los quimiorreceptores del cuerpo carotídeo y el aumento reflejo de la ventilación suele corregir (o sobre corregir) cualquier aumento de la PaCO₂ causado por el problema primario. Por lo tanto, cuando la hipoxia arterial es causada por cualquier cosa que no sea hipoventilación, la PaCO₂ suele ser normal o baja, a menos que algo (por ejemplo, el agotamiento en un ataque de asma grave) esté interfiriendo con la respuesta ventilatoria normal a la hipoxia (375).

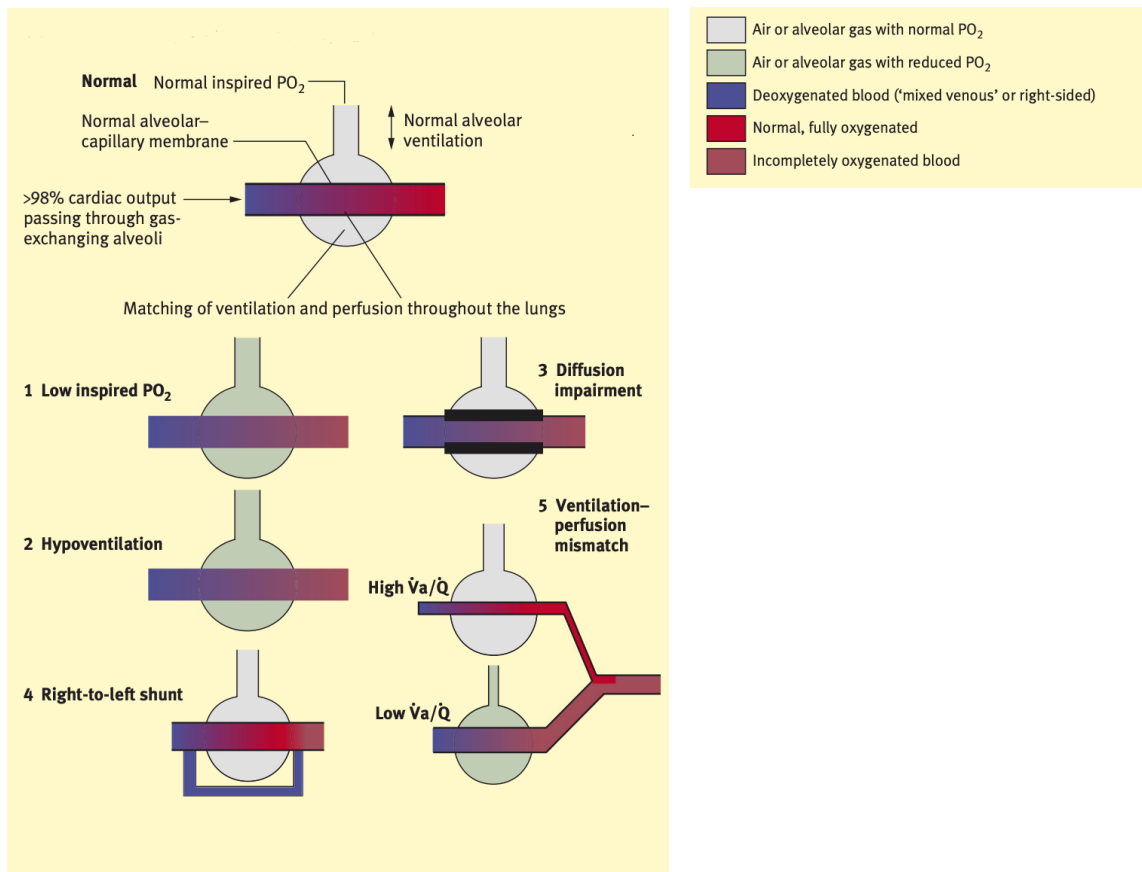


Figura 9. Mecanismos de hipoxia arterial (baja PaO_2). Modificada de Ward et al. (375).

Si la PO_2 capilar media cae por debajo de una presión crítica, la PO_2 en las mitocondrias de algunas células (normalmente las más alejadas de un capilar) caerá por debajo de aquella a la que puede continuar la respiración aeróbica. Esta PO_2 capilar crítica aumenta si se incrementa el metabolismo tisular o si aumentan las distancias de difusión debido al edema. El aporte de oxígeno a un tejido es el producto del flujo sanguíneo (min/L) y el contenido de oxígeno arterial (mL/L). El valor normal del suministro de oxígeno en todo el cuerpo (DO_2) en un adulto en reposo es de unos 1000-1200 mL/min (gasto cardíaco 5-6 min/L ; contenido de oxígeno arterial 200 mL/L) (Figure 10). Esto equivale a unos 550-650 mL/min/m^2 . Las reducciones moderadas de la DO_2 en relación con el metabolismo son bien toleradas, con el VO_2 mantenido inicialmente por el aumento de la extracción de oxígeno. Se alcanza una DO_2 crítica (Figura 10) por debajo de la cual la extracción de oxígeno no aumenta, o aumenta lo suficiente, para compensar la reducción de la entrega, y el VO_2 cae. En esta situación, la hipoxia tisular y el metabolismo anaeróbico son cada vez más intensos a medida que el suministro de oxígeno cae por debajo de este punto crítico (375).

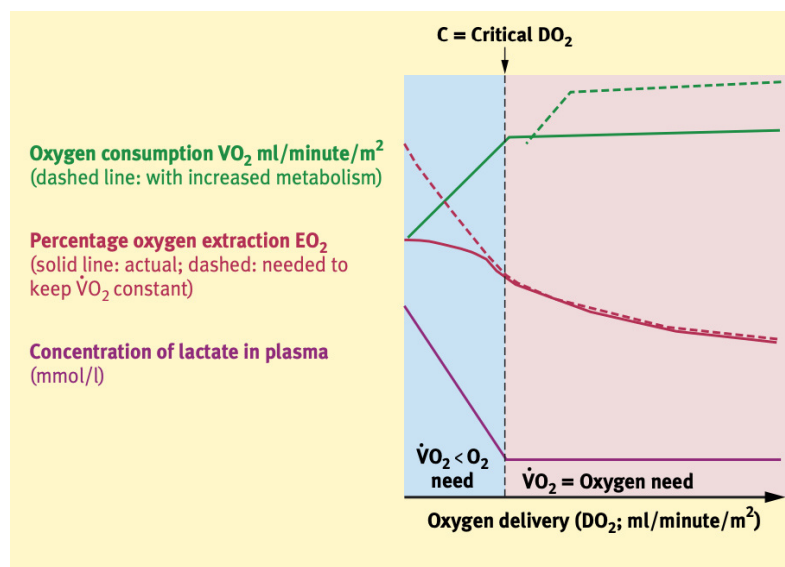


Figura 10. Efecto del suministro de oxígeno en el consumo de oxígeno ($\dot{V}O_2$). Modificada de Ward et al. (375).

Con el aumento de la tasa metabólica, la curva de consumo de oxígeno-entrega de oxígeno se desplaza hacia arriba y hacia la derecha, y aumenta la $\dot{V}O_2$ crítica por debajo de la cual caería el $\dot{V}O_2$ (Figura 10, línea verde discontinua). El gasto cardíaco y el suministro de oxígeno aumentan normalmente durante el ejercicio, pero lo hace proporcionalmente menos que el $\dot{V}O_2$, por lo que se requiere una mayor extracción de oxígeno (2.5 veces en este ejemplo). El $\dot{V}O_{2\text{MAX}}$ se alcanza cuando ni el gasto cardíaco ni la extracción de oxígeno pueden aumentar más (375). Aproximadamente la mitad de los atletas de resistencia bien entrenados muestran una reducción de su SaO_2 con respecto a los valores en reposo durante intensidades de ejercicio que se aproximan al $\dot{V}O_{2\text{MAX}}$, mientras que este no es el caso de los sujetos no entrenados (377).

Una reducción de la tensión arterial de O_2 inducida por el ejercicio se denomina hipoxemia arterial inducida por el ejercicio (EIAH), y reduce tanto el $\dot{V}O_{2\text{MAX}}$ como la capacidad de rendimiento en el ejercicio de los atletas altamente entrenados (378). El EIAH puede producirse de forma más profunda y frecuente durante la carrera en cinta rodante en comparación con la ergometría en bicicleta (379). Asimismo, Gavin y Stager (380) demostraron que el $\dot{V}O_2$ era mayor y la SaO_2 era menor en el ejercicio máximo durante la carrera en cinta rodante en comparación con la ergometría en bicicleta en un grupo heterogéneo de atletas entrenados en resistencia (Figura 11), un hallazgo confirmado por Rice et al. (381), que también examinaron un grupo heterogéneo (siete corredores, seis ciclistas) al 95% del $\dot{V}O_{2\text{MAX}}$. En ambos estudios, la EIAH estaba

relacionada con un equivalente ventilatorio para el dióxido de carbono (VE/VCO_2) más bajo durante la carrera en comparación con el ciclismo.

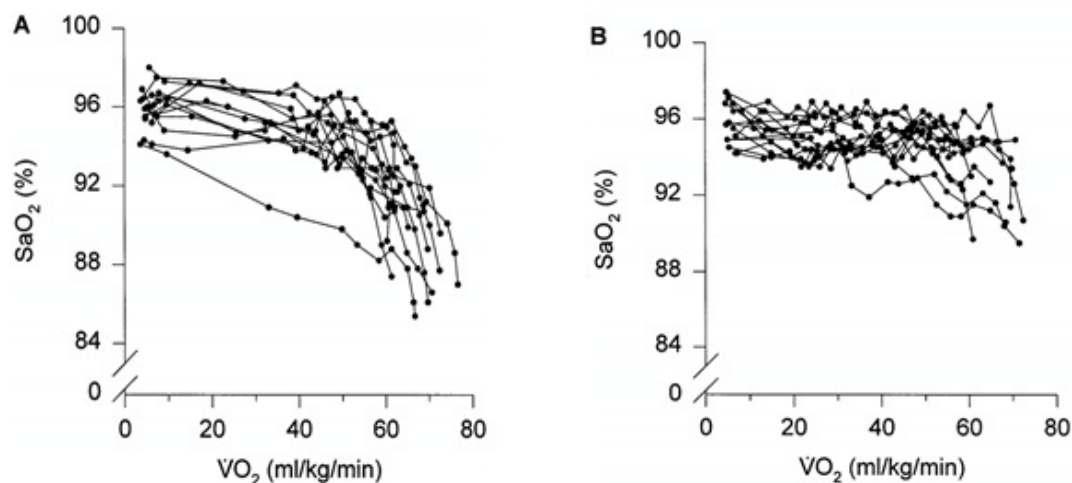


Figura 11. Saturación arterial de oxígeno (SaO_2) durante el reposo y el durante un maximal. A. Datos individuales de la carrera en cinta rodante; B. Datos individuales de la ergometría en bicicleta. Modificada de Gavin et al. (380).

Sin embargo, Rice et al. (381) demostraron que la disciplina de entrenamiento puede influir en el EIAH. Durante 5 minutos de ejercicio al 95% del VO_{2MAX} , los corredores y los ciclistas mostraron una tendencia hacia una interacción para la PaO_2 entre la disciplina de entrenamiento y la modalidad de ejercicio. En otro grupo de mujeres atletas ($VO_{2MAX} = 51 \pm 2 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$), Hopkins et al. (379) mostraron que la PaO_2 era menor durante la carrera en cinta rodante en comparación con la ergometría en bicicleta, y esto se debía a un mayor gradiente alveolar-arterial de oxígeno ($AaDpO_2$) durante la carrera en comparación con la bicicleta. Sin embargo, en este estudio no se controló la fase del ciclo menstrual de este estudio, y la progesterona puede mejorar la ventilación (382).

No obstante, los autores concluyeron que correr debería ser la modalidad de ejercicio preferida para examinar la EIAH (379). Otro autor encontró un menor VE/VCO_2 durante un test submáximo en cicloergómetro en comparación a correr, lo que sugiere que el EIAH podría desarrollarse a intensidades de ejercicio más bajas durante el ciclismo en comparación con la carrera. No hubo diferencias en la presentación de la EIAH entre triatletas y corredores (Figura 12) (383).

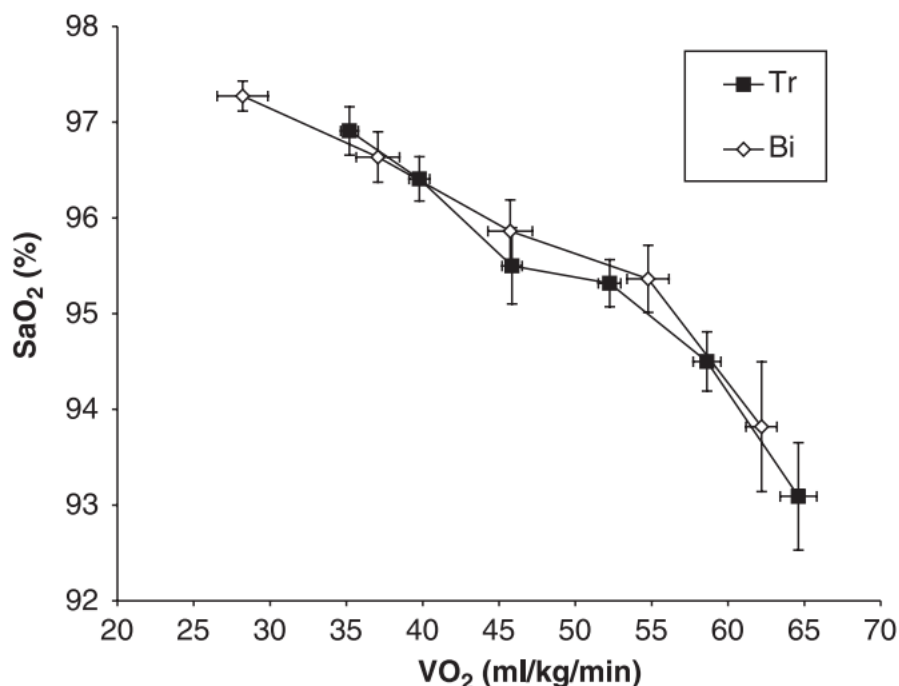


Figura 12. Cambios medios en la saturación de oxihemoglobina (SaO₂) a lo largo de intensidades de ejercicio relativas progresivas entre la ergometría (Bi) y la carrera en cinta rodante (Tr). Modificada de Laursen et al. (383).

2.6.5. Estado ácido-base

Los productos finales del metabolismo incluyen ácidos inorgánicos como el ácido clorhídrico (HCl), el ácido sulfúrico (H₂SO₄) y el ácido fosfórico (H₃PO₄); y varios ácidos orgánicos (por ejemplo, aminoácidos, ácido láctico), que finalmente se oxidan a CO₂ y H₂O. La mayor parte de los 13 moles de CO₂ producidos por un adulto cada día se convierte en ácido carbónico (H₂CO₃) mediante la anhidrasa carbónica. El ácido carbónico se disocia con iones H⁺ y bicarbonato (HCO₃⁻) (384).

El CO₂ es transportado a los pulmones como:

- Bicarbonato disuelto en el plasma (60%).
- Bicarbonato dentro de los eritrocitos (29%).
- Hemoglobina-carbamato (Hb-NH.COO⁻) en los eritrocitos.
- CO₂ disuelto en el plasma.

Los ácidos fijos como el HCl, el H₂SO₄ y el H₃PO₄ son excretados por los riñones como NH₄⁻, HSO₄⁻ y H₂PO₄⁻ respectivamente.

En condiciones de reposo, los fluidos corporales tienen más bases (HCO_3^- , fosfatos (P_i) y las proteínas) que ácidos, lo que produce un pH en los tejidos que oscila entre 7.1 en los músculos y 7.4 en la sangre arterial. Los límites tolerables para el pH de la sangre arterial van desde 6.9 hasta 7.5, aunque estos extremos pueden tolerarse solamente durante unos pocos minutos. Una concentración de H^+ por encima de lo normal recibe la denominación de acidosis, mientras que una reducción de los H^+ por debajo de su concentración normal recibe la denominación de alcalosis. El pH de los fluidos corporales intra y extracelulares está mantenido dentro de una amplitud relativamente limitada por (385):

- Amortiguadores químicos
- Ventilación pulmonar
- Función de los riñones

Los 3 amortiguadores químicos más importantes del cuerpo son el HCO_3^- , los P_i y las proteínas. Además de estos, la hemoglobina de los glóbulos rojos también actúa como un potente amortiguador. La Tabla 7. muestra las contribuciones relativas de estos amortiguadores al control de los ácidos en la sangre. Como se ha comentado anteriormente, el HCO_3^- se combina con los H^+ para formar ácido carbónico, eliminando con ello su influencia acidificante, a su vez, el ácido carbónico forma CO_2 y agua en los pulmones, y finalmente, el CO_2 será eliminado por la vía respiratoria (385).

Tabla 7. Capacidad de amortiguación de los componentes de la sangre.

Amortiguador	Slykes ^a
<i>Bicarbonato</i>	18.0
<i>Hemoglobina</i>	8.0
<i>Proteínas</i>	1.7
<i>Fosfatos</i>	0.3
Total	28.0

^a Miliequivalentes de iones de hidrógeno tomados por cada litro de sangre desde un pH. de 7.4 hasta 7.0.

El HCO_3^- en sangre disminuye en la acidosis metabólica aguda porque el HCO_3^- actúa como tampón. En la acidosis metabólica crónica, el aumento de la reabsorción

renal de HCO_3^- da lugar a un aumento de la HCO_3^- en sangre. Por otro lado, una alcalosis metabólica pura secundaria a la pérdida de H^+ (por ejemplo, vómitos prolongados, tratamiento diurético crónico) provoca un aumento de HCO_3^- en sangre. Además, el exceso de bases es negativo en una acidosis metabólica (exceso de bases < -2 mmol/L) y positivo en una alcalosis metabólica (exceso de bases $> +2$ mmol/L) (Tabla 8). También tenemos el Anion Gap ($\text{Na}^+ + \text{K}^+ - \text{Cl}^- - \text{HCO}_3^-$) que se utiliza para diferenciar las causas de acidosis y alcalosis metabólica. Los aniones no medidos (p. ej., proteínas, lactato, cetonas, fosfatos y sulfatos) representan un Anion Gap de 8-16 mmol/L. Y factores como la cetosis, la insuficiencia renal, la acidosis láctica, los fármacos ácidos (penicilina, salicilatos) y la intoxicación por alcohol (metanol, etanol) aumentan este marcador metabólico. La acidosis metabólica con un Anion Gap normal suele indicar una acidosis hiperclorémica: pérdida de álcali (por ejemplo, HCO_3^-) o acumulación de cloruro (por ejemplo, HCl , NH_4Cl). Las causas de la acidosis con Anion Gap normal incluyen la diarrea excesiva, las fístulas gastrointestinales y la acidosis tubular renal (372). Se ha establecido que el Anion Gap de una persona normal es de unos 12-20mmol/L, y si está ≥ 20 mmol/L, hay un 67% de probabilidad de acidosis metabólica y si está ≥ 30 mmol/L, hay un 100% de probabilidad de acidosis metabólica, por ejemplo, en los casos en que hay acumulación de ácidos como el salicílico, el láctico, el pirúvico, el acetoacético y sulfúrico (373).

Tabla 8. Caracterización de la gravedad de la acidosis y la alcalosis. Modificada de et al. (372).

	<----- Acidosis ----->				<----- Normal ----->			<----- Alkalosis ----->			
Base excess	-13	-9	-6	-4	-2	0	+2	+4	+6	+9	+13
PaCO₂ (kPa)	8.3	7.3	6.7	6.1	5.7	5.3	4.9	4.4	4.0	3.3	2.4
PaCO₂ (mmHg)	62	55	50	46	43	40	37	33	30	25	18
	Severe	Marked	Moderate	Mild	Minimal	Normal	Minimal	Mild	Moderate	Marked	Severe

Base excess = exceso de bases; PaCO₂ = presión arterial de dióxido de carbono.

2.6.6. Efectos ácido-base del metabolismo celular durante el ejercicio

Los principales iones que intervienen en la determinación de SID ($(\text{Na}^+) + (\text{K}^+) - ((\text{Cl}^-) + (\text{lactato}))$) son: 1) Cl^- (típicamente en equilibrio electroquímico a través del sarcolema debido a la alta conductancia de Cl^- de esta membrana); 2) Na^+ y (3) K^+ cuyas concentraciones están determinadas principalmente por la $\text{Na}^+\text{-K}^+$ ATPasa y

secundariamente por el cotransportador $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ y otros canales catiónicos; 4) PC que está determinado por sus tasas simultáneas y continuas de degradación y resíntesis; y 5) el lactato, cuya concentración está determinada por la tasa de producción de piruvato glucolítico, la conversión de piruvato en lactato (en parte determinada por la conversión de piruvato en acetil CoA y la tasa metabólica aeróbica), y los efectos netos del transportador de monocarboxilato (MCT) facilitaron la captación y el eflujo (386).

Los cambios en las variables ácido-base independientes a al H^+ muscular en un estudio en el que los hombres realizaron un test en bici de muy alta intensidad (30 s seguido de 90 min de recuperación), mostro cambios en las variables ácido-base independientes PCO_2 , Atot (albumina y fosfato) y SID (387, 388). Inmediatamente después del ejercicio, el lactato intracelular aumentó a 47 meq/L, explicando casi por completo la reducción de la diferencia intracelular de iones fuertes SID de 154 a 106 meq/L. Al mismo tiempo, la PCO_2 venosa femoral aumentó a 100 Torr y la plasmática a 9,7 meq/L; sin embargo, la SID plasmática no cambió debido a un aumento concomitante de la SID inorgánica secundaria a los aumentos de K^+ , Na^+ y Ca_2^+ . En la Figura 13, aparecen las interacciones ácido-base en todo el cuerpo durante ejercicio de alta intensidad.

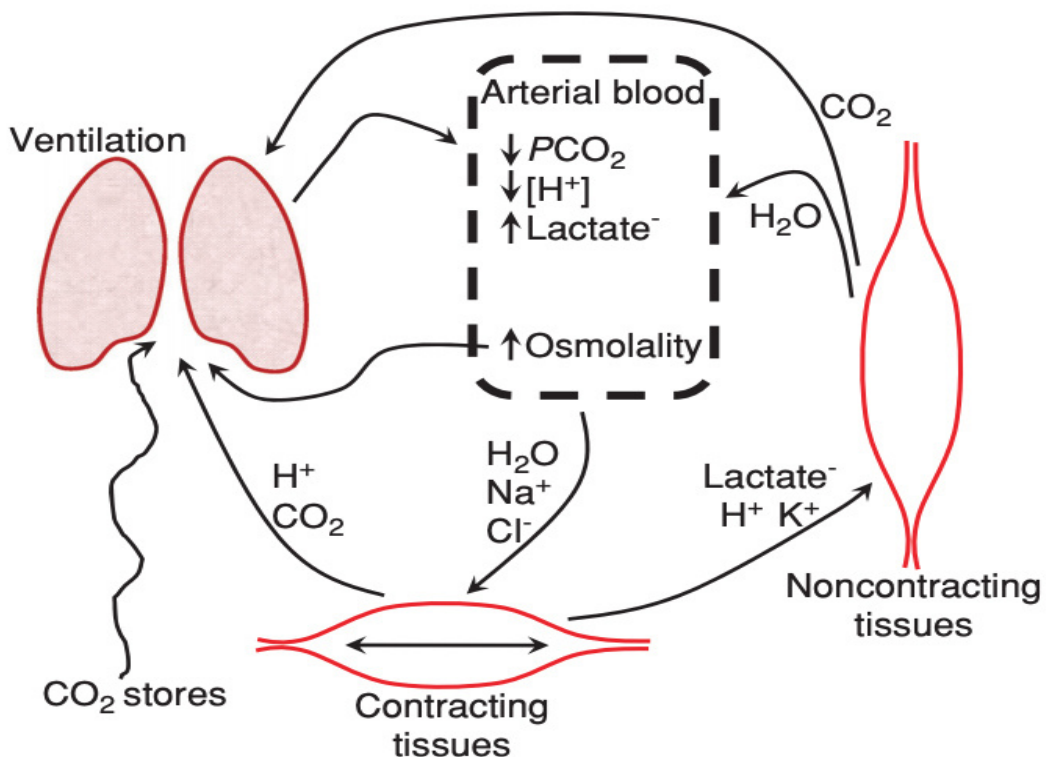


Figura 13. *Esquema de las interacciones ácido-base en todo el cuerpo durante ejercicio de alta intensidad. CO₂ = dióxido de carbono; H⁺ = hidrogenión; Cl⁻ = cloro; K⁺ = potasio; Na⁺ = sodio; PCO₂ = presión parcial de dióxido de carbono. Modificada de Stickland et al. (386).*

Esto es debido, a que el suministro de ATP en el músculo esquelético durante la transición del reposo al ejercicio, durante el aumento de la intensidad del trabajo y durante la realización de ejercicio de alta intensidad, depende en gran medida de la degradación de PC y en la glucólisis con la consiguiente producción de lactato. La degradación de la PC da lugar a un aumento de la SID intracelular (389), por lo tanto, tiene un efecto alcalinizante inicial en el equilibrio ácido-base intracelular (386). El almacén de PC es muy limitado y la degradación neta se ralentiza rápidamente (en 2 min) cuando la demanda de ATP es máxima, por lo que la alcalinización que se produce es breve. Es breve porque la glucólisis, suministrada por las reservas de glucógeno dentro del músculo y la capacidad del músculo para extraer glucosa de la circulación, se activa rápida y a una alta magnitud. El aumento del flujo glucolítico da lugar a la producción de piruvato, que se convierte rápidamente en lactato, ambos aniones ácidos fuertes. La producción y acumulación de lactato dentro del músculo puede ser rápida (390, 391), con tasas de producción de aproximadamente 60 mEq/L/min y concentraciones intracelulares que superan los 45 mEq/L (391). Simultáneamente se produce el aumento del lactato intracelular, que conlleva a una rápida y considerable pérdida de K⁺ intracelular hacia los fluidos extracelulares y el plasma como resultado de las repetidas despolarizaciones y repolarizaciones del sarcolema. La disminución de la K⁺ contribuye aproximadamente en un 50% a la disminución de la SID intracelular. La disminución de la SID puede ocurrir muy rápidamente y ser muy grande, y el aumento asociado de H⁺ intracelular es indicativo de la pronunciada acidificación intracelular (392).

El H⁺ en los tejidos en contracción y en la circulación venosa aumentará durante el período de ejercicio de alta intensidad y producirá un modesto aumento del H⁺ arterial al final del ejercicio y en la recuperación posterior al mismo (387, 388). Dentro del músculo en contracción, el aumento de la H⁺ es muy rápido, lo que da lugar a la recuperación de las reservas de CO₂ que, junto con el aumento de la producción mitocondrial de CO₂, da lugar a un aumento pronunciado (hasta 100 mmHg) de la PCO₂ muscular y venosa. Para entonces, la VE es ya tan alta que la PCO₂ arterial cae por debajo (hasta 30 mmHg) de los valores de reposo (388, 392).

Incluso a intensidades de ejercicio más bajas, la contracción del músculo esquelético provoca un aumento progresivo de las actividades del ciclo de los ácidos tricarbónicos (TCA), la β-oxidación y las enzimas de la cadena respiratoria, de forma que el aumento del flujo de carbonos de los carbohidratos y los ácidos grasos a través de los sistemas oxidativos da lugar a la activación de la piruvato deshidrogenasa (393)

y al consiguiente aumento de la producción de CO_2 mitocondrial. Esto hace necesaria la rápida eliminación del CO_2 de las células para minimizar la acidificación celular. Para lograrlo, hay una serie de isoformas de anhidrasa carbónica presentes en las membranas mitocondriales (394), el sarcolema, las membranas del sistema T, dentro del citoplasma y en el líquido intersticial y el plasma sanguíneo (395). Las isoformas de anhidrasa carbónica también son abundantes en la membrana plasmática de los eritrocitos y dentro de los eritrocitos y los pulmones (396, 397). Los eritrocitos desempeñan un profundo papel en la amortiguación de los aumentos de H^+ , lactato y pCO_2 (390, 398-400), de manera que en ausencia de eritrocitos las concentraciones de estos metabolitos en el plasma serían probablemente tóxicas (394) (Figura 14). El cuerpo está así dotado de un sistema físico-químico para la rápida transferencia de CO_2 de las células a pulmones y al medio ambiente (atmósfera).

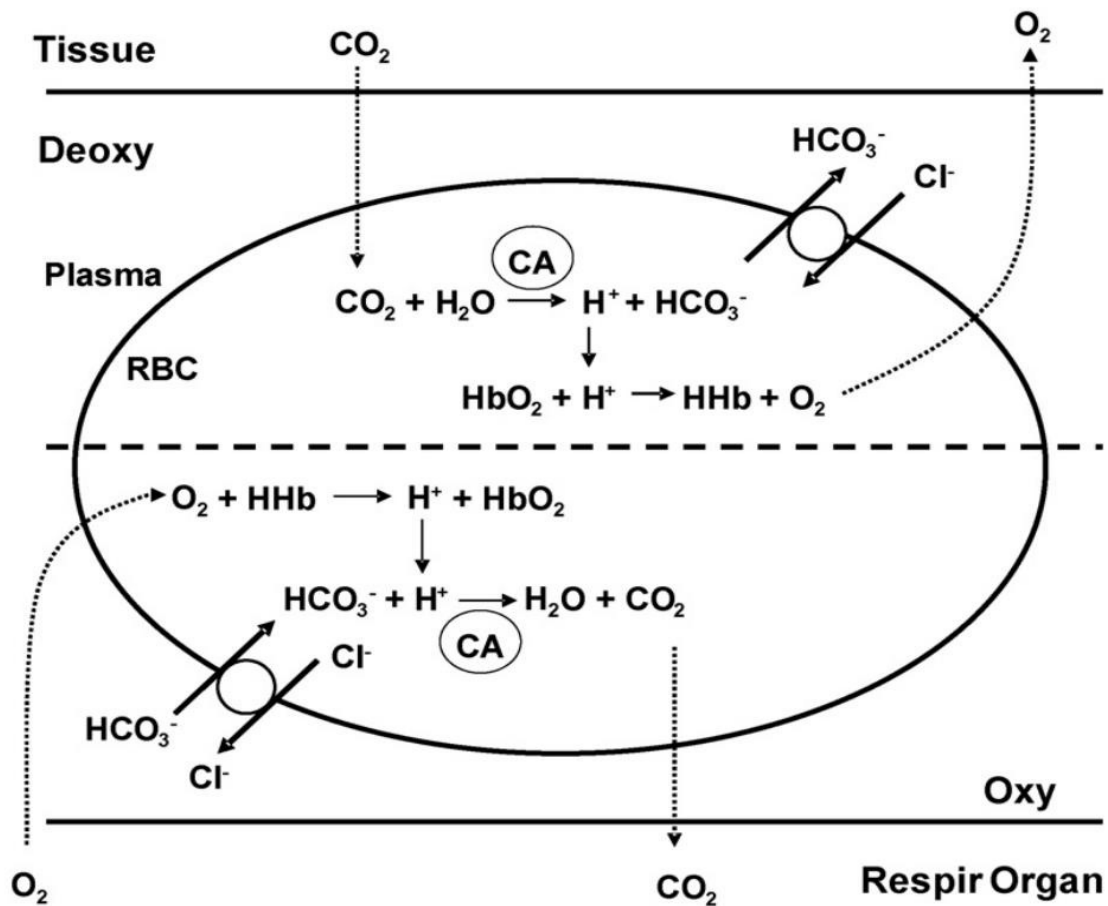


Figura 14. El papel de la anhidrasa carbónica de los glóbulos rojos en el transporte de gases de los vertebrados. Modificada de Esbaugh et al. (394).

La relación entre la H^+ muscular y la PCO_2 durante 30 s de ejercicio de muy alta intensidad y 9,5 min de recuperación se muestra en la Figura 15. Debido al elevado

Atot (fuerte amortiguación de protones sin bicarbonato) dentro del músculo esquelético ($A_{tot} = 140 \text{ mEq/L}$) se requieren grandes cambios en la PCO_2 para producir cambios significativos en H^+ y HCO_3^- . Además de estos mecanismos no aeróbicos, también se produce un rápido aumento simultáneo de la liberación de CO_2 del músculo debido tanto a la titulación ácida de los "almacenes" de CO_2 (bicarbonato, carbonato, compuestos carbamínicos dentro de los tejidos (388, 400-402) como al aumento del metabolismo aeróbico (390, 402). Esta liberación de CO_2 almacenado también es fácilmente evidente en los tejidos no contráctiles durante y después de períodos de ejercicio de alta intensidad (387, 400, 401). Una indicación de la tasa de producción y liberación total de CO_2 por el músculo en contracción y los tejidos sin contracción puede obtenerse de la diferencia de PCO_2 venosa-arterial (Figura 15).

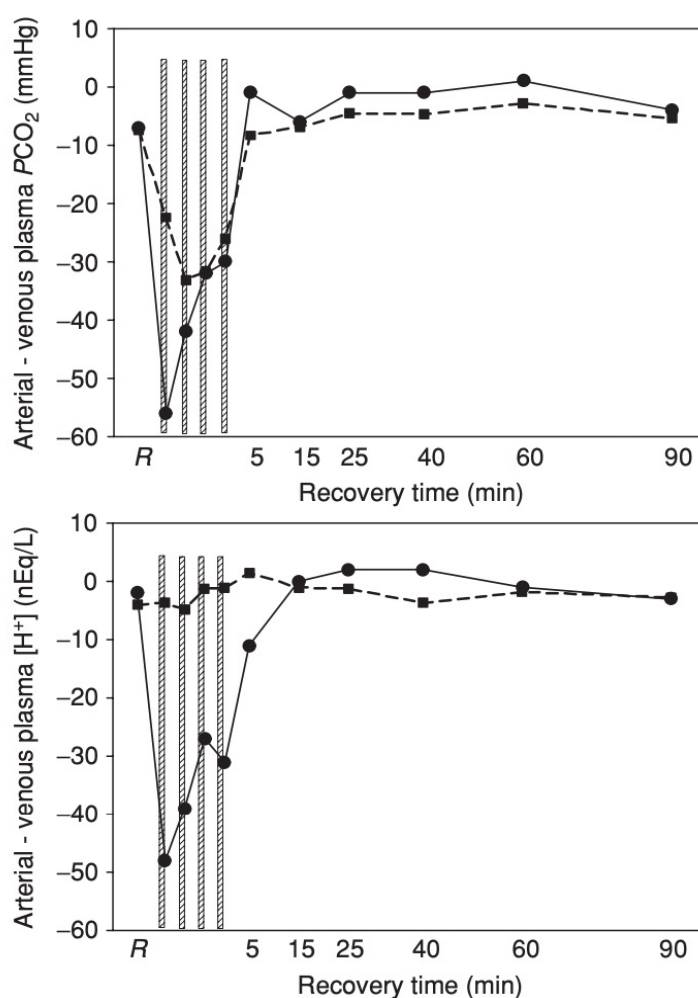


Figura 15. Diferencias de PCO_2 = presión de dióxido de carbono (panel superior) y H^+ = hidrogeniones (inferior) entre la arteria y la vena antero cubital (▪) y la arteria y la vena femoral (•) durante 4 ejercicios repetidos de 30 s de muy alta intensidad intercalados con 4 minutos de descanso, seguidos de 90 minutos de recuperación. Modificada de Stichkland et al. (386).

A medida que la intensidad del ejercicio aumenta de baja a alta, también habrá una contribución creciente de la deshidratación de HCO_3^- resultante de la acidificación, de manera que la valoración de las reservas de CO_2 (principalmente bicarbonato) dentro de los tejidos no contráctiles y dentro del músculo contráctil genera CO_2 que contribuye a la elevación de la PCO_2 plasmática y de la H^+ durante el ejercicio, y que ambos contribuyen al impulso ventilatorio para aumentar el VE (386).

El efecto principal de los cambios en las concentraciones de iones sobre el H^+ muscular durante la contracción es una disminución del K^+ intracelular que reduce la SID intracelular (ya que el Na^+ y el Cl^- se acumulan en grados similares). Con el ejercicio de alta intensidad, la reducción neta de K^+ se produce rápidamente y, a través de su efecto sobre la SID intracelular, contribuye a los aumentos de H^+ . Sin embargo, este cambio coincide con la hidrólisis simultánea de la PC (386). La rápida hidrólisis de la PC reduce su concentración, lo que tiene un efecto directo en el aumento de la SID intracelular y, por lo tanto, contribuye a la disminución de los H^+ . En los primeros segundos de la transición de reposo a trabajo, la disminución de la PC eleva efectivamente la SID y por lo tanto la H^+ debe disminuir. También debe reconocerse que la hidrólisis de PC resulta en la producción de creatina, que es electroneutral, y que la subsiguiente hidrólisis de ATP de la reacción de la creatina quinasa resulta en la producción del ácido débil fosfato inorgánico. La hidrólisis de la PC es, por tanto, un potente medio para aumentar el SID y para reducir el H^+ intracelular, aunque aumentando modestamente la A_{tot} (386). Con el aumento de la actividad glucogenolítica y glucolítica, el anión ácido fuerte lactato aumenta progresivamente su concentración y su acumulación neta no se equilibra eficazmente por los cambios simultáneos en otros iones fuertes intracelulares. Así, las disminuciones concurrentes de K^+ intracelular y los aumentos de lactato resultan en disminuciones progresivas de la SID intracelular porque la PC no cambia más o puede aumentar si se reduce la demanda de ATP. La acumulación y/o eliminación desigual de cationes y aniones durante el ejercicio conduce a una disminución de la SID intracelular (equilibrio alterado de iones fuertes y débiles en la solución) que contribuye directa y fisicoquímicamente al aumento de la H^+ durante el ejercicio de intensidad moderada a alta. La magnitud de este aumento es proporcional a: 1) las concentraciones de ácidos débiles disociados (es decir, la capacidad estructural de amortiguación de protones no bicarbonatada del músculo; 2) la velocidad a la que los equivalentes ácidos (aniones ácidos fuertes como PC, piruvato y lactato) se acumulan o se eliminan del músculo o se consumen dentro del músculo; y 3) la velocidad a la que los cationes de base fuerte se añaden o se eliminan del músculo (386).

2.7. EFECTOS DEL EJERCICIO DE RESISTENCIA EN A NIVEL METABÓLICO-BIOQUÍMICO

2.7.1. Efectos de la intensidad y la duración del ejercicio en el metabolismo energético

Las grasas y los hidratos de carbono son los principales sustratos que alimentan la síntesis de ATP aeróbica en el músculo esquelético humano. La utilización relativa de las grasas y los hidratos de carbono durante el ejercicio puede variar enormemente y depende en gran medida de la intensidad del mismo (403). A medida que la intensidad del ejercicio pasa de moderada (es decir, 65% del VO_{2MAX}) a alta intensidad (85% del VO_{2MAX}), la glucogenólisis muscular, la glucogenólisis hepática y la captación de glucosa aumentan de manera que predomina el metabolismo de los hidratos de carbono (HC) (403, 404). Por el contrario, se produce una reducción de la oxidación de lípidos en todo el cuerpo debido a una reducción de los ácidos grasos libres (FFA) en plasma y de la oxidación de triglicéridos intramusculares. Se considera que las tasas máximas de oxidación de lípidos se producen en torno al 65% del VO_{2MAX} , aunque esto depende de otros factores, como el estado de entrenamiento, el sexo y la dieta (405).

Además, a intensidades bajas-moderadas de ejercicio, tenemos que diferenciar 2 términos claramente diferentes que son la máxima de oxidación de grasas (MFO) durante el ejercicio y la intensidad a la que produce la MFO (FatMax) (Figura 16), estos pueden ser afectados por el tipo de entrenamiento y dieta.

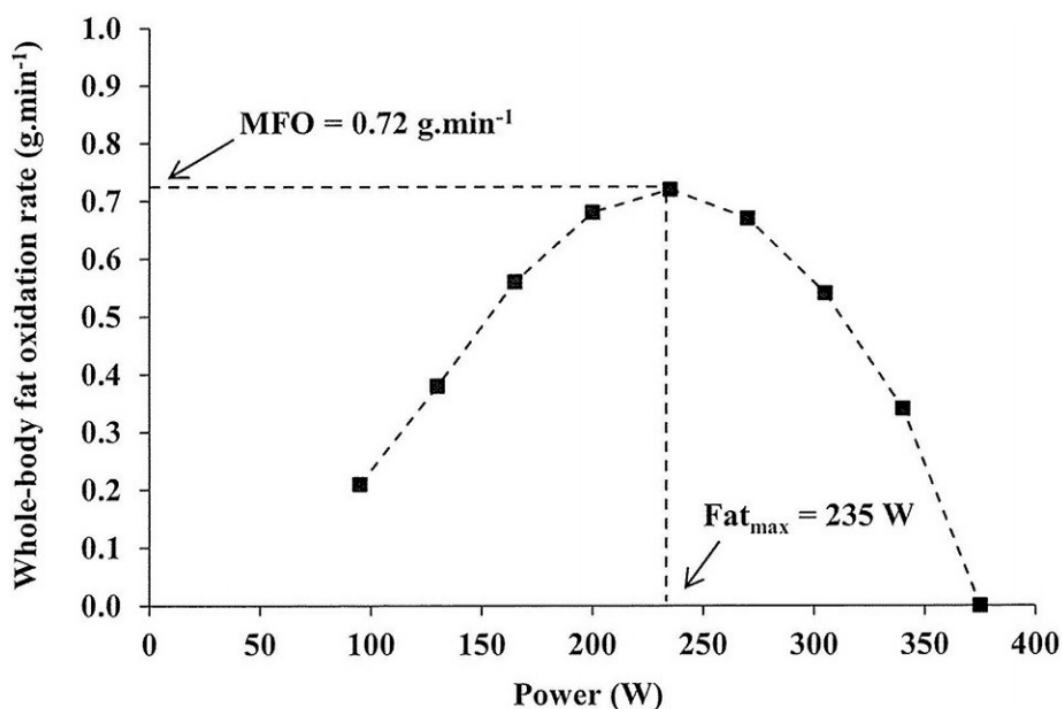


Figura 16. Ilustración representativa de la oxidación de las grasas ($\text{g}\cdot\text{min}^{-1}$) frente a la carga del ejercicio (W) durante una prueba FatMax gradual, en la que MFO, tasa máxima de oxidación de las grasas ($\text{g}\cdot\text{min}^{-1}$) y FatMax, la carga a la que se produce la MFO (W). Modificada de Maunder et al. (406).

Se ha demostrado que las tasas máximas de oxidación de grasas se alcanzan a intensidades entre el 59% y el 64% del $\text{VO}_{2\text{MAX}}$ en individuos entrenados y entre el 47% y el 52% del $\text{VO}_{2\text{MAX}}$ en una amplia muestra de la población general. Además, el modo de ejercicio también puede afectar a la oxidación de las grasas, siendo ésta mayor durante la carrera que durante el ciclismo (405). Donde la MFO llega alcanzar la media de $0.59 \pm 0.18 \text{ g}\cdot\text{min}^{-1}$, oscilando entre 0.17 y $1.27 \text{ g}\cdot\text{min}^{-1}$ (407).

En contraste con el ejercicio de alta intensidad, el ejercicio prolongado en estado estacionario que dura varias horas (57% del $\text{VO}_{2\text{MAX}}$ durante 240 min) se caracteriza por un cambio hacia una mayor oxidación de lípidos y una reducción de las tasas de oxidación de HC (408) (Figura 17). Este cambio en las tasas de oxidación va acompañado de un aumento de la contribución de los FFA plasmáticos al gasto energético (Figura 17) y una menor dependencia del glucógeno muscular y de los triglicéridos intramusculares (IMTG) (408). Los estudios que examinan los mecanismos reguladores que subyacen a este cambio en la utilización de sustratos han sugerido que una reducción en la disponibilidad de glucógeno muscular (debido al agotamiento progresivo del glucógeno y, por lo tanto, a una reducción del flujo glucolítico) regula a la baja la actividad de la enzima piruvato deshidrogenasa (PDH), lo que conduce a una reducción de la oxidación del HC. Además, el aumento progresivo de la disponibilidad de FFA en plasma (debido a la lipólisis continua en el tejido adiposo) estimula la oxidación de lípidos. La regulación a la baja de la actividad de la PDH a medida que avanza la duración del ejercicio puede deberse a la reducción del flujo de piruvato, reduciendo así la producción de sustrato necesaria para la reacción de la PDH (408).

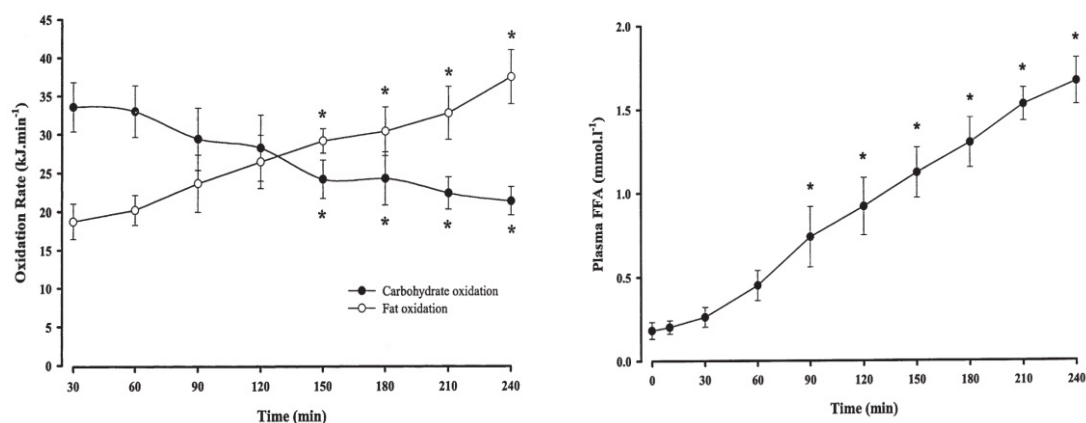


Figura 17. *Tasas de oxidación de carbohidratos y grasas en todo el cuerpo de oxidación de grasas durante 240 minutos de ejercicio moderado en hombres (izquierda). Concentración de FFA en plasma antes y durante 240 minutos de ejercicio moderado en hombres (derecha). Diferencias significativas ($P < 0.05$) comparado con valores de reposo. Modificado de Watt et al. (408).*

Además, datos más recientes demuestran la disminución del flujo glucolítico y el aumento crónico de la actividad de la piruvato deshidrogenasa quinasa (PDK) atenúa la actividad de la PDH y la oxidación de hidratos de carbono al final de un ejercicio prolongado (4 horas de ejercicio de al 55% del VO_{2MAX}) (409).

Con el aumento de la intensidad del ejercicio, además del glucógeno muscular, la contribución de la glucosa plasmática a la producción de ATP también aumenta. La explicación más probable es que esto se debe al aumento del flujo sanguíneo muscular (y, por lo tanto, del suministro de sustrato), además del aumento del reclutamiento de las fibras musculares (410). Por otro lado, la entrega de glucosa al músculo en contracción es, por supuesto, también un reflejo del aumento de las tasas de glucogenólisis hepática de acuerdo con el aumento de la intensidad del ejercicio (411). Dentro de los mecanismos celulares, aunque la captación de glucosa también está regulada por el contenido en el transportador de glucosa específico del músculo esquelético (GLUT4), es poco probable que éste desempeñe un papel en esta situación, dado que la translocación de GLUT4 a la membrana plasmática no aumenta con la intensidad del ejercicio (412). Una vez que la glucosa es transportada al citosol, es fosforilada a glucosa-6-fosfato bajo el control de la hexoquinasa. Las pruebas sugieren que la actividad de la hexoquinasa tampoco es limitante, dado que los pacientes con diabetes tipo 2 (que tienen una actividad máxima reducida de la hexoquinasa) muestran patrones normales de captación de glucosa inducida por el ejercicio, probablemente debido a una perfusión normal y a la translocación de GLUT4 (413). Por el contrario, durante el ejercicio intenso a una intensidad casi máxima o supra-máxima, la fosforilación de la glucosa puede limitar la tasa de utilización de la glucosa dado que las altas tasas de glucosa-6-fosfato, secundarias a la descomposición del glucógeno muscular, pueden inhibir directamente la actividad de la hexoquinasa (414). Una vez que la glucosa entra en la vía glucolítica, se considera que la enzima limitante de la glucólisis es la fosfofructoquinasa (PFK). La PFK es activada alostéricamente por el adenosín difosfato (ADP), el adenosín monofosfato (AMP) y el Pi, y es probable que este mecanismo explique las altas tasas de glucólisis durante el ejercicio intenso, incluso ante la acidosis metabólica, cuando la PFK podría estar inhibida (Figura 18) (415).

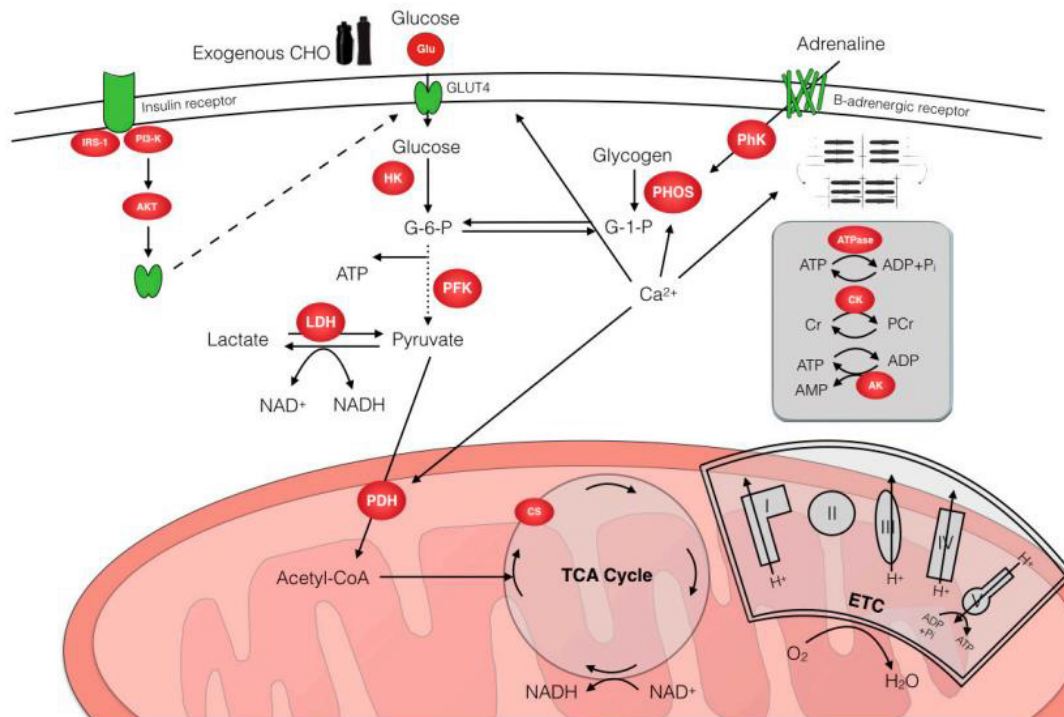


Figura 18. Esquema general del metabolismo del HC y los puntos de control. Las enzimas reguladoras clave son bien reconocidas como la fosforilasa (PHOS), hexoquinasa (HK), fosfofructoquinasa (PFK), lactato deshidrogenasa (LDH) y piruvato deshidrogenasa (PDH). Además, la tasa de captación de glucosa muscular también puede determinar el flujo a través de la glucólisis. Modificada de Hearn et al (415). Abreviaturas: ADP, adenosín difosfato; AK, adenilato quinasa; AKT, proteína quinasa B; AMP, adenosín monofosfato; ATP, adenosín trifosfato; Ca^{2+} , calcio; HC, hidratos de carbono; CK, creatina quinasa; Cr, creatina; CS, citrato sintasa; ETC, cadena de transporte de electrones; G-1-P, glucosa-1-fosfato; G-6-P, glucosa-6-fosfato; Glu, glucosa; GLUT4, transportador de glucosa 4; H^+ , ion hidrógeno; H_2O , agua; IRS-1, sustrato del receptor de insulina 1; O_2 , oxígeno; NAD, dinucleótido de adenina nicotinamida; Ciclo TCA, ciclo del ácido tricarbóxico; Pi, fosfato; PC, fosfocreatina; PhK, fosforilasa cinasa; PHOS, glucógeno fosforilasa; PI3-K, fosfoinositol 3 quinasa

2.8. NUTRICIÓN DEPORTIVA

La optimización de la nutrición del deportista es determinante para obtener el máximo rendimiento en todas las especialidades deportivas. Ésta depende de diferentes factores que hay que tener en cuenta como: el estado de la fase del organismo, las características individuales, la edad y el sexo y otras características. Pero es de mayor importancia para los atletas que practican deporte de resistencia, por ejemplo, ciclismo y maratón (416).

Durante más de 50 años de investigación se han estudiado estrategias para preparar la competición (la alimentación previa al ejercicio), promover el rendimiento durante la competición (la ingesta de líquidos y la alimentación con carbohidratos) y la recuperación después de la competición (por ejemplo, la alimentación con carbohidratos y proteínas para promover la recuperación muscular) (417). Además, muchos investigadores han estudiado las ayudas ergogénicas que pueden mejorar el rendimiento durante el ejercicio y/o la fatiga mediante la modulación de aspectos centrales o periféricos de la fatiga (417). Por lo tanto, la utilización de estrategias de nutrición en competición que se centran en una ingesta suficiente de macronutrientes y de ayudas ergogénicas para promover la disponibilidad de energía y retrasar los determinantes bioquímicos de la fatiga, tienen como objetivo una mejora del rendimiento del deportista.

En la última década, se han publicado una gran cantidad de trabajos científicos que han demostrado un potente papel de la disponibilidad de macro y micronutrientes en la regulación de las vías de señalización celular inducidas por el ejercicio que se cree que regulan las adaptaciones del músculo esquelético en el entrenamiento (417). Por ello, tanto los investigadores como los profesionales que se dedican al mundo de la nutrición, están empezando a tratar la nutrición para la competición y la nutrición para el entrenamiento como dos entidades distintas. La primera con un enfoque obvio de rendimiento y la segunda con un enfoque de adaptación. Por ejemplo, en el caso del ejercicio de resistencia, los datos emergentes sugieren que los períodos de reducción de carbohidratos (y alta disponibilidad de grasas) pueden mejorar las adaptaciones fundamentales para el rendimiento de la resistencia, como la biogénesis mitocondrial, el aumento de la oxidación de los lípidos y una mayor resistencia a la fatiga (418, 419). Estas adaptaciones son debidas a que la restricción de carbohidratos y/o el agotamiento del glucógeno durante el ejercicio conducen a una mayor aumento de la actividad de la proteína quinasa activada por adenosín monofosfato (AMPK), quinasa activada por mitógenos p38 y sirtuina 1 (SIRT1) (Figura 19) (417).

Del mismo modo, también están apareciendo muchos nuevos compuestos (epicatequinas y resveratrol (polifenoles), nicotinamida, etc.) (aunque de estudios con roedores) que también pueden regular las vías de señalización inherentes al entrenamiento de resistencia (417). Los polifenoles (hesperidina, resveratrol, naringina, catequinas, etc.), son moléculas que tienen potentes efectos de eliminación de radicales libres y/o quelación de metales (193). Los mecanismos antioxidantes indirectos de los polifenoles incluyen la inducción de la expresión génica antioxidante endógena y la regulación de las enzimas productoras de especies reactivas de oxígeno (ROS)/nitrógeno (RNS) y de los factores de transcripción relacionados con el sistema redox. Además, algunos tienen la capacidad de capturar radicales libres de forma directa (193). Aunque, actualmente y debido a la escasa evidencia encontrada en relación a la ingesta de polifenoles y el rendimiento deportivo, no está claro si su recomendación por parte de los nutricionistas deportivos y otros profesionales deportivos puede ejercer un efecto positivo sobre el rendimiento.

Debido a la falta de evidencia científica sobre algunos suplementos, existen varios organismos que han categorizado y clasificados a estos. Nosotros nos vamos a centrar en el sistema de clasificación ABCD que clasifica los alimentos para deportistas y los ingredientes de los suplementos en 4 grupos atendiendo a las pruebas científicas encontradas, según la última actualización (2021) del “Australian Institute of Sports” (AIS) (420). La clasificación que establece AIS es la siguiente:

- **Categoría A:** evidencia científica sólida para su uso en situaciones específicas en el deporte utilizando protocolos basados en la evidencia.
 - Alimentos para el deporte: bebidas deportivas, geles deportivos, confitería deportiva, suplementos con electrolitos, proteínas de suero de leche, macronutrientes mixtos y barritas energéticas.
 - Suplementos médicos: hierro, calcio, vitamina D, multivitamínico, probióticos y zinc.
 - Suplementos para mejorar el rendimiento: cafeína, β -alanina, bicarbonato, zumo de remolacha/nitratos, creatina y glicerol.
- **Categoría B:** apoyo científico emergente, merecedor de más investigación.
 - Polifenoles: compuestos alimentarios que pueden tener bioactividad, incluyendo propiedades antioxidantes y antiinflamatorias. Puede consumirse en forma de alimentos (enteros o concentrados) o como extractos aislados (hesperidina, hesperetina, resveratrol, epicatequinas, ect.).
 - Antioxidantes: compuestos que a menudo se encuentran en los alimentos y que protegen contra el daño oxidativo de los radicales libres (Vitamina C, N-Acetilcisteína).

- Saborizantes: compuestos derivados de los alimentos que interactúan con los receptores de la boca/intestino para activar el sistema nervioso central (mentol, agonistas de los receptores de potencial transitorio y quinina).
- Otros: colágeno, curcumina, cuerpos cetónicos, aceite de pescado y carnitina.
- **Categoría C**: la evidencia científica no apoya el beneficio entre los atletas o no se ha realizado investigación para orientar una opinión informada.
 - Productos de las categorías A y B utilizados fuera de los protocolos aprobados (polifenoles derivados de las frutas).
 - Otros productos: suplementos que han sido trasladados a una nueva categoría (magnesio, ácido alfa-lipoico, HMB, BCAA/Leucina, fosfato, prebióticos, vitamina E y tirosina).
 - El resto: si no encuentra un ingrediente/ producto en los grupos A, B o D, probablemente merezca estar aquí.
- **Categoría D**: productos prohibidos o con alto riesgo de contaminación con sustancias que podrían dar lugar a un control de dopaje positivo.
 - Estimulantes: efedrina, estricnina, sibutramina, metilhexanamina (DMAA), 1,3-dimetilbutilamina (DMBA) y otros estimulantes de origen vegetal.
 - Prohormonas y potenciadores hormonales: dehidroepiandrosterona (DHEA), androstenediona, 19-norandrostenediona/ol, otras prohormonas, tribulus terrestris y otros potenciadores de la testosterona y raíz de maca en polvo.
 - Liberadores de GH y péptidos
 - Beta-2 agonistas: Higenamina.
 - Moduladores selectivos de los receptores de andrógenos (SARMS): andarine, ostarine, ligandrol.
 - Moduladores metabólicos: GW1516 (Cardarine).
 - Otros: Consulte la lista de la AMA para más información, <https://www.wada-ama.org/>; por ejemplo, el calostro no es recomendado por la AMA debido a la inclusión de factores de crecimiento en su composición.

Puede haber varios factores de motivación diferentes para cada categoría de suplementos que son usados por los atletas; donde los alimentos y los suplementos que contienen nutrientes esenciales (por ejemplo, vitaminas) se utilizan principalmente por razones de salud, siendo los efectos sobre el rendimiento secundarios a la mejora de la salud, mientras que las ayudas ergogénicas se utilizan con la intención de maximizar el rendimiento (421). Los suplementos relacionados con el efecto en el rendimiento tienen

un impacto directo sobre la mejora de uno o de varios mecanismos fisiológicos que afectan a diferentes vías metabólicas (422). Sin embargo, otros suplementos pueden tener un impacto indirecto en el rendimiento a través de su capacidad para mejorar el proceso del entrenamiento, a través de su influencia en factores como la modulación del sistema inflamatorio, el estrés oxidativo, las vías de señalización para la adaptación al entrenamiento, o su capacidad para apoyar el rendimiento repetitivo mediante la restauración de la homeostasis entre dos sesiones de ejercicio (422).

Dentro del segundo grupo (impacto indirecto), se encuentran los polifenoles y N-Acetilcisteína, que a su vez están encuadrados en el grupo B del AIS, otorgándole una cierta evidencia científica en relación a sus propiedades antioxidantes. Sin embargo, en relación al rendimiento deportivo, no está tan claro un posible beneficio. Esto es debido a que ciertas sustancias con actividad antioxidante (vitamina C, E, β -caroteno y N-Acetilcisteína) han sido evaluadas mediante una ingesta crónica, para intentar mejorar el rendimiento, y en general, han sido ineficaces, debido a una atenuación de las adaptaciones bioquímicas inducidas por el ejercicio (216, 423). La utilización de este tipo de sustancias (antioxidantes) para mejorar el rendimiento, está basada en la hipótesis de que la rápida elevación en la concentración de las especies reactivas de oxígeno y nitrógeno (RONS; oxidantes) durante el ejercicio puede ser un factor que contribuye a la fatiga muscular (216, 424). Pero también hay que tener en cuenta, que los RONS son necesarios para generar adaptaciones al entrenamiento, ya que, actúan como mensajeros intracelulares para estimular cambios en la función celular y regular la expresión génica (425). Por otro lado, cuando un estudio ha evaluado si la ingesta crónica de polifenoles puede mejorar el rendimiento, se ha encontrado que algunos flavonoides (subgrupo de polifenoles) como la quercetina o picnogenol mejoran el rendimiento a pesar de tener también un efecto directo antioxidante debido a su estructura molecular (53, 426-431). Sin embargo, también existen estudios donde la ingesta de polifenoles no ha mostrado una mejora del rendimiento (432-434).

Por otro lado, hay que diferenciar, de forma clara, las sustancias antioxidantes (micronutrientes) de acción directa como la vitamina C y E (435), de los polifenoles que tienen una acción también directa e indirecta, ya que, son moléculas que interactúan con elementos de transcripción genética. Estos elementos son el NRF1y 2, proteína quinasa activada por adenosínmonofosfato (AMPK), óxido nítrico sintasa endotelial (eNOS), sirtuina 1 desacetilasa dependiente de nicotinamida adenina dinucleótido (SIRT1), coactivador 1 α del receptor activado gamma del proliferador de peroxisomas (PGC-1 α). Estos factores de transcripción activan genes que modulan el sistema antioxidante, biogénesis mitocondrial, flujo sanguíneo y oxidación de grasa e hidratos de carbono (436). La activación de estas vías moleculares permite la mejora de funciones fisiológicas que mejoran el rendimiento deportivo (437, 438).

Revisiones bibliográficas recientes muestran como la ingesta de suplementos con polifenoles pueden ejercer un efecto positivo sobre el rendimiento deportivo (430, 433, 439, 440). Sin embargo, a pesar de estas evidencias científicas, el efecto general de los polifenoles sobre el rendimiento no es concluyente, ya que la mayoría de los estudios tienen un tamaño de muestra pequeño, diferentes tipos de muestra (desentrenados y entrenados) y existen diferencias en el tipo de molécula y cantidades utilizadas, el protocolo de ejercicio utilizado para evaluar el rendimiento y (27). Hasta donde alcanza nuestro conocimiento, solo existe un meta-análisis y una revisión sistemática sobre la quercetina (polifenol), se ha informado de un aumento del rendimiento (\uparrow VO₂MAX y tiempo de ejercicio en VO₂MAX) entre el 0.74 y el 3.0% (430, 440). En contra, existe poca evidencia del efecto de otros polifenoles en el rendimiento, en concreto, dentro de los flavonoides (27), que son un subgrupo de polifenoles y específicamente en relación a la hesperidina. Aunque esta molécula sí ha mostrado mejoras en ensayos clínicos con pacientes con glaucoma (disminución del estrés oxidativo en el ojo) (441), reducción de la presión arterial sistólica en personas pre-hipertensas (modulación expresión de genes relacionados con la hipertensión) (442) y la mejora de la disfunción endotelial en humanos (incremento del NO y descenso inflamación) (31).

En animales, también se ha encontrado una mejora en el déficit de memoria emocional en el envejecimiento en ratas viejas (443), efecto protector sobre hepatocitos durante la lesión inducida por hipoxia/reoxigenación (reducción del antioxidante y antiinflamatorio) (444), efecto antiosteoporótico en ratas ovariectomizadas (reducción del estrés oxidativo e inflamación) (445) y efecto antivírico (COVID-19) (446). Además, la hesperidina también ha mostrado un efecto preventivo y/o terapéutico sobre las enfermedades relacionadas con la obesidad, la diabetes mellitus (antiinflamatorio) (447); efecto preventivo contra la resistencia a la insulina inducida por una dieta alta en grasa en ratas (regulación de la glucólisis y la gluconeogénesis) (448); mejora de la colitis ulcerosa en modelo de ratón (efecto antioxidantes y antiinflamatorio) (449); efecto antidepresivo en ratas (efecto antiinflamatorio) (450); disminución de esteatosis hepática en ratones (incremento de la biogénesis mitocondrial mediada activación de AMPK y PGC-1 α) (451) y un efecto radioprotectivo (reducción del estrés oxidativo e inflamación) (452). Como se puede observar, las mejoras en los diferentes modelos patológicos tras la ingesta de hesperidina, viene por su efecto antioxidante y antiinflamatorio. Además, la hesperetina (un metabolito de la hesperidina) ha mostrado potenciar la producción del adenosín trifosfato (ATP) a nivel intracelular en un 33% y la capacidad mitocondrial en un 25%, mejorando también el estado antioxidante y el rendimiento en animales (35).

Por todo ello, a continuación, vamos a profundizar sobre dicho compuesto.

2.9. HESPERIDINA

2.9.1. Estructura molecular de la hesperidina y sus formas

Los polifenoles son sustancias biológicamente activas que proporcionan color y sabor a diferentes partes de las plantas y las protegen contra patógenos, radiaciones y toxinas (453). Los flavonoides son uno de los polifenoles más comunes, y un gran número de estos compuestos han sido evaluados por sus efectos beneficiosos en la salud humana, en su estado libre o como glucósidos (453, 454). La estructura de los flavonoides se basa en el compuesto principal, la flavona (2-fenilcromona o 2-fenilbenzopirona), caracterizado por un esqueleto de carbono C6-C3-C6 donde los componentes C6 son anillos aromáticos (455). La hesperidina es un glucósido de la flavanona que consiste en la aglicona flavona hesperetina unida a un disacárido (453, 454, 456). La hesperetina tiene la siguiente fórmula molecular $C_{16}H_{14}O_6$ y químicamente es 3',5,7-trihidroxil-4'-metoxiflavonona (455). La unidad de disacárido tiene una fórmula molecular $C_{12}H_{22}O_{10}$, y está compuesta por una molécula de ramnosa y otra de glucosa y puede adoptar 2 formas isoméricas, rutinosa o neohesperidosa (455, 457). La rutina (flavonoide) es químicamente O- α -L-ramnosil-(1 \rightarrow 6)-glucosa y la neohesperidosa es O- α -L-rhamnosil-(1 \rightarrow 2)-glucosa, diferenciándose únicamente en el enlace glucosídico. Por lo tanto, la hesperetina está generalmente glicosilada por disacáridos, como la rutina o la neohesperidosa, y en los cítricos, la forma glicosídica más extendida es el rutinósido (455, 457). Se sabe que existe una relación entre los isómeros de los disacáridos y la presencia o ausencia de amargor en el compuesto. Los rutinósidos presentes en la naranja y el limón son insípidos mientras que los neohesperidosidos presentes en el pomelo son intensamente amargos (455, 458). Además, la hidrólisis ácida de un mol de hesperidina produce un mol de la aglicona hesperetina, D-glucosa y L-ramnosa. Por otro lado, la hesperidina (molécula anterior a la hesperetina) (Figura 19) es un β -7-ramnoglucósido de hesperetina, tiene una fórmula molecular $C_{28}H_{34}O_{15}$, un peso molecular de 610,57 Da y químicamente es 3',5,7-trihidroxil-4'-methoxyflavanone-7-rutinoside (454, 455). En la estructura de la hesperidina, la glucosa está unida a la hesperetina y la ramnosa (455) (Figura 20).

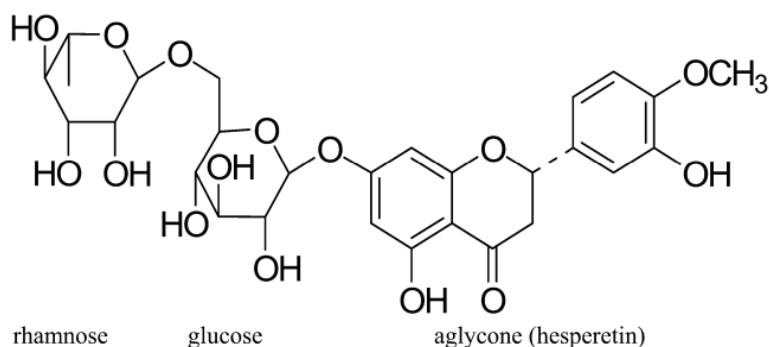


Figura 20. Estructura química de la hesperidina. Modificada de Nielsen et al. (459).

Hay que mencionar, que la hesperidina es el principal flavonoide presente en las naranjas dulces (*Citrus sinensis*) y en el zumo de naranja, pero que también puede encontrarse en otros cítricos como el limón, la lima y la mandarina y en algunas hierbas (460). En concreto, en el zumo de naranja podemos encontrar la 2*S*-hesperidina en una proporción S:R de al menos 92:8 a favor del epímero 2*S*, la 2*S*-hesperidina predomina de forma natural en los cítricos. Aunque en la naturaleza predomina el epímero 2*S* de la hesperidina y, por tanto, el enantiómero *S*-hesperetina, actualmente la hesperetina y la hesperidina sólo se comercializan como una mezcla de ambos estereoisómeros (Figura 21) (461).

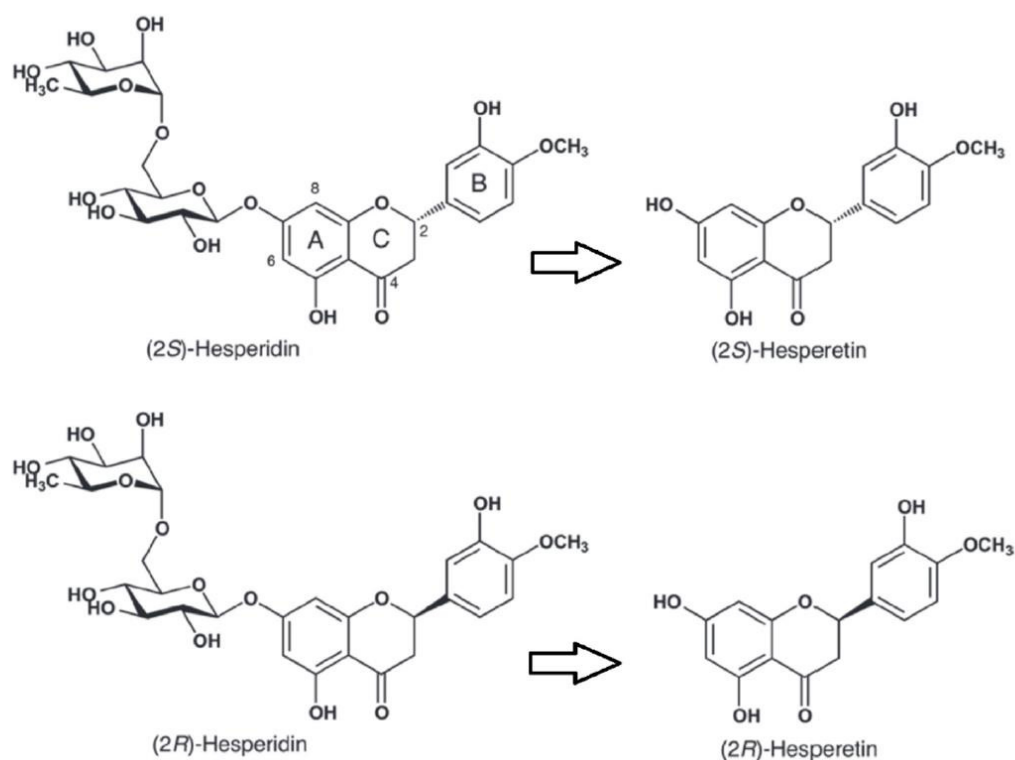


Figura 21. Estructura de los enantiómeros *S* y *R* de la hesperidina y de sus metabolitos hesperetina, producidos por la microbiota intestinal. Modificada de Li et al (462).

2.9.2. Farmacocinética, seguridad y toxicidad de la hesperidina

2.9.2.1. Absorción

La capacidad de las flavanonas cítricas para ejercer efectos beneficiosos depende en gran medida de su biodisponibilidad, que puede verse afectada por la estructura del compuesto, la matriz alimentaria y los factores del huésped (463). Para evaluar la absorción oral de la hesperidina de los productos cítricos, se administró a varones blancos sanos, de 25 años de edad, 500 mg del fármaco en agua y cantidades equivalentes de zumo de pomelo y naranja. Se absorbió en el tracto gastrointestinal tras la administración oral en cualquier forma, pero la recuperación urinaria acumulada indicó una baja biodisponibilidad (25%). La aglicona hesperetina, se detectó tanto en la orina como en el plasma. Se cree que las flavanonas cítricas absorbidas sufren una glucuronidación antes de su excreción urinaria (464). Además, se investigó la permeabilidad intestinal en los glucósidos de hesperidina utilizando una monocapa cultivada de células Caco-2 como modelo del epitelio del intestino delgado. Mientras que la hesperidina no permeó a través de la monocapa Caco-2, probablemente debido a su baja solubilidad, sus glucósidos sí lo hicieron, dependiendo del tiempo y la dosis. Se cree que esta permeación se produce a través de una vía paracelular (465).

El metabolismo intestinal de las flavanonas de los cítricos está determinado principalmente por su grado de conjugación con los azúcares (466) y la eliminación de éstos por las bacterias intestinales (467). Se considera que las flavanonas de los cítricos, como la hesperidina y naringina, son muy resistentes a la descomposición enzimática en el estómago y el intestino delgado y, por tanto, llegan intactas al colon. Aquí, la hesperidina se expone a las α -ramnosidasas secretadas por la microbiota intestinal, que eliminan la fracción de ramnosa, seguida de la eliminación de la glucosa por las β -glucosidasas (466) y una vez dentro del epitelio intestinal, la hesperetina se libera en el torrente sanguíneo en forma de conjugados de glucurónido y sulfato (Figura 22) (468). Aunque la mayor parte se convierte en el colon, puede producirse ya una cierta descomposición en la parte distal del intestino delgado (469). Una vez liberada, la aglicona hesperetina se absorbe a través del epitelio intestinal mediante difusión pasiva y transporte activo acoplado a protones, o bien se metabolizan en ácidos fenólicos y fenólicos simples mediante la escisión del anillo C, la desmetilación y la deshidroxilación por enzimas bacterianas (469).

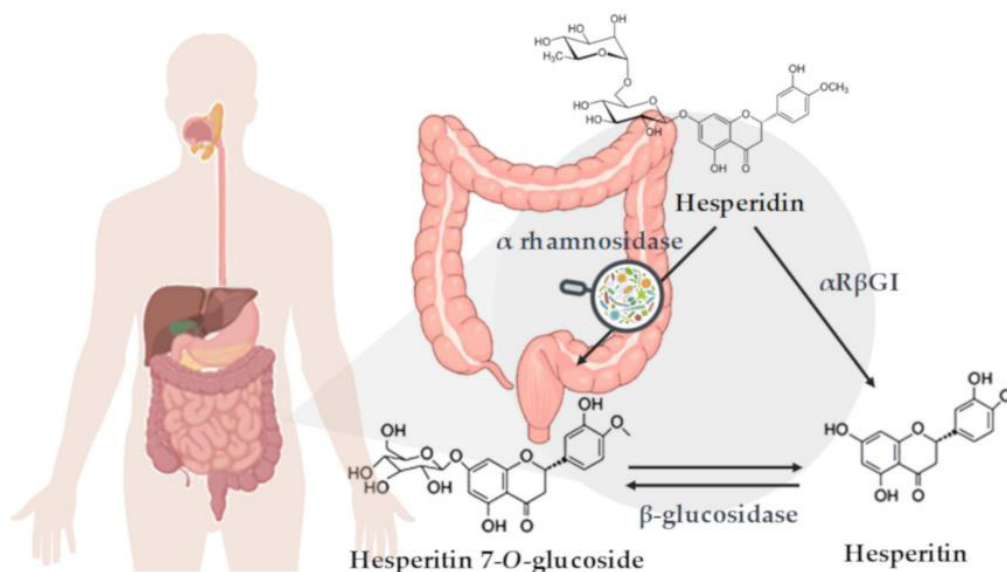


Figura 22. Representación esquemática de la metabolización de la hesperidina en el colon. Desglucosilación enzimática de la hesperidina para dar lugar a la hesperetina: a través de la hesperetina-7-O-glucósido por 2 monoglucosidasas específicas, la α -ramnosidasa y la β -glucosidasa, y a través de la deglicosilación en un solo paso por medio de la α -ramnosil- β -glucosidasa (α R β GI). Modificada de Mas-Capdevila et al. (470).

Los estudios en humanos que realizaron análisis en plasma, orina y/o heces también mostraron la formación de sus formas agliconas, así como de muchos metabolitos fenólicos más pequeños tras el consumo de flavanonas cítricas (469). Estos datos indican que las flavanonas de los cítricos, al igual que muchos otros polifenoles, sufren un amplio metabolismo *in vivo* por parte de la microbiota intestinal. Se ha observado una gran variación interindividual, que es consistente para cada individuo después de la ingesta de las diferentes bebidas a base de zumo de naranja a diferentes concentraciones (29.2-70.3 mg de flavanonas/100 mL), lo que sugiere que la biodisponibilidad de las flavanonas también depende de la existencia de una microbiota específica que es capaz de eliminar los rutinósidos de los glucósidos del zumo, lo que da lugar a agliconas que luego se absorben en el intestino (471).

Varios de los metabolitos identificados en las simulaciones *in vitro* mencionadas anteriormente, también se encontraron en estas muestras *in vivo*, lo que sugiere que las simulaciones *in vitro* tienen la capacidad de imitar el metabolismo *in vivo* (469). Además, como la mayoría de estos estudios se realizaron en voluntarios sanos o con donaciones fecales de voluntarios sanos, no está claro cómo los estados de enfermedad, los factores dietéticos y el uso de medicamentos pueden afectar a estos resultados. Aunque los datos son escasos, algunos estudios han investigado los efectos de la

matriz alimentaria o el uso de medicamentos en la biodisponibilidad de las flavanonas cítricas (466).

2.9.2.2. Metabolismo y excreción urinaria

A la hora de analizar el metabolismo de la hesperidina se han encontrado bajas concentraciones plasmáticas de aglicona de hesperetina (hesperidina) (<2 mmol/L) tras la ingestión de 0.5-1 L de zumo de naranja, indicando una biodisponibilidad limitada en voluntarios humanos (467, 472). Además, se realizó un análisis profundo del metabolismo de la hesperidina en humanos, utilizando 3 tipos de tratamiento: 1) tratamiento con dosis bajas de hesperidina (zumo de naranja con hesperidina natural que proporciona 2 mg/kg de peso corporal de hesperidina); 2) ingesta en zumo de naranja tratado con la enzima hesperidinasasa para producir hesperetina-7-glucósido y que proporciona 1.52 mg/kg de peso corporal de hesperidina (hesperetina-7-glucósido); y 3) tratamiento con altas dosis de hesperidina en zumo de naranja que contiene hesperidinasasa desactivada para proporcionar 6 mg/kg de peso corporal de hesperidina (459). El área bajo la curva de la hesperetina total (metabolito de la hesperidina) en plasma después de que los sujetos consumieran el zumo de hesperetina-7-glucósido fue 2 veces mayor en comparación con el consumo del zumo de hesperidina en dosis bajas (Tabla 9). Además, tras el consumo del zumo de hesperetina-7-glucósido, el área mejoró hasta ser equivalente al del zumo de hesperidina de dosis alta.

Tabla 9. Mediciones farmacocinéticas de la hesperetina total en humanos sanos tras el consumo de 3 tratamientos de zumo de naranja.

	Tratamientos		
	<i>Dosis baja de hesperidina</i>	<i>Hesperetina-7-glucosido</i>	<i>Dosis alta de hesperidina</i>
Sujetos	10	10	10
Dosis (mg)	61 ± 10 (47-81)	80 ± 13 (60-105)	192 ± 30 (145-253)
Mg/kg peso corporal	0.93 ± 0.06 (0.92-0.95)	1.21 ± 0.08 (1.20-1.22)	2.92 ± 0.18 (2.89-2.95)
AUC _(0-10h) (mmol/L·h)	1.16 ± 0.52	3.45 ± 1.27	4.16 ± 1.50

	(0.39-2.10)	(2.16-5.84)	(2.13-6.65)
C_{MAX} (mmol/L)	0.48 ± 0.27 (0.10-1.00)	2.60 ± 1.07 (1.44-4.36)	2.60 ± 1.07 (1.44-4.36)
T_{MAX} (h)	7.00 ± 3.00 (2.00-10.00)	0.60 ± 0.10 (0.50-0.80)	7.40 ± 0.20 (5.00-10.00)
Excreción urinaria relativa (% ingesta)	4.06 ± 1.77 (1.80-6.96)	14.40 ± 6.75 (10.19-33.31)	8.90 ± 3.83 (3.91-15.58)

AUC = área bajo la curva; C_{MAX} = concentración máxima; T_{MAX} = tiempo que tarda desde la ingesta hasta alcanzar C_{MAX} .

Las curvas cinéticas mostraron que cuando los sujetos consumieron el zumo de naranja de dosis baja y el zumo de hesperetina-7-glucósido, la hesperetina total en plasma volvió a la línea de base a las 10 h, pero no durante el consumo del zumo de naranja de dosis alta (Figura 23). Por lo tanto, el área bajo la curva puede estar subestimado; 20% para el zumo de naranja con dosis altas de hesperidina basándose en la forma de la curva. Sin embargo, esto no cambia el resultado de que la biodisponibilidad mejoró con el hesperetin-7-glucósido en comparación con la hesperidina natural del zumo de naranja (459) (Figura 23).

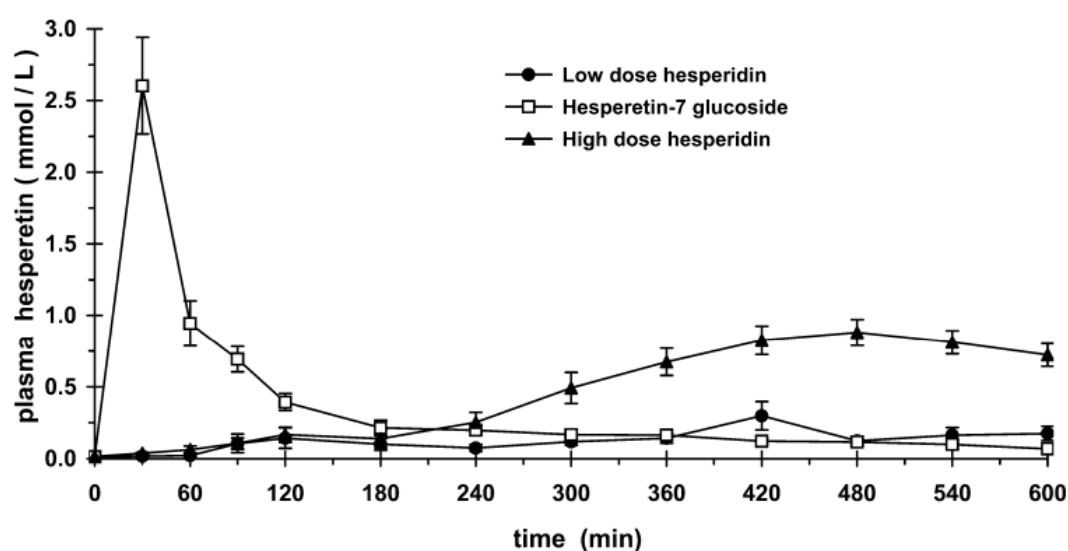


Figura 23. Curva de concentración plasmática frente al tiempo de la hesperetina total en humanos sanos tras el consumo de 3 tratamientos de zumo de naranja ($n=16$). Modificada de Nielsen et al. (459).

Las concentraciones plasmáticas máximas (C_{MAX}) de hesperetina fueron 4 veces mayores cuando los sujetos consumieron zumo de hesperetina-7-glucósido en comparación con el zumo de hesperidina de dosis baja y 1,5 veces mayores que el zumo de hesperidina de dosis alta. El tiempo correspondiente para alcanzar la concentración plasmática máxima (T_{MAX}) en los sujetos se alcanzó significativamente más rápido tras el consumo de zumo de hesperetina-7-glucósido. Aunque hubo una diferencia significativa entre la $C_{máx}$ de los sujetos después de consumir los zumos de naranja con alta y baja dosis de hesperidina, su T_{MAX} no difirió (Tabla 9) (459).

Por otro lado, el volumen total de orina producido por los sujetos después de cada uno de los 3 tratamientos no difirió. La excreción total de hesperetina a lo largo de 24 h se calculó agrupando las 3 fracciones de orina recogidas, expresadas como porcentaje del consumo de hesperetina. La excreción urinaria relativa de hesperetina total de los sujetos fue significativamente mayor tras el consumo de hesperetina-7-glucósido que tras el consumo de dosis bajas de hesperidina y dosis altas de hesperidina (Tabla 9). Los sujetos también tuvieron una excreción urinaria significativamente mayor después de consumir el jugo de hesperidina de dosis alta en comparación con el jugo de hesperidina de dosis baja (459).

En relación al metabolismo de la hesperidina (glucósido), existen dos hipótesis sobre el motivo por el que los glucósidos de flavonoides se ven favorecidos por la absorción en el intestino delgado. En primer lugar, el glucósido es hidrolizado por la lactasa-clorizina-hidrolasa (473), y la aglicona libre se difunde a través de las células epiteliales de forma pasiva o por difusión facilitada. El proceso de deglicosilación no sólo es específico, sino que tiene una gran capacidad, de ahí la rápida detección de la concentración plasmática máxima de la aglicona hesperetina. Alternativamente, la molécula de glucósido puede ser transportada al enterocito a través de un transportador de azúcares (SGLT1) y, a continuación, ser deglicosilada por la enzima b-glucosidasa presente en las células intestinales (474). Ambas vías de absorción dan lugar a agliconas intracelulares, que se conjugadas con glucurónidos o sulfatos. La Figura 24 indica las posibles vías de absorción de la hesperidina y el hesperetin-7-glucósido.

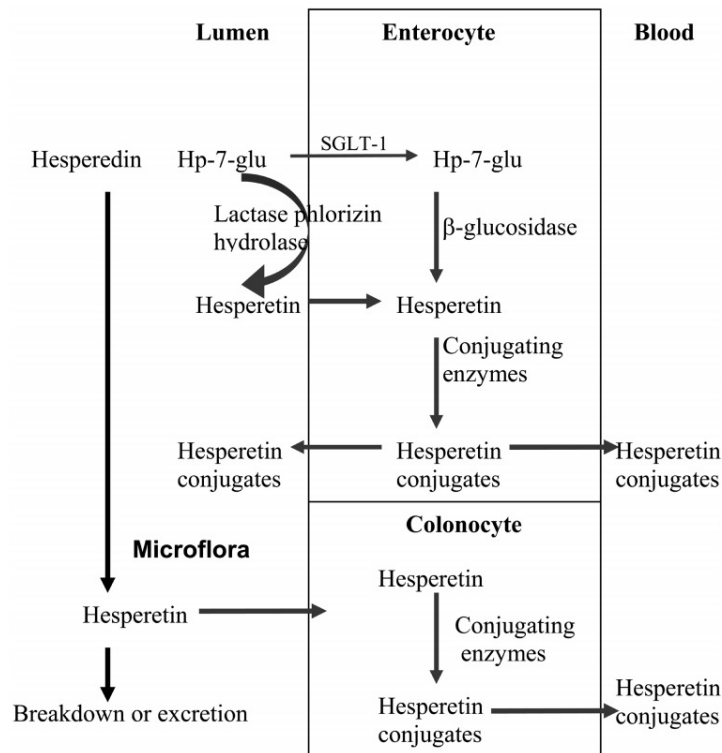


Figura 24. Posible vía de absorción de la hesperidina y del 7-glucósido de hesperetina que muestra el cambio propuesto en el lugar de absorción de la hesperetina del colon al intestino delgado. Modificada de Nielsen et al. (459).

2.9.2.3. Seguridad y toxicidad

En general, los bioflavonoides cítricos, incluida la hesperidina parecen ser extremadamente seguros y sin efectos secundarios incluso durante el embarazo (475). Además, un experimento en ratones estableció que la hesperidina fosforilada (PH) no era tóxica ni para el organismo ni para los tejidos, fácil de asimilar, no se acumulaba y no provocaba reacciones alérgicas (476). Se ha realizado un estudio en humanos informando que la hesperidina fosforilada podía administrarse clínicamente a los humanos como agente antifertilidad, junto con otros factores de sustitución como vitaminas, endocrinas, derivados de anfetaminas y derivados del ácido dechólico. Además, los traumatismos, las enfermedades infecciosas o sistémicas no inhibieron su efecto antifertilidad (476).

Por otro lado, en un estudio en ratas, cuando se administró Daflon® 500 mg (una fracción flavonoide purificada, compuesta por un 90% de diosmina y un 10% de hesperidina) por intubación gástrica durante 26 semanas, no se produjeron muertes, cambios en el peso o anomalías en las pruebas funcionales estándar (477). También se ha investigado la metil-hesperidina, cuando se administró por vía oral a

ratones a un nivel tan alto como el 5% en la dieta, no se encontraron efectos mutagénicos, ni carcinogénicos, sin efectos tóxicos evidentes en ratones de ambos sexos (478). Además, la ingestión de hesperidina no afectó a la ingesta diaria de alimentos, al aumento de peso corporal o la eficiencia alimentaria (479). En un estudio realizado en seres humanos se observó que la administración de hesperidina provocaba efectos secundarios leves en sólo el 10% de los sujetos, en comparación con el 13.9% de los tratados con placebo (480). Sin embargo, se han notificado algunas interacciones entre la aglicona hesperetina (481), la hesperidina (482) y los medicamentos convencionales.

2.9.3. Metabolización de la hesperidina por la flora intestinal

La microbiota intestinal es un ecosistema complejo que varía según los individuos (483). La interacción entre la microbiota intestinal y los polifenoles se considera bidireccional: además de la capacidad de las bacterias intestinales para metabolizar los polifenoles, también se han acumulado pruebas de que los polifenoles pueden inducir cambios en la microbiota hacia una composición y actividad más favorables, incluida la producción de ácidos grasos de cadena corta (AGCC) en el colon. Estos metabolitos tienen muchos efectos biológicos beneficiosos conocidos, por ejemplo, actuar como combustible para los enterocitos, mejorar la función de barrera e inhibir la inflamación. Los estudios que investigan el efecto de las flavanonas cítricas o de los productos alimentarios derivados de los cítricos sobre la microbiota intestinal o fecal se han centrado principalmente en su capacidad para inhibir el crecimiento de patógenos, aumentar las bacterias comensales beneficiosas (como las especies de *Bifidobacterium* y *Lactobacillus*) y estimular la producción de AGCC (466).

El efecto de la suplementación con hesperidina sobre la composición de la microbiota y los AGCC se ha estudiado en humanos. En un ensayo aleatorizado y controlado con placebo en sujetos sanos con características de síndrome metabólico, la suplementación diaria con 500 mg de extracto de cítricos (con >80% de 2S-hesperidina y >4% de naringina) durante 12 semanas, dio lugar a un aumento de la proporción entre butirato y AGCC totales, pero no de los niveles absolutos de AGCC fecales (466). Por otro lado, en voluntarios sanos, el consumo de un zumo de naranja pasteurizado con un contenido desconocido de flavanonas durante 2 meses dio lugar a un aumento significativo de *Lactobacillus spp.* y anaerobios totales en las muestras fecales. Además, se encontró una reducción significativa en la concentración de amonio y un aumento en la relación entre acetato y AGCC total en comparación con los valores de inicio del estudio (484). Además, la suplementación diaria de 2 zumos de naranja con diferentes contenidos de flavanona (consumo diario de 500 mL de zumo de cara cara o bahía) durante siete días en voluntarios sanos dio lugar a cambios en la composición de la microbiota, de los cuales el más notable fue un aumento en la abundancia de unidades

taxonómicas operativas de clostridia de las familias mogibacteriaceae, tissierellaceae, veillonellaceae, odoribacteraceae y ruminococcaceae (485).

En este sentido, algunos estudios clínicos han demostrado el papel de los polifenoles, suplementados en varios productos alimenticios, en el mantenimiento de la salud intestinal y la preservación de la homeostasis microbiana, promoviendo el crecimiento de las bacterias beneficiosas e inhibiendo la progresión de las bacterias patógenas (486). De hecho, los polifenoles y la hesperidina pueden modular la composición o la funcionalidad del microbioma intestinal, lo que afecta a la liberación de metabolitos derivados del microbioma (487). Los flavonoles son inhibidores activos contra algunas bacterias Gram negativas, como *Prevotella spp.*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *E. coli*, *Pseudomonas aeruginosa* y *Clostridium spp.* (488, 489) (Figura 25). Además, la hesperidina y otros flavonoles también inhiben el crecimiento de algunas bacterias Gram-positivas, como *Staphylococcus aureus* y *Lactobacillus acidophilus* (488, 489). Además de su capacidad inhibidora, los compuestos fenólicos pueden modificar la microbiota intestinal promoviendo selectivamente el crecimiento de bacterias beneficiosas de los géneros *Lactobacillus* o *Bifidobacterium* (490, 491).

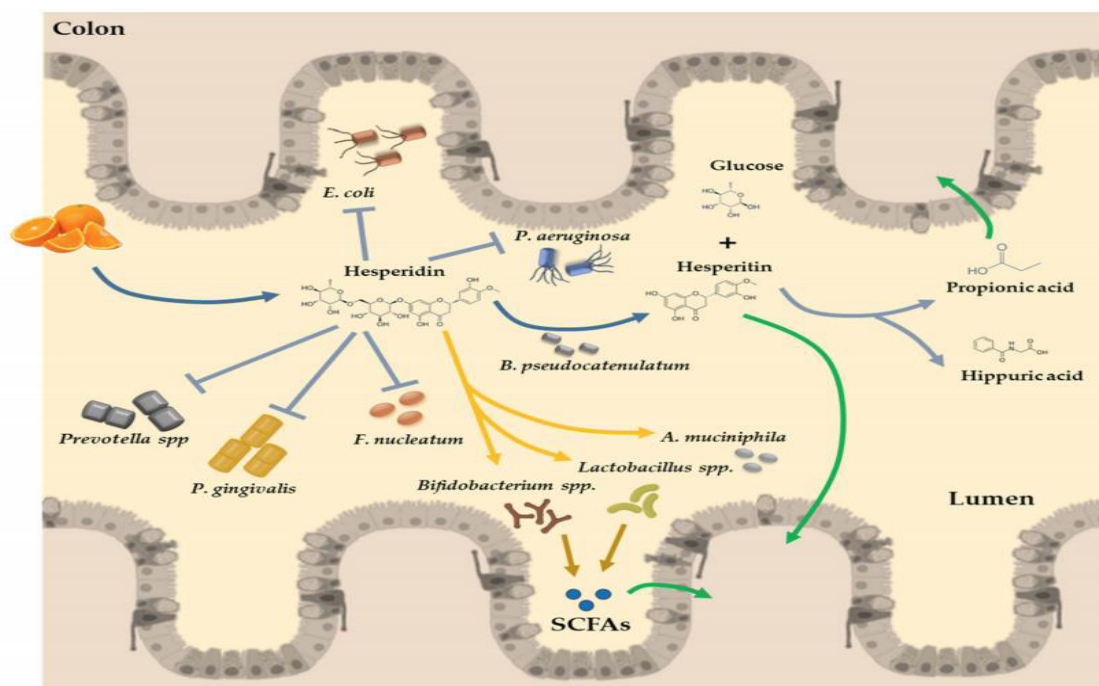


Figura 25. Diagrama ilustrativo de la absorción de hesperidina en el colon. Las flavonas presentes en las naranjas llegan al colon casi sin cambios en su estructura. En el lumen del colon, la hesperidina es convertida en su forma activa por la actividad α -ramnosidasa de la microbiota (*Bifidobacterium pseudocatenulatum*), liberando la fracción de rutina y la hesperetina para su posterior absorción por los colonocitos. En el colon, la hesperidina promueve el crecimiento de algunas especies de bacterias beneficiosas, con un papel clave en la producción

de AGCC (*Bifidobacterium spp.*, *Lactobacillus spp.*, o *Akkermansia muciniphila*). Los AGCC se absorben con efectos saludables en la permeabilidad de la barrera intestinal y en los órganos y tejidos distales. Además, la hesperidina tiene otros efectos beneficiosos al inhibir la proliferación de bacterias perjudiciales, como *Escherichia coli*, *Pseudomonas aeruginosa*, *Prevotella spp.* otros. AGCC: ácidos grasos de cadena corta. Modificada de Mas-Capdevila et al. (470).

Hay que añadir que algunas cepas bacterianas han sido estudiadas con el objetivo de averiguar si estas mejoran la metabolización de la hesperidina a sus metabolitos de hesperetina. Se ha visto que la ingesta oral (4 semanas) de *Bifidobacterium longum* R0175 junto a zumo de naranja (alto en flavanonas), no mostraba ningún cambio a las 0-24h en la excreción urinaria de metabolitos de hesperidina (principales metabolitos: hesperetina-O-glucurónida, la naringenina-O-glucurónida y la hesperetina-3'-O-sulfato). La excreción urinaria global de estos metabolitos tras la ingestión del zumo de naranja y la ingesta aguda de probióticos correspondió al 22% de la ingesta, mientras que la excreción de ácidos fenólicos y aromáticos derivados del colon fue equivalente al 21% de los polifenoles del zumo de naranja ingerido. Además, el consumo agudo de zumo de naranja tras la ingesta crónica de probióticos durante 4 semanas dio lugar a la excreción del 27% de la ingesta de flavanonas, y la excreción de ácidos fenólicos seleccionados también aumentó significativamente hasta el 43% de la ingesta de polifenoles, lo que corresponde a una biodisponibilidad global del 70% (492). Por lo tanto, la modulación de las especies bacterianas que forman la microbiota intestinal pueden modular la absorción de los metabolitos de la hesperidina y sería una herramienta eficaz para maximizar la absorción de esta.

2.9.4. Efectos fisiológicos-bioquímicos de la hesperidina en el organismo

2.9.4.1. Efectos de la hesperidina en el sistema inmune

En algunos estudios clínicos, se ha evaluado la influencia de la ingesta crónica con zumo de naranja (alto porcentaje de hesperidina) sobre los marcadores inflamatorios. En un estudio no controlado realizado en 12 adultos jóvenes, comprobaron que el consumo de 2 vasos diarios de zumo de naranja (500 mL) durante 14 días reducía las concentraciones plasmáticas de prostaglandina E2 y 8-epi-prostaglandina F2 α y tendía a reducir la concentración de la PCR (493). Por otro lado, en hombres sanos con sobrepeso, el consumo de 500 mL de zumo de naranja al día durante 4 semanas no afectó a las concentraciones séricas de varios marcadores inflamatorios (PCR, IL6, ICAM1, molécula de adhesión celular vascular soluble 1 (sVCAM1), aunque la presión arterial se redujo y la función vascular mejoró (494). Además, la intervención con zumo de naranja ha sido capaz de modular la expresión de 3.422 genes, muchos de los cuales están implicados en la quimiotaxis, la adhesión y

la infiltración celular (495). Otro estudio, encontró una reducción de las concentraciones plasmáticas de PCR, IL6 y TNF α en individuos no diabéticos con mayor riesgo cardiovascular tras una semana de consumo diario de 500 mL de zumo de naranja roja (496). Además, la función endotelial, mejoró significativamente en estos sujetos. También se ha visto como el consumo de 500 mL de zumo de naranja fresco y comercial 2 veces al día mediante un estudio cruzado (22 adultos por 4 semanas), tiene la capacidad de disminuir las concentraciones séricas de PCR, sVCAM1 y selectina, con ambos tipos de zumo sin que hubiera diferencias entre ellos, pero no de IL6 (497). En otro estudio, tomaron 750 mL de zumo de naranja diarios durante 8 semanas, reduciendo la PCR circulante y aumentaron la interleucina 12 (IL12), pero no afectaron a la IL4, la IL10, el TNF α o el interferón γ (IFN γ), tanto en adultos con peso normal como con sobrepeso (498). Un segundo estudio con el mismo diseño (750 mL de zumo de naranja de pulpa roja al día durante 8 semanas) también informó de una reducción de la concentración de PCR tanto en individuos con peso normal como con sobrepeso (499). En línea con los resultados del estudio anterior, los pacientes con hepatitis C que consumieron 500 mL de zumo de naranja al día durante 8 semanas mostraron una reducción de la concentración plasmática de PCR, aunque el valor inicial fue superior al del grupo de control (500). Un reciente metaanálisis de los efectos del zumo de naranja sobre los factores de riesgo de las enfermedades cardiovasculares informó de que el zumo de naranja disminuyó significativamente los niveles de PCR (7 ensayos) en comparación con el placebo (501).

2.9.4.2. Efectos de la hesperidina en el metabolismo de los lípidos.

El tejido adiposo almacena lípidos en forma de triglicéridos, que secretan y regulan una serie de adipoquinas y citoquinas. Durante la obesidad, para compensar la carga excesiva de lípidos, el tejido adiposo se expande rápidamente (502). En la búsqueda de moléculas que puedan luchar contra la obesidad, la hesperidina ha mostrado que puede mejorar el metabolismo de los lípidos (Figura 26) (503). La hesperidina (0.08%) reduce la esteatosis hepática, el peso del tejido adiposo y del hígado, y disminuye las concentraciones séricas de colesterol total y de la proteína de unión al retinol 4 (RBP4) en dietas ricas en grasas (504). Se cree que la proteína de unión a los ácidos grasos del corazón (H-FABP) y la proteína de unión a los ácidos grasos cutánea (C-FABP) desempeñan funciones clave en el metabolismo de los ácidos grasos, como su almacenamiento y transporte (505). La hesperidina puede mejorar la hipercolesterolemia y el hígado graso al inhibir la síntesis y la absorción del colesterol, regulando la expresión del ARNm de la RBP, la C-FABP y la H-FABP (504). La hesperidina ha mostrado una reducción la presión arterial sistólica (PAS) (506), los niveles de colesterol total y TG en plasma en ratas obesas hipertensas, atenuó la sintasa de FFA en plasma a través de su actividad anti-lipolítica, aumentó significativamente

el colesterol de las lipoproteínas de alta densidad (HDL-C), y disminuyó el colesterol de las lipoproteínas de baja densidad (LDL-C) y el colesterol de las lipoproteínas de muy baja densidad (VLDL-C) en plasma (Figura 26) (507).

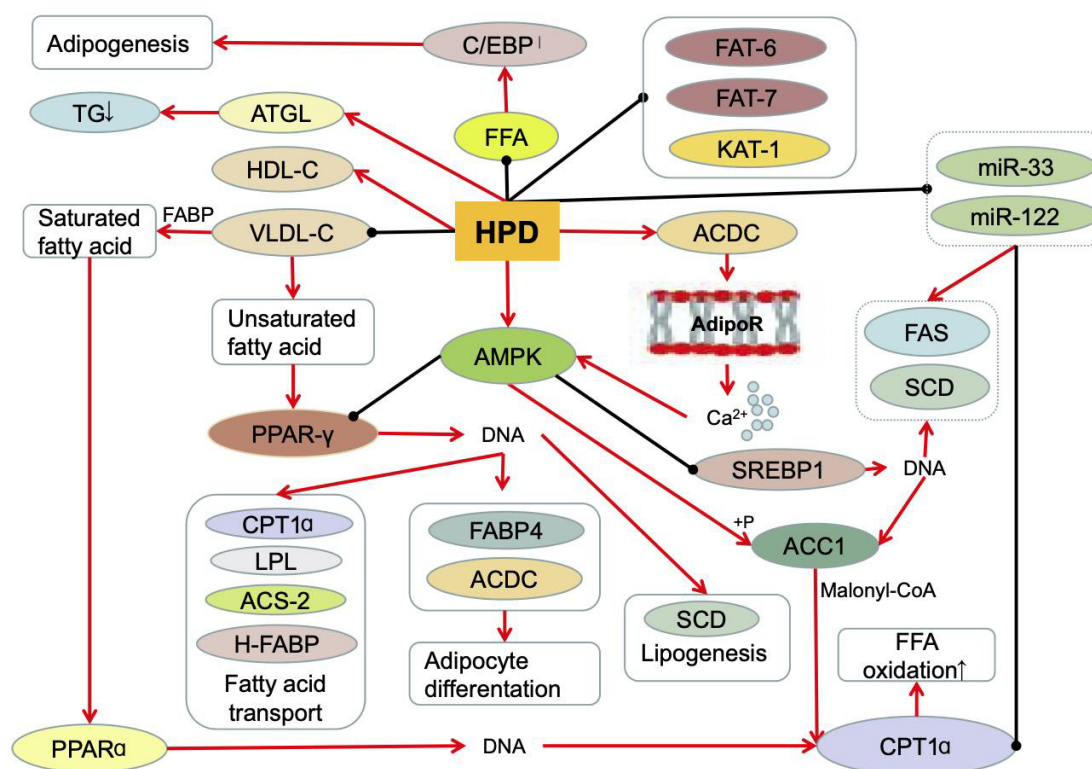


Figura 26. El efecto de la hesperidina en el metabolismo de los lípidos. Línea roja activación/promoción y línea negra inhibición/reducción. Modificada de Xiong et al. (508).

En animales tratados con hesperidina mostraron una disminución de los niveles de expresión de tres genes clave relacionados con la adipogénesis, factor de transcripción de unión a elementos reguladores de esteroides 1 (SREBP1), FAS (509) y esteroil-CoA desaturasa (SCD) (510), y una normalización de la expresión del gen PKLR (510). La hesperidina, además mostró una actividad inhibidora específica sobre los preadipocitos 3T3-L1 en la fase intermedia de diferenciación (511) y un aumento de la expresión del ARN mensajero de las lipasas sensibles a las hormonas y estimula la descomposición de los adipocitos maduros (512). Por otro lado, la hesperidina ha mostrado una regulación significativa de la expresión de la esteroil-CoA desaturasa, la desaturasa de ácidos grasos 6 y 7 (FAT6 y FAT7) y reduce la expresión de otros genes implicados en el metabolismo de los lípidos, como la acetil-CoA carboxilasa 2 (POD2), la subunidad mediadora 15 (MDT15), la acil-CoA sintetasa-2 (ACS2) y la 3-cetoacil-CoA tiolasa-1 (KAT1), reduciendo así la acumulación de grasa (513).

2.9.4.3. Efectos de la hesperidina en el metabolismo de la glucosa

En relación al metabolismo de la glucosa, la hesperidina ha mostrado una actividad inhibidora moderada y selectiva de la alfa-glucosidasa (514), pudiendo inhibir la digestión de la amilosa y la amilopectina y reducir significativamente la actividad de la glucosa-6-fosfatasa en las células hepáticas (515). Las simulaciones de acoplamiento (docking) mostraron que la hesperetina y la hesperidina bloquean la entrada de enzimas en el canal, impidiendo la producción de piruvato, alfa-cetoglutarato y oxaloacetato, e inhibiendo la gluconeogénesis hepática, impidiendo así la progresión de la diabetes (516). Además, la hesperidina estimula la glucogenólisis y la glucólisis en hígado de rata perfundido aislado (517) y reduce los niveles de glucosa inducidos en modelos de ratas diabéticas e inducida por estreptozotocina porcina (518). La respuesta glucémica postprandial tras la ingesta del zumo de naranja es regulada a la baja mediante la inhibición parcial del transportador intestinal de glucosa en función de la concentración de azúcar y hesperidina (519), lo que indica que la hesperidina puede utilizarse para prevenir la hiperglucemia postprandial (515).

El receptor activado por proliferadores peroxisomales γ (PPAR γ) es un factor de transcripción de proteínas nucleares que regula el metabolismo de los lípidos y la glucosa, y la hesperidina mantiene el metabolismo de la glucosa regulando la activación del PPAR γ e inhibiendo la acumulación de grasa (520). Se ha demostrado en ratas destetadas que la hesperidina y la hesperetina encapsulada en ciclodextrina normalizan los niveles de glucosa en sangre alterando la actividad de las enzimas reguladoras de la glucosa y reduciendo los niveles de lípidos séricos y hepáticos. Estos efectos hipoglucémicos e hipolipidémicos en ratas diabéticas de tipo 2 se ven parcialmente alterados por la modificación de la expresión de los genes que codifican el PPAR, la 3-hidroxi-3-metil-glutamil coenzima A (HMG-CoA) reductasa y los receptores de LDL (521). Por otro lado, se ha identificado la RBP4 como una adipocina implicada en la regulación del metabolismo de la glucosa (505). La activación de GLUT4 mejora la captación de glucosa y aumenta la cantidad de glucosa intracelular disponible para la conversión metabólica, promoviendo así una mayor proliferación celular (522). La hesperidina puede reducir la expresión de RBP4 y afectar a GLUT4 (504). La insulina puede promover la síntesis de ácidos grasos en el hígado, promover la entrada de glucosa en las células grasas y convertirla en triacilglicerol para su almacenamiento, al tiempo que inhibe la actividad de la lipasa y reduce la descomposición de la grasa (523). Otro efecto, es que la hesperidina afecta indirectamente a la resistencia a la insulina y estimula el crecimiento microbiano intestinal para aumentar la producción de AGCC, regulando así la función del tejido adiposo, el músculo esquelético y el tejido hepático, y mejorando la homeostasis de la glucosa y la sensibilidad a la insulina (484). Por lo tanto, la hesperidina regula directa o indirectamente el metabolismo de la glucosa y la insulina para mejorar la interacción

entre la obesidad y los trastornos del metabolismo de la glucosa (como la hiperglucemia, la diabetes, etc.) (Figura 27) (508).

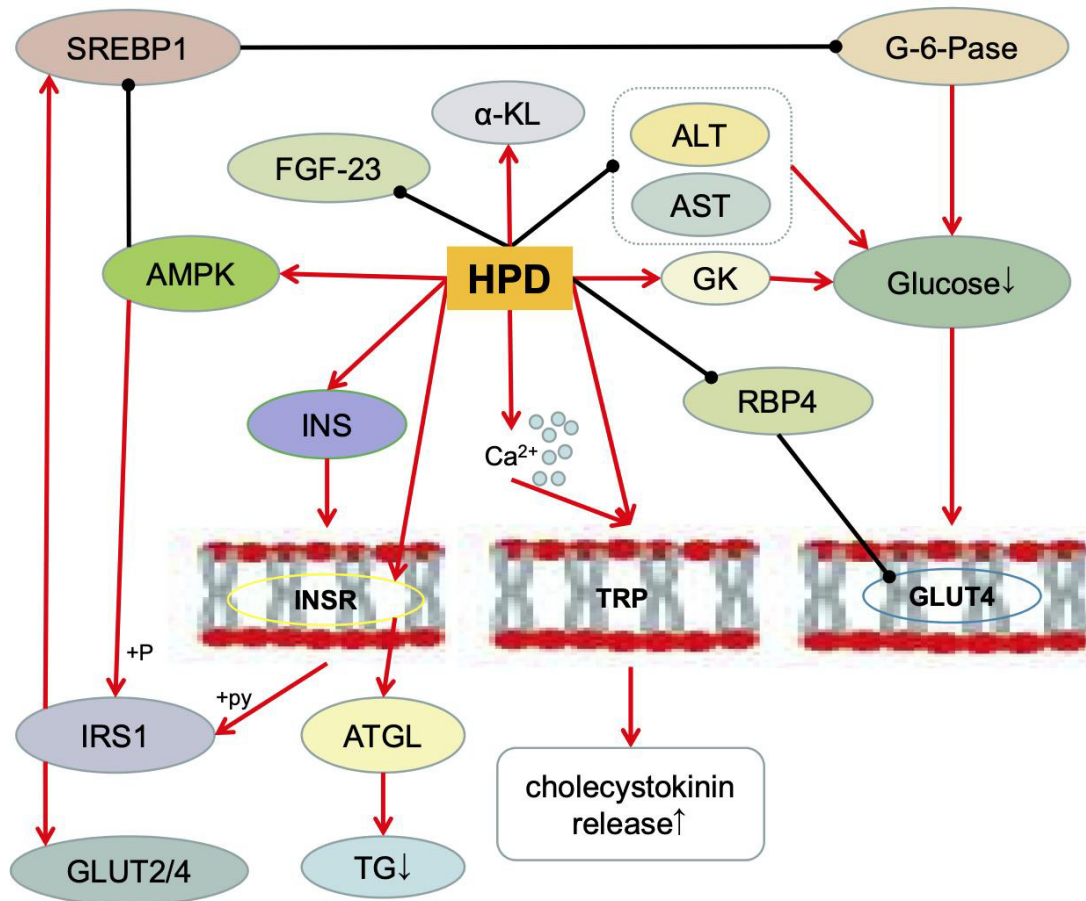


Figura 27. El efecto de la hesperidina en el metabolismo de la glucosa. Línea roja activación/promoción y línea negra inhibición/reducción. Modificada de Xiong et al. (508).

2.9.4.4. Efectos de la hesperidina en el sistema antioxidante endógeno

En relación entre la conexión del estrés oxidativo y el sistema antioxidante endógeno, se ha visto que la hesperidina disminuye los contenidos de glucosa, hemoglobina glucosilada (HbA1c%), malondialdehído (MDA) y NO en ratas diabéticas, y aumenta los niveles de insulina sérica, GSH, vitamina C y vitamina E, lo que indica un efecto protector sobre el daño oxidativo inducido por la hiperglucemia (524). Además, la hesperidina puede prevenir el aumento de la producción de ROS en ratas mediada por el ejercicio exhaustivo, y evita la disminución de la actividad de SOD y CAT en el timo y el bazo (33), lo que puede inhibir eficazmente la formación del radical superóxido (525). Por otro lado, se ha visto como la hesperidina reduce los

2.9.4.5. Efectos de la hesperidina en el óxido nítrico

La hesperidina, además, se ha visto que tiene la capacidad de mejorar la síntesis del NO. Rizza et al (31) evaluó la afirmación anterior con 2 estudios. En el primero utilizó células endoteliales aórticas de ovejas, donde observó que la administración de hesperetina (metabolito de la hesperidina) aumentó la producción de NO. En el mismo estudio, se encontró que el consumo diario de hesperidina (500 mg/d) por vía oral durante 3 semanas, mejoró la función endotelial en sujetos con síndrome metabólico (31). De la misma forma que el autor anterior, Liu et al (531), también observó una mejora de la síntesis de NO en células endoteliales de la vena umbilical humana, habiendo un aumento exponencial de las concentraciones de NO desde bajas a altas dosis de hesperidina (12.5-100 μ M).

2.9.5. Efectos de la hesperidina en el rendimiento deportivo

En relación al rendimiento deportivo, se ha estudiado el efecto de la ingesta crónica de hesperidina en humanos. Se ha observado que la ingesta de 250 mL de zumo de naranja roja 3 veces al día durante 4 semanas en mujeres mayores, estas aumentaban significativamente su capacidad de trabajo (prueba incremental) en un 9.0% en comparación con el placebo (-1.5%) (532). Otro estudio, que evaluó el efecto de una suplementación de 4 semanas de 2S-hesperidina (500 mg/día) en ciclistas entrenados, observó aumentos significativos en la potencia media (14.9 W = 5.0%) en una prueba de contrarreloj (cicloergómetro) de 10 minutos, mientras que los que consumieron placebo tuvieron un aumento no significativo de la potencia media (3.8 W = 1.3%), además, se encontraron diferencias al comparar los grupos.

Además, en animales también se ha investigado la ingesta de hesperidina a largo plazo, observando que seis semanas de suplementación con hesperetina (principal metabolito de la hesperidina) (50 mg·kg⁻¹·d⁻¹) mejoraban el rendimiento en carrera en un 28.8% (tiempo de ejercicio hasta el agotamiento) en comparación con el placebo en ratones de edad avanzada (35). Este estudio también encontró una mejora en las enzimas antioxidantes endógenas, como el glutatión reducido (GSH), el glutatión oxidado (GSSG) y la relación GSH:GSSG. En la misma línea, un estudio reciente en animales entrenados mostró de que la ingesta de hesperidina durante 4 semanas mejoraba el rendimiento y prevenía las alteraciones inmunológicas inducidas por el ejercicio agotador en comparación con el placebo (34). Recientemente, un estudio con grupos paralelos ha mostrado mejoras en el tiempo hasta el agotamiento (58%) en un test incremental a las 3 semanas de suplementación con 2S-hesperidina (200 mg/kg), pero no en el grupo de placebo (33). En el mismo estudio, se observó una mejora del estado antioxidante (SOD y GPx) en el tejido linfóide y hepático después del test

incremental hasta el agotamiento en las ratas que consumieron 2S-hesperidina en comparación con el placebo.

Debido a la poca evidencia en relación a la suplementación con hesperidina y la mejora del rendimiento en humanos, se hace necesario realizar más estudios que profundicen en los posibles mecanismos fisiológicos y bioquímicos que pueden conllevar una mejora del rendimiento, lo cual podría facilitar un buen posicionamiento a la hesperidina como una nueva ayuda ergogénica.

CAPÍTULO III.
JUSTIFICACIÓN E HIPÓTESIS

CAPÍTULO III. JUSTIFICACIÓN E HIPÓTESIS

Tras la revisión de la literatura y en base al efecto antioxidante, antiinflamatorio, regulador del metabolismo de los lípidos y glucosa, además de modular la síntesis de óxido nítrico, tras la administración de 2S-hesperidina tanto en animales como en humanos, unido a la escasa evidencia de la relación entre la ingesta de 2S-hesperidina y rendimiento deportivo en humanos, nos surgen los siguientes problemas de investigación:

3.1. PROBLEMA PRINCIPAL

- ¿Existen diferencias en el rendimiento, componentes del sistema antioxidante endógeno y la composición corporal entre ciclistas profesionales y amateurs?
- ¿La suplementación aguda (una sola dosis) y crónica (500 mg/d para 8 semanas) de 2S-hesperidina puede mejorar el rendimiento aeróbico y anaeróbico de ciclistas amateur?

3.2. PROBLEMAS ESPECÍFICOS

ESTUDIO 1

- ¿Existen diferencias entre ciclistas profesionales y amateur en los componentes del sistema antioxidante endógeno y rendimiento, que pueden afectar a los resultados de una intervención con 2S-hesperidina?

ESTUDIO 2

- ¿Existen diferencias entre ciclistas profesionales y amateur en los componentes de la composición corporal, que pueden afectar a los resultados de una intervención con 2S-hesperidina?

ESTUDIO 3

- ¿Puede la ingesta aguda (una dosis) de 500 mg de 2S-hesperidina mejorar el rendimiento anaeróbico (potencia anaeróbica) y la oxidación de grasas de ciclistas amateur?

ESTUDIO 4

- ¿Puede la ingesta crónica de 500 mg/d de 2S-hesperidina durante 8 semanas mejorar el rendimiento aeróbico (test incremental) y anaeróbico (potencia

anaeróbica), y la oxidación de grasas mediante un test rectangular de ciclistas amateur?

ESTUDIO 5

- ¿Puede la ingesta crónica de 500 mg/d de 2S-hesperidina durante 8 semanas mejorar el sistema endógeno antioxidante y el estado inflamatorio antes, durante y después de un test rectangular de ciclistas amateur?

ESTUDIO 6

- ¿Puede la ingesta crónica de 500 mg/d de 2S-hesperidina durante 8 semanas mejorar la masa grasa de ciclistas amateur?

ESTUDIO 7

- ¿Puede la ingesta crónica de 500 mg/d de 2S-hesperidina durante 8 semanas mejorar el metabolismo del oxígeno y el estado ácido-base antes, durante y después de un test rectangular de ciclistas amateur?

Atendiendo a los problemas de investigación presentados anteriormente, se plantean las siguientes hipótesis:

3.3. HIPÓTESIS GENERAL

La hipótesis inicial está basada en el efecto antioxidante/antiinflamatorio y modulación del metabolismo de la 2S-heperidina, el cual ha sido mostrado en numerosos estudios. Como ya es conocido, el ejercicio físico a intensidad submáxima y máxima produce altas cantidades de radicales libres que pueden ser una de las causas por las cuales puede aparecer la fatiga en deportes de resistencia (ciclismo, atletismo, etc.), y posteriormente estos radicales libres pueden aumentar el estado inflamatorio. Por lo tanto, la ingesta de 2S-hesperidina podría mejorar el rendimiento al retrasar la fatiga, principalmente mediante una mejora del estado antioxidante y una posible modulación del óxido nítrico, y de forma secundaria, mejorando el estado inflamatorio, por lo que, también mejoraría la recuperación tras el ejercicio de alta intensidad.

3.4. HIPÓTESIS ESPECÍFICAS

ESTUDIO 1

- Los niveles superiores de componentes del sistema endógeno en ciclistas profesionales podrían conducir a una mejora en el rendimiento comparado a ciclistas amateur.

ESTUDIO 2

- Las diferencias en los componentes de la composición corporal entre ciclistas profesionales y amateur pueden afectar al rendimiento independientemente del nivel practicado.

ESTUDIO 3

- La ingesta aguda (una dosis) de 500 mg de 2S-hesperidina puede mejorar el rendimiento anaeróbico (potencia anaeróbica) y la oxidación de grasas en ciclistas amateur, mediante un efecto antioxidante y modulación del metabolismo de los ácidos grasos.

ESTUDIO 4

- La ingesta crónica de 500 mg/d de 2S-hesperidina durante 8 semanas puede mejorar el rendimiento aeróbico (test incremental) y anaeróbico (potencia anaeróbica), y la oxidación de grasas mediante un test rectangular en ciclistas amateur, mediante un efecto antioxidante y modulación del metabolismo de los ácidos grasos.

ESTUDIO 5

- La ingesta crónica de 500 mg/d de 2S-hesperidina durante 8 semanas puede mejorar el sistema endógeno antioxidante y el estado inflamatorio antes, durante y después de un test rectangular en ciclistas amateur, mediante un efecto antioxidante.

ESTUDIO 6

- La ingesta crónica de 500 mg/d de 2S-hesperidina durante 8 semanas puede disminuir la masa grasa en ciclistas amateur, mediante la modulación del metabolismo de los ácidos grasos.

ESTUDIO 7

- La ingesta crónica de 500 mg/d de 2S-hesperidina durante 8 semanas puede mejorar el metabolismo del oxígeno a intensidades de ejercicio submáximas y máximas en ciclistas amateur, mediante la modulación de marcadores del metabolismo del oxígeno y del estado ácido-base, en parte afectados por una acción sobre el óxido nítrico.

CAPÍTULO IV.
OBJETIVOS

CAPÍTULO IV. OBJETIVOS

4.1. OBJETIVOS GENERALES

Los objetivos generales que se persiguen con este proyecto son:

- Determinar si existen diferencias a nivel del sistema antioxidante endógeno, rendimiento y composición corporal entre ciclistas profesionales y amateur, se llevaron a cabo el estudio **1** y **2**.
- Evaluar si la ingesta aguda (una dosis) de 500 mg de 2S-heperidina puede mejorar el rendimiento deportivo y/o modular el metabolismo y el estado antioxidante/oxidante en ciclista amateur, para tal fin, se llevó a cabo el estudio **3**.
- Evaluar si la ingesta de 500/d de 2S-heperidina durante 8 semanas puede mejorar el rendimiento y modular el estado antioxidante, inflamatorio, la composición corporal, y el metabolismo del oxígeno y el estado ácido base en ciclistas amateur, para tal fin, se llevó a cabo los estudios **4**, **5**, **6** y **7**.

4.2. OBJETIVOS ESPECÍFICOS

Los objetivos específicos que se persiguen con este proyecto son:

ESTUDIO 1

- Determinar si existen diferencias en el sistema antioxidante endógeno (catalasa (CAT), superóxido dismutasa (SOD), glutatión reducido (GSH) y glutatión oxidado (GSSG)) entre ciclistas profesionales y amateur, y averiguar si estas tienen relación con el rendimiento deportivo.

ESTUDIO 2

- Determinar si existen diferencias en la composición corporal (masa ósea y muscular) entre ciclistas profesionales y amateur, y averiguar si estas tienen relación con el rendimiento deportivo.

ESTUDIO 3

- Evaluar el efecto de la ingesta aguda (una dosis) de 500 mg de 2S-heperidina en el rendimiento anaeróbico (potencia máxima, potencia media, tiempo hasta potencia máxima, velocidad máxima y energía total del Σ de 4 esprines) medido mediante un test de Wingate en ciclistas amateur.

- Evaluar el efecto de la ingesta aguda (una dosis) de 500 mg de 2S-hesperidina en marcadores metabólicos y energéticos (consumo de oxígeno (VO_2), producción de dióxido de carbono (CO_2), ratio de intercambio gaseoso (RER), eficiencia, oxidación de hidratos de carbono y grasa) durante un ejercicio en el VT1 antes y después del test de Wingate en ciclistas amateur.
- Evaluar el efecto de la ingesta aguda (una dosis) de 500 mg de 2S-hesperidina en el estado antioxidante/oxidativo (CAT, SOD, GSH, GSSG y las sustancias reactivas del ácido tiobarbitúrico (TBARS)) durante todo el protocolo de ejercicio y después de 24 horas en ciclistas amateur.

ESTUDIO 4

- Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores de rendimiento aeróbico (producción de potencia en FatMax, umbral ventilatorio 1 y 2 (VT1 y 2) y potencia máxima) mediante un test incremental, y el rendimiento anaeróbico (potencia máxima, potencia media, tiempo hasta potencia máxima, velocidad máxima y energía total de 1 esprint) en ciclistas amateur.
- Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores metabólicos (VO_2 , consumo de oxígeno relativo al peso (VO_{2R}), eficiencia energética, oxidación de hidratos de carbono y grasas) en zonas de ejercicio de FatMax, VT1 y VT2 (10 min en cada zona) mediante un test rectangular en ciclistas amateur.

ESTUDIO 5

- Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores del estado antioxidante/oxidante endógeno (CAT, SOD, GSH, GSSG, hemoxigenasa 1 (HO1) y TBARS) y marcadores inflamatorios (interleucina 6 (IL6, factor de necrosis tumoral α ($\text{TNF}\alpha$), proteína quimioatrayente de monocitos 1 (MCP1) y proteína C reactiva (CRP)) pre, después del test rectangular (FatMax, VT1, VT2 y potencia máxima) y 30 min después de finalizar el test rectangular en ciclistas amateur.

ESTUDIO 6

- Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores de la composición corporal mediante densitometría (DXA) (masa grasa, % masa grasa, masa grasa en los miembros inferiores, masa magra y % masa magra) y antropometría (masa grasa, % masa grasa, Σ 8 pliegues, masa muscular y % masa muscular), además, de la tasa metabólica en reposo (VO_2 , CO_2 , VO_{2R} , RER,

equivalentes metabólicos (MET), oxidación de grasas e hidratos de carbono y kcal/d) pre y post intervención en ciclistas amateur .

ESTUDIO 7

- Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores del metabolismo del oxígeno y del estado ácido-base mediante gasometría (ABL-90) de sangre capilar pre, durante (FatMax1, VT1, VT2, potencia máxima y FatMax2), tras finalizar y 30 min después de finalizar el test rectangular en ciclistas amateur.

CAPÍTULO V.
VISIÓN GENERAL DE LOS
ESTUDIOS

CAPÍTULO V. VISIÓN GENERAL DE LOS ESTUDIOS

Durante la realización de esta Tesis Doctoral, y con el fin de alcanzar los objetivos establecidos para dar respuestas a las hipótesis planteadas se publicaron los siguientes estudios:

1. Martínez-Noguera, F. J., Alcaraz, P. E., Ortolano-Ríos, R., Dufour, S. P., & Marín-Pagán, C. (2021). Differences between Professional and Amateur Cyclists in Endogenous Antioxidant System Profile. *Antioxidants*, 10(2), 282. <https://doi.org/10.3390/antiox10020282>. JCR (IF:6.312; 2020). Q1 (Biochemistry & Molecular Biology).
2. Martínez-Noguera, F. J., Alcaraz, P. E., Ortolano-Ríos, R., Dufour, S., & Marín-Pagán, C. (2021). Professional cyclists have lower levels of bone markers than amateurs. Is there a risk of osteoporosis in cyclist?. *Bone*, 116102. <https://doi.org/10.1016/j.bone.2021.116102>. JCR (IF:4.398; 2020). Q1 (Endocrinology & Metabolism).
3. Martínez-Noguera, F. J., Marín-Pagán, C., Carlos-Vivas, J., Rubio-Arias, J. A., & Alcaraz, P. E. (2019). Acute effects of hesperidin in oxidant/antioxidant state markers and performance in amateur cyclists. *Nutrients*, 11(8), 1898. <https://doi.org/10.3390/nu11081898>. JCR (IF:5.717; 2020). Q1 (Nutrition & Dietetics).
4. Martínez-Noguera, F. J., Marín-Pagán, C., Carlos-Vivas, J., & Alcaraz, P. E. (2020). Effects of 8 Weeks of 2S-Hesperidin Supplementation on Performance in Amateur Cyclists. *Nutrients*, 12(12), 3911. <https://doi.org/10.3390/nu12123911>. JCR (IF:5.717; 2020). Q1 (Nutrition & Dietetics).
5. Martínez-Noguera, F. J., Marín-Pagán, C., Carlos-Vivas, J., & Alcaraz, P. E. (2021). 8-Week Supplementation of 2S-Hesperidin Modulates Antioxidant and Inflammatory Status after Exercise until Exhaustion in Amateur Cyclists. *Antioxidants*, 10(3), 432. <https://doi.org/10.3390/antiox10030432>. JCR (IF:6.312; 2020). Q1 (Biochemistry & Molecular Biology).
6. Noguera, F. J. M., Alcaraz, P. E., Vivas, J. C., Chung, L. H., Cascales, E. M., & Pagán, C. M. (2021). 8 weeks of 2S-Hesperidin supplementation improves muscle mass and reduces fat in amateur competitive cyclists: randomized controlled trial. *Food & Function*, 12(9), 3872-3882. <https://doi.org/10.1039/D0FO03456H>. JCR (IF:5.396; 2020). Q1 (Food Science & Technology).

7. Martínez-Noguera, F. J., Marín-Pagán, C., Carlos-Vivas, J., & Alcaraz, P. E. (2021). 8-Week Supplementation of 2S-Hesperidin Improves Acid-base Status and Decreases Lactate at Low-moderate and Submaximal Intensities, Enhancing Recovery After an Incremental Test in Amateur Cyclists. *The American Journal of Clinical Nutrition*. JCR (IF:7.045; 2020). Q1 (Bajo revisión).

CAPÍTULO VI.

ESTUDIO N° 1

CAPÍTULO VI. ESTUDIO N° 1: DIFERENCIAS ENTRE CICLISTAS PROFESIONALES Y AFICIONADOS EN EL PERFIL DEL SISTEMA ANTIOXIDANTE ENDÓGENO

6.1. INTRODUCTION

Competitive cycling is highly stressful on both aerobic and anaerobic metabolisms. Road cycling races require the riders to produce high relative power output (W/kg) for short duration (i.e., less than 1 min at the start, during steep climb and at the end of the race) while also sustain efforts that last for several minutes to several hours (1). Overall, professional cyclists (PRO) perform high training volumes (~32,500 Km) during the competitive season, which include 90–100 race days (18). On the other hand, amateur competitive cyclists (AMA) can be defined as cyclists that train 3–7 times per week, with daily training volumes of 60–120 min and that compete about 20 times in a year (11). During training sessions and competitions (aerobic and anaerobic exercise), there is a rise in reactive oxygen species (ROS) and subsequent oxidative stress, which can lead to a favorable adaptation in the body's antioxidant defense system (533). This improvement in the endogenous antioxidant system is generally associated with lower levels of oxidative stress biomarkers (534).

Within the endogenous antioxidant system, superoxide dismutase (SOD) is the first line of enzymatic defense that transforms the superoxide radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2) (535). Then, H_2O_2 , which is also harmful to cells, can be metabolized in a couple of ways: (1) conversion into water by glutathione peroxidase (GPx) with the reduced glutathione consumption (GSH) being converted into oxidized glutathione (GSSG) and (2) when the production of H_2O_2 exceeds the capacity of glutathione peroxidase, catalase (CAT) takes over to remove the excess H_2O_2 (536). However, un-neutralized H_2O_2 can interact with transition metals, such as Fe^{2+} and Cu^+ , and result in the production of the hydroxyl radical ($\cdot OH$; i.e., Fenton reaction), which is an extremely powerful oxidizing agent that reacts with all biological macromolecules. However, currently it is not clear what the end product of the Fenton reaction $\cdot OH$ or FeO_2^+ is (537). Finally, $\cdot OH$ can elicit damage to DNA, oxidation of the thiol group of proteins and peroxidation of lipids (538).

Exercise increases oxygen uptake and almost 0.15% of the oxygen consumed can be converted into ROS, which can be harmful to muscle and mitochondrial function (539). Recent findings show that the main source of ROS during exercise is nicotinamide adenine dinucleotide phosphate oxidases (540). It is known that long-duration strenuous exercise and extensive sprint training can exceed our ability to detoxify the action of reactive oxygen species within the blood cells, as well as at the muscle level

(541). Conversely, prevention of oxidative stress to enhance performance in professional athletes can be done by the adaptation mechanisms (hormesis) and detoxifying function of antioxidant enzymes (SOD, CAT, GPx, glutathione reductase (GR), glutathione-s-transferase), as well as via non-enzymatic antioxidants (such as vitamins E, A, C, and GSH and GSSG (542)). Therefore, it has been suggested that higher levels of the endogenous antioxidant system may improve performance of skeletal muscle contraction (534). Cordova et al. (543) analyzed antioxidant markers and showed an average GSH of $3.24 \mu\text{mol g}^{-1} \text{Hb}$, GSSG of $1.54 \mu\text{mol g}^{-1} \text{Hb}$, GSSG/GSH ratio of 0.56 %, catalase (CAT) of $172.0 \text{ mmol min}^{-1} \text{g}^{-1} \text{Hb}$ and superoxide dismutase (SOD) of $1983.0 \text{ U g}^{-1} \text{Hb}$ activity. In a previous study 15, average values of GSH of $\sim 4.7 \mu\text{mol g}^{-1} \text{Hb}$, GSSG of $\sim 0.7 \mu\text{mol g}^{-1} \text{Hb}$ and GSSG/GSH ratio of 0.15% were observed in PRO cyclists in February in of the same competitive season, suggesting that training season can modify the levels of these components of the endogenous antioxidant system (534, 541, 544).

At rest, endogenous antioxidant enzymes (EAE) levels are generally lower in athletes than in sedentary subjects, although higher or unchanged levels have been observed (535, 545). Several factors may explain this discrepancy, the most important being differences in the methods used to estimate the state of oxidative stress, the characteristics of the sample population (high-level athlete, sedentary, etc.) and the time of measurement (period of the sport's season) (542). However, what is clearly evident is that acute exercise can lead to an imbalance between ROS and endogenous antioxidants, causing what is known as oxidative stress (536). A recent study has shown that acute exercise at low, moderate or high intensity has the capacity to reduce GSH concentration and increase SOD and CAT activity compared to baseline, in addition to increasing F2-isoprostanes (markers of oxidative stress) at all levels of exercise. (546). In addition, it is generally known that chronic exercise causes an increase in enzymatic and non-enzymatic antioxidant defense, leading to adaptations to the training response and improving the protection against ROS (547). Chronic exercise (6-week cycling training) has the ability to increase the concentration and activity of GSH, SOD and CAT, while maintaining the levels of F2-isoprostanes (546). This same study also found that moderate and high intensity exercise promoted greater adaptations in antioxidant markers than low intensity exercise at baseline.

Despite the large amount of information on the EAE status and their relationship to the effects of acute and chronic exercise, to our knowledge, there are no studies that have compared the status of antioxidant enzymes between professional and amateur cyclists and their relationship to performance. Therefore, the main objective of this research was to determine the differences in endogenous antioxidants enzymes and hemogram levels between PRO and AMA, and whether these might be related to differences in performance (power output at VT1, VT2 and $\text{VO}_{2\text{MAX}}$) in an incremental

test. Finally, the secondary objective was to assess whether differences between endogenous antioxidant enzymes and hematological were associated with differences in performance between PRO and AMA.

6.2. METHODOLOGY

6.2.1. Selection of participants

A total of 26 male cyclists (11 PRO, 15 AMA) were recruited and completed the study. The PRO were competing at the Union Cycliste Internationale (UCI) PRO TOUR level and have participated in UCI major stage races (Vuelta a España, Giro d'Italia, Tour de France). The 15 AMA were from the southeast region of Spain. The PRO riders were selected based on the following criteria: (1) 20 to 40 years of age, (2) enrolled in a professional licensed team and (3) competed in at least one of the main 3-week stage races in the last years. Subjects for the AMA group had to meet the following inclusion criteria: (1) 20 to 40 years of age, (2) had at least 3 years of cycling experience and (3) performed specific training 6–12 h/week.

All subjects signed the informed consent document before their participation. The study was performed following the guidelines of the Helsinki Declaration for Human Research (548) and was approved by the Ethics Committee of the Catholic University of Murcia (CE091802).

6.2.2. Study Protocol

The experimental design of the study required each rider to visit the laboratory twice between the end of October and December (i.e., post-season period). In the first visit, a medical exam and blood analysis were completed to check their state of health. In the second visit (post-48 h), the cyclists performed a maximal incremental test. The 2 h prior to this latter test, they ingested a standardized breakfast, which was based relative to body mass (557.7 kcal) and composed of 95.2 g of carbohydrates (68%), 19.0 g of protein (14%) and 11.3 g of lipids (18%), established by a sports nutritionist. All subjects were instructed to refrain from high-intensity training 48 h before each visit.

6.2.3. Incremental Test

An incremental step test with final ramp until exhaustion was performed on a cycle ergometer (Cyclus 2TM, RBM elektronik- automation GmbH, Germany) using a metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine VT1, VT2 and VO_{2MAX}, as well as the associated levels of power output. The testing protocol started with 35 W and increased by 35 W every 2 min until RER > 1.05 was reached, from which the final

ramp (+35 W min⁻¹) until exhaustion was initiated (549). To ensure that VO_{2MAX} was achieved, at least 2 of the following criteria had to be met: plateau in the final VO₂ values (increase 2.0 mL kg⁻¹·min⁻¹ in the two last loads), maximal theoretical HR (220-age) 0.95) (550), RER 1.15 and lactate 8.0 mmol L⁻¹ (551, 552). Ventilatory thresholds were obtained using the ventilatory equivalents method described by Wasserman (553).

6.2.4. Blood Analysis

A total of 21.5 mL of blood were withdrawn from the antecubital vein for analyses: one 3.0 mL tube with ethylenediaminetetraacetic acid (EDTA) for hemogram and another 3.5 mL tube with polyethylene terephthalate (PET) for biochemical parameters. For the measurement of antioxidant parameters, five 3.0 mL EDTA tubes were obtained, where one tube was immediately centrifuged at 3500 rpm at 4 °C for 10 min. All tubes were temporarily stored at 2–4 °C and then sent to an external laboratory for analysis. Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, hemoglobin, hematocrit and hematocrit indexes (mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC)) were estimated.

6.2.5. Oxidative Stress and Antioxidant Status Markers

6.2.5.1. Catalase

The activity of catalase was measured in the whole blood using a UV-VIS spectrophotometer. The catalase enzyme extracts the peroxides from the region of the gel it occupies, following the isolation of the native protein. The removal of peroxide does not cause potassium ferricyanide (yellow substance) to be reduced to potassium ferrocyanide, which reacts with ferric chloride to form a blue Prussian precipitate. The catalase positive control activity is defined in international unit equals (1 unit) to the amount of catalase necessary to decompose 1.0 μM of H₂O₂ per minute at pH 7.0 at 25 °C while H₂O₂ concentration falls from 10.3 mM to 9.2 mM. The absorbance of H₂O₂ decreases at 240 nm proportional to its decomposition so that the concentration of H₂O₂ is critical in this determination. The decrease in absorbance per time unit is the measure of catalase activity (554). Results were expressed in U/g of Hb.

6.2.5.2. SOD

Superoxide dismutase (SOD) activity was measured using an SD125 Ransod kit

(Randox Ltd. Crumlin, Reino Unido) in whole blood. Xanthine and xanthine oxidase were used to produce superoxide anion ($O_2^{\bullet-}$), which responded with the 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) reactive and formed a red complex that is detectable at 420 nm. The SOD activity was measured as the inhibition degree of this reaction (555). Results were expressed in U/g of Hb.

6.2.5.3. Glutathione

The analysis of reduced glutathione (GSH) was performed using the glutathione-S-transferase assay described by Akerboom and Sies (556). Calculation of GSH was performed from lymphocytes treated with perchloric acid at a final concentration of 6%, collecting the supernatant after vortexing and subsequent centrifugation for 10 min at 10,000 rpm. Following collection of the supernatants in vials, high-performance liquid chromatography (HPLC) coupled to a Waters NH2 ODS S5 column (0.052, 25 cm) was conducted. Oxidized glutathione (GSSG) was analyzed using a similar method described by Asensi. (557).

6.2.6. Statistical Analyses

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 21.0, International Business Machines Chicago, IL, USA). Descriptive statistics are presented as mean standard deviation (SD). Levene and Shapiro–Wilks tests were performed to check for homogeneity and normality of the data, respectively. A Student's t-test for unpaired data was used to evaluate differences between groups. Additionally, the standardized mean differences were calculated using Cohen's effect size (ES) (95% confidence interval) for all comparisons. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large (558). The different correlations between the parameters were evaluated using Pearson's correlation (r). Significance level was set at $p \leq 0.05$.

6.3. RESULTS

6.3.1. Subject Characteristics

The general characteristics and hemogram results are presented in Table 1. Age, body mass and height were not different between PRO and AMA groups. Interestingly, PRO had higher MCH (4.8%, $p < 0.001$) and MCHC (3.6%, $p < 0.001$) compared to AMA. There were no group differences in RBC, Hb, HCT and MCV (Table 10). No correlation was found between age and antioxidant markers in both groups. However, there were correlations found between age and Hb, HCT and MCHC ($r \leq -0.597$, $p < 0.05$).

6.3.2. Antioxidant Parameters

Table 11 shows the outcomes of CAT, SOD, GSSG, GSH, %GSSG/GSH and GSSG+GSH, which were measured at baseline before the incremental tests. Higher levels in CAT (30.0%, $p < 0.001$), GSSG (63.2%, $p < 0.001$) and %GSSG/GSH (70.1%, $p < 0.001$), and lower levels in SOD (16.2%, $p = 0.009$) were found in PRO compared to AMA. However, no differences in GSH (4.3%, $p = 0.216$) and GSSG+GSH (3.5%, $p = 0.317$) values were observed between PRO and AMA.

Table 10. Baseline general characteristics and hemogram variables of professional and amateur cyclists.

	PRO	AMA	<i>p</i> -Value	Cohen's <i>d</i>	Effect Size
Age (years)	28.3 (4.65)	29.3 (6.54)	0.671	0.17	Trivial
Body mass (kg)	68.5 (4.43)	69.9 (5.50)	0.488	0.28	Small
Height (cm)	178.0 (6.93)	175.0 (6.71)	0.274	0.44	Small
HEMOGRAM					
RBC ($10^6 \cdot \mu\text{L}^{-1}$)	5.06 (0.281)	5.15 (0.260)	0.441	0.08	Trivial
Hb ($\text{g} \cdot \text{dL}^{-1}$)	15.6 (0.827)	15.1 (0.676)	0.107	0.49	Small
HCT (%)	44.5 (2.28)	44.6 (1.57)	0.866	0.13	Trivial
MCV (fl)	87.9 (2.19)	86.8 (2.92)	0.305	1.10	Large
MCH (pg)	30.8 (0.35)	29.4 (1.03)	<0.001	1.44	Large
MCHC (%)	35.0 (0.74)	33.8 (0.60)	<0.001	1.19	Large

Values are expressed as mean (SD). Abbreviations: CAT = catalase; SOD = superoxide dismutase; GSH = reduced glutathione; GSSG = oxidized glutathione; % GSSG/GSH = oxidized/reduced glutathione ratio and SD = standard deviation.

Table 11. Endogenous antioxidant enzymes from professional and amateur cyclists.

	PRO	AMA	<i>p</i> -Value	Cohen's <i>d</i>	Effect Size
CAT (U/g Hb)	32.5 (5.34)	25.0 (4.51)	<0.001	1.55	Large
SOD (U/g Hb)	1213 (233.0)	1447 (184.4)	0.009	1.13	Large
GSSG (nmol/mg protein)	0.524 (0.103)	0.321 (0.077)	<0.001	2.28	Large
GSH (nmol/mg protein)	24.4 (2.00)	25.5 (2.17)	0.216	0.50	Moderate
GSSG/GSH	2.16 (0.436)	1.27 (0.279)	<0.001	2.52	Large
GSSG+GSH (nmol/mg protein)	24.9 (2.02)	25.8 (2.19)	0.317	0.41	Small

Values are expressed as mean (SD). Abbreviations: CAT = catalase; SOD = superoxide dismutase; GSH = reduced glutathione; GSSG = oxidized glutathione; % GSSG/GSH = oxidized/reduced glutathione ratio and SD = standard deviation.

6.3.3. Physiological and Metabolic Parameters at VT1

VO_2 , W, WR, $\%VO_{2MAX}$, HR and RER values at VT1 are shown in Table 12. Significant group differences in VO_2 (76.0%, $p < 0.001$), W (90.4%, $p < 0.001$), WR (92.5%, $p < 0.001$), $\%VO_{2MAX}$ (53.3%, $p < 0.001$) and HR (12.9%, $p = 0.004$), but not for RER (0.78%, $p = 0.707$) were observed. GSSG/GSH was significantly correlated with W_{VT1} and VO_{2VT1} ($r = -0.657$ and $r = -0.651$; $p < 0.05$, respectively) in PRO (Figure 29) (Table 13).

Table 12. Metabolic and performance variables of professional and amateur cyclists.

	PRO	AMA	<i>p</i> -value	Cohen's d	Effect size
VT1					
VO_2 (mL·min ⁻¹)	3593 (271.0)	2041 (401.0)	< 0.001	4.40	Large
W	299 (32.9)	157 (36.1)	< 0.001	4.07	Large
WR (W·kg ⁻¹)	4.37 (0.42)	2.27 (0.56)	< 0.001	4.14	Large
$\%VO_{2max}$	76.2 (3.91)	49.7 (5.58)	< 0.001	5.36	Large
HR (beats·min ⁻¹)	149 (14.7)	132 (13.2)	0.004	1.25	Large
RER	0.906 (0.05)	0.899 (0.04)	0.707	0.15	Trivial
VT2					
VO_2 (mL·min ⁻¹)	4259 (234.0)	3389 (505.0)	< 0.001	2.10	Large
W	379 (34.0)	286 (45.1)	< 0.001	2.28	Large
WR (W·kg ⁻¹)	5.54 (0.41)	4.13 (0.74)	< 0.001	2.28	Large
$\%VO_{2max}$	90.3 (2.36)	84.7 (5.67)	0.005	1.24	Large
HR (beats·min ⁻¹)	168 (11.1)	171 (9.4)	0.467	0.29	Small
RER	1.01 (0.05)	1.03 (0.03)	0.323	0.40	Small
VO_{2max}					
VO_2 (mL·min ⁻¹)	4714 (241.0)	4066 (580.7)	0.002	1.38	Large
VO_2/R (mL·kg ⁻¹ ·min ⁻¹)	69.0 (3.94)	58.7 (9.58)	0.003	1.34	Large
W	474 (31.5)	383 (49.2)	< 0.001	2.13	Large
WR (W·kg ⁻¹)	6.93 (0.44)	5.51 (0.81)	< 0.001	2.09	Large
HR (beats·min ⁻¹)	186 (7.42)	186 (7.62)	0.966	0.02	Trivial
RER	1.22 (0.04)	1.14 (0.06)	0.001	1.49	Large

Values are expressed as mean (SD). Abbreviations: VO_2 = oxygen uptake; VO_{2max} = maximum oxygen consumption; VO_2/R = maximum oxygen consumption relative to weight; W = power out-put; WR = power output relative to weight; $\%VO_{2max}$ = percentage of VO_{2max} ; HR = heart rate (beats·min⁻¹); RER = respiratory exchange ratio; VT1 = ventilatory threshold 1; VT2 = ventilatory threshold 2 and SD = standard deviation.

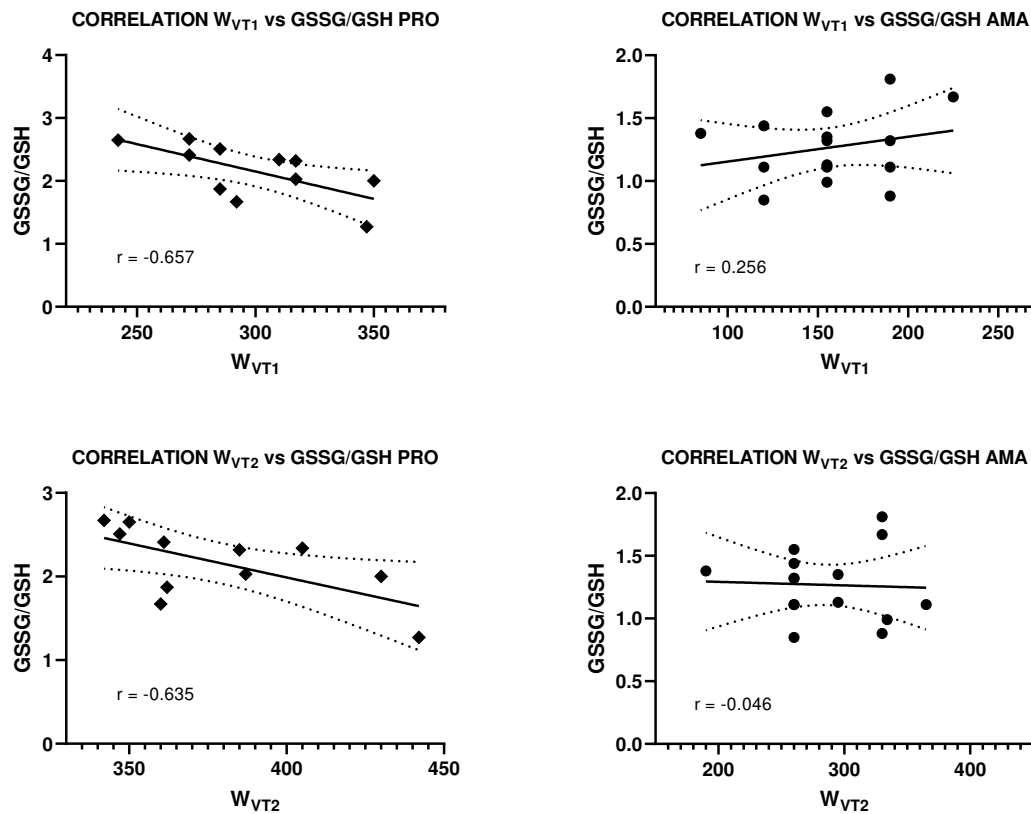


Figure 29. Correlations between the power generated at the ventilation threshold 1 and 2 between the GSSG/GSH ratio in PRO and AMA.

Table 13. Correlation between endogenous antioxidant enzymes and performance-metabolic variables from professional and amateur cyclists.

		CAT	SOD	GSSG	GSH	%GSSG/GSH	GSSG + GSH
PRO (n = 11)							
W_{VT1}	r	-0.120	0.305	-0.449	0.425	-0.657	0.397
	p-value	0.72	0.36	0.17	0.19	0.03	0.23
VO_{2VT1}	r	0.001	0.378	-0.442	0.457	-0.651	0.429
	p-value	0.998	0.252	0.173	0.157	0.030	0.188
W_{VT2}	r	-0.253	0.183	-0.575	0.116	-0.635	0.085
	p-value	0.45	0.59	0.06	0.73	0.04	0.80
VO_{2VT2}	r	-0.319	0.423	-0.518	0.277	-0.622	0.247
	p-value	0.34	0.20	0.10	0.41	0.04	0.46
W_{MAX}	r	-0.045	0.186	-0.342	0.239	-0.443	0.219
	p-value	0.90	0.58	0.30	0.48	0.17	0.52
VO_{2MAX}	r	-0.375	0.422	-0.312	0.304	-0.414	0.284
	p-value	0.26	0.20	0.35	0.36	0.21	0.40
AMA (n=15)							
W_{VT1}	r	0.181	0.172	0.206	-0.102	0.256	-0.098
	p-value	0.52	0.54	0.46	0.72	0.36	0.73
VO_{2VT1}	r	0.360	0.159	0.182	-0.108	0.230	-0.105
	p-value	0.19	0.57	0.52	0.70	0.41	0.71

W _{VT2}	r	0.414	0.113	-0.002	0.047	-0.046	0.040
	p-value	0.13	0.69	0.99	0.87	0.87	0.89
VO _{2VT2}	r	0.358	0.234	-0.104	-0.068	-0.097	-0.077
	p-value	0.19	0.69	0.71	0.81	0.73	0.78
W _{MAX}	r	0.180	0.173	-0.136	-0.379	0.009	-0.386
	p-value	0.52	0.54	0.63	0.16	0.97	0.16
VO _{2MAX}	r	0.289	0.278	-0.118	-0.334	0.001	-0.339
	p-value	0.30	0.32	0.66	0.22	0.10	0.22

Values are expressed as mean (SD). Abbreviations: CAT = catalase (U/g Hb); SOD = superoxide dismutase (U/g Hb); GSH = reduced glutathione (nmol/mg protein); GSSG = oxidized glutathione (nmol/mg protein); % GSSG/GSH = oxidized/reduced glutathione ratio; VO₂ = oxygen uptake; VO_{2MAX} = maximum oxygen consumption; VT1 = ventilatory threshold 1; VT2 = ventilatory threshold 2 and W = power output.

6.3.4. Physiological and Metabolic Parameters at VT2

Table 12 demonstrates the VT2 results of VO₂, W, WR, %VO_{2MAX}, HR and RER. Significant group differences in VO_{2VT2} (25.6%, p = <0.001), W_{VT2} (32.5%, p = <0.001), WR_{VT2} (34.1%, p < 0.001) and %VO_{2MAXVT2} (6.6%, p = 0.005) were observed.

GSSG/GSH was significantly correlated with W_{VT2} and VO_{2VT2} (r = -0.635 and r = -0.622; p < 0.05, respectively) in PRO (Figure 1). GSSG tended to correlate with W_{VT2} (r = -0.575; p = 0.06) in PRO (Table 13).

6.3.5. Physiological and Metabolic Parameters at VO_{2MAX}

Maximal values of VO₂, VO_{2/R}, W, WR, HR and RER are presented in Table 12. Significant group differences in VO_{2MAX} (15.9%, p = 0.002), VO_{2/RMAX} (17.5%, p = 0.002), W_{MAX} (23.8%, p < 0.001), WR_{MAX} (25.8%, p < 0.001), and RER_{MAX} (7.0%, p = 0.001), but not for HR_{MAX} (p = 0.966) were found.

In VO_{2MAX}, no correlation with any antioxidant marker was observed (Table 13).

6.4. DISCUSSION

This study provides the first direct comparison of endogenous antioxidant, hematological, performance and metabolic biomarkers (VT1, VT2 and VO_{2MAX}) between PRO and AMA cyclists. Our results demonstrate that: i) PRO have higher values in MCH, MCHC, CAT, GSSG and GSSG/GSH but lower values in SOD than AMA; ii) PRO have higher levels of absolute and relative power output and oxygen consumption in all intensity zones (VT1, VT2 and VO_{2MAX}) than in AMA, with the largest differences found at VT1; iii) inverse correlations were identified in W_{VT1}, VO_{2VT1}, W_{VT2} and VO_{2VT2} with GSSG/GSH in PRO.

6.4.1. Differences in Antioxidant Enzymes and Hemogram

When intense physical exercise is performed (especially in untrained or those not familiar with the exercise), there is an increase in the production of reactive oxygen species, which are neutralized by our complex endogenous antioxidant defense system (GSH, GSSG, CAT, SOD, GPx and GR) and by exogenous antioxidants (vitamin C, vitamin E, carotenes) (253).

Regarding EAE, we observed higher levels of CAT activity, GSSG and GSSG/GSH, but lower levels of SOD activity in PRO versus AMA. Mena et al. (559) found higher resting levels of SOD, CAT and GPx in a sample of PRO cyclists compared to sedentary people. Tauler et al. (560) also showed differences in antioxidant enzyme activity in erythrocyte between PRO and AMA at rest. In the same study, a decrease in CAT (-12%), GPx (-14%) and GR activity (-16%) but an increase in SOD activity of about 25% after a submaximal test (80% VO_{2MAX} ; 1 h 30 min) was reported (560).

Long distance runners have been shown to have a three-fold higher CAT activity compared to short distance runners (561). Similarly, it was observed that marathon runners had twice as high catalase activity compared to sprinters (541). In this study, we also demonstrated higher levels of CAT in PRO than in AMA, and this may be largely explained by the fact that PRO perform greater volume, intensity and competitions (higher aerobic load and prolonged periods of exercise) than AMA, which induces higher levels of exposure to ROS and, consequently, adaptations of EAE (535). When CAT levels increase, it is possible that GPx activity is not sufficient to neutralize high levels H_2O_2 (endurance exercise) (536).

Regarding SOD, Mena et al. (559) observed lower levels of SOD activity (-32.1%) in PRO than in elite cyclist, but in the case of CAT (80.0%) and GPx (149.0%), the levels were higher, reporting an ascending behavior of SOD during a stage race (2800 km in 17 stages) in PRO. Tauler et al. (560) has also found lower levels of SOD activity in PRO (-19.8%) than in AMA at baseline, which are in line with the results of our study. Antioxidant enzyme activity can be modified either by an initial increase (adaptation) or a decrease if the oxidative stress of long duration (utilization) (248). Therefore, the low basal levels of SOD activity in professional cyclists could be overwhelmed and the high concentration of superoxide anions could activate CAT, allowing compensated metabolism of H_2O_2 . This may be the reason why PRO has lower levels of SOD activity than AMA, as PRO have higher levels of exercise exigency that sometimes get close to exhaustion, which can lead a decrease in the working capacity of SOD.

On the other hand, there is evidence to suggest that GSH or GSH/GSSG decreases during exercise because of its utilization against ROS (248). Ultra-endurance exercise depletes erythrocyte GSH levels by ~66% for 24 h and levels remain ~33%

lower than normal 1 month later (562). PRO frequently compete in longer distance events than AMA, which can lead to lower levels of GSH in PRO than in AMA, although no differences were observed in GSH between PRO vs. AMA in our study. In addition, the muscle can import GSH from plasma during exercise, and as a result, there is a change in the GSH/GSSG ratio after exercise with a decrease in the GSH/GSSG ratio at the time of exhaustion (563). Furthermore, it is important to mention that tissues are not only capable of importing GSH but also exporting GSSG under oxidative stress (563). Moreover, GSH is a molecule that is key in cellular redox status regulation, and consequences of prolonged GSH depletion may include a compromise in immunity, where lower GSH is associated with decreased lymphocyte proliferation and increased viral reactivation (562).

GSSG levels are a biomarker of cellular oxidative stress, since GSH is an important antioxidant in many tissues and oxidizes in the catalyzed reduction of H_2O_2 to H_2O to become GSSG (564). The increase in GSH (mainly) and GSSG in plasma after exercise could be explained by an efflux from the liver to other tissues, including skeletal muscle (565). GSSG levels in skeletal muscle have previously been shown to increase by ~50% in rats after running on a treadmill at moderate intensity (566) and by ~20% after cycling in humans (workload corresponding to 90% of VO_{2peak} ; 10×4 min) (567). Leonardo et al. (568) observed an increase in both GSSG and GSSG/GSH after a period of intense PRO training, which returned to their baseline levels after a period of tapering. We found similar baseline values of GSSG in our study. In addition, we found higher levels of GSSG and GSSG/GSH in PRO than in AMA.

The efforts made during cycling competitions produce oxidative stress in lymphocytes, leading to a reduction in GSH levels and an increase in GSSG levels. The decrease in GSH and increase in GSSG during exercise may be explained by an increase in H_2O_2 formation, as reported by Wang et al. who found that high-intensity exercise (80% VO_{2MAX}) decreased GSH levels while lipid peroxidation increased immediately and after 24h of exercise (569). Furthermore, in this study, lymphocytes were incubated with H_2O_2 for 2 and 4 h, promoting an increase in DNA fragmentation immediately and 24 h after high intensity exercise. Thus, H_2O_2 would cause a failure of the endogenous antioxidant system leading to DNA damage in lymphocytes. Ferrer et al. (570) found that high intensity exercise (swimming) increased GPx activity (converts GSH to GSSG) in lymphocytes, in the same way as other authors found after a cycling stage (571, 572). This supports the decrease in GSH and increase in GSSG after high intensity exercise. Therefore, the higher levels of GSSG and GSSG/GSH in PRO vs. AMA in our study may be due to a higher production of ROS, which leads to a higher production of GSSG and, consequently, of GSSG/GSH together with a decrease in GSH.

In addition, our study is the first to show correlations between GSSG/GSH with

W_{VT1} ($r = -0.657$) and W_{VT2} ($r = -0.635$) in PRO. This is also supported by a trend towards a significant correlation between GSSG and WVT2 ($r = -0.575$; $p = 0.06$) in PRO. These relationships suggest that cyclists who generate more power at VT1 and VT2 have lower GSSG/GSH levels, and therefore, less oxidative stress, as GSSG/GSH ratio is known to be a marker of antioxidant status (549).

In response to strenuous physical working conditions, the body's antioxidant capacity may be temporarily diminished, as its components are used to scavenge the harmful radicals that are produced (573). It is well known that exercise-induced ROS are detrimental to physiological function, including decreased performance and immune function and increased fatigue (573). Moreover, it has been shown that the response of antioxidant capacity to exercise responds in a similar way to the activity of EAE (573). Therefore, the antioxidant defense system may be temporarily reduced in response to increased ROS production but may increase during the recovery period as a result of the initial prooxidant insult (574). However, contradictory findings have been reported where increases in GPx, SOD, and CAT, as well as decreases in GPx, GR, SOD have been observed (573). Evidently, this controversy may depend on the moment of sampling (i.e., period of the season), as well as on the duration and intensity of the exercise, which varies considerably between studies.

It could be that there is an undefined optimal level of ROS production and oxidative damage required for adaptations in antioxidant defenses and other physiological parameters, leading to health and performance improvements (573). However, overproduction of ROS and oxidative damage due to chronic long-term exercise and/or overtraining may exceed the above-mentioned optimal level, resulting in irreparable oxidative damage, which can lead to the development or progression of poor health and/or disease (575). Therefore, the measurement of the antioxidant capacity (CAT, SOD, GSH, GSSG and GSGG/GSH) of the body is used as a marker of oxidative stress and can provide us insight on how it affects performance. Given the results of our research and the evidence shown in the scientific literature, there is no endogenous antioxidant profile defined in PRO compared to AMA.

There are also other antioxidant proteins, such as peroxiredoxin (PRX) and thioredoxin (TRX) containing thiol groups, with a high capacity to neutralize reactive oxygen and nitrogen species and decrease oxidative stress (576). One study showed how moderate and high-intensity exercise and a low volume high intensity interval training trial increased TRX (85%, 64% and 206%, respectively); however, PRX only increased during high intensity exercise (moderate: -6229%; high: 203% and low volume high intensity interval: -23%, respectively) in peripheral blood mononuclear cells (576). In addition, an increase in nuclear transcription factor kappa B was found during all exercises, suggesting an activation of the inflammatory system, probably due to increased oxidative stress. Future studies should examine whether there are

differences in these antioxidant proteins between PRO and AMA and their relationship with performance.

Regarding hematological parameters, no significant differences were found except for MCH and MCHC between PRO vs. AMA. Schumacher et al. found hematological values in elite cyclists from the German national team (blood samples collected between November and January) and the values were similar to ours in Hb (~15.5 g/dL), Hct (~45.0%) and RBC ($\sim 5.0 \times 10^6/\text{mm}^3$) in PRO (577). In addition, other studies have found hematological values of approximately 15.0 g/dL of Hb and 45% of Hct in professional cyclists (578-580). Well-trained cyclists have found values of 14.3 g/dL in Hb and 43.1% in Hct, values lower than PRO (581). However, Bejder et al. (582) observed amateur competitive cyclist values of 14.8 g/dL Hb, 42.8% Hct, $4.92 \times 10^6 \cdot \mu\text{L}^{-1}$ RBC, 87.1 fl MCV, 30.1 pg MCH and 34.6 g/dL MCHC, lower than those reported in PRO.

MCH indicates the amount of hemoglobin contained in an erythrocyte and MCHC is the average hemoglobin concentration (583). Therefore, the red blood cells of PROs will have a higher oxygen transport capacity due to the higher levels of MCH and MCHC. Currently, no study on cyclists has examined the differences in MCH and MCHC, so we cannot draw many conclusions in this regard. These hematological parameters have mainly been used as markers of anemia both in athletes and in the general population (584), but so far, they are not associated with an athlete's performance level in this study.

6.5. LIMITATIONS

Our study had limitations with regards to the sample number, since it was more difficult to recruit PRO athletes than lower-level athletes (AMA).

Differences in endogenous antioxidant marker between this study and previous works may be influenced by the instrumentation and methodology used, the timing of the season at which the measurements were made, and the training status of the cyclists.

6.6. CONCLUSIONS

Regarding the endogenous antioxidants profile, PRO had higher values of CAT, GSSG and GSSG/GSH compared to AMA. An inverse correlation was found for the first time between W_{VT1} and W_{VT2} with GSSG/GSH at rest only in PRO. This indicates better antioxidant status that allow for higher performance with regard to power output. Future studies should examine how training adaptations affect the studied variables and how antioxidant enzymes evolve during a race stage (e.g. Tour de

France), in order to see their association with performance, recovery and fatigue, thereby helping to develop monitoring tools for medical doctors, nutritionists and coaches.

CAPÍTULO VII.

ESTUDIO N° 2

CAPÍTULO VII. ESTUDIO Nº 2: LOS CICLISTAS PROFESIONALES TIENEN NIVELES MÁS BAJOS DE MARCADORES ÓSEOS QUE LOS AFICIONADOS. ¿EXISTE RIESGO DE OSTEOPOROSIS EN LOS CICLISTAS?

7.1. INTRODUCTION

There are several differences between professional (PRO) and amateur competitive (AMA) cyclists, among them are the large volumes of training during the season (~32,500 km) with ~100 days of competition in PRO (18), however, AMA perform between 3 and 7 days of training per week (60–120 min per training) participating about 20 competitions per year (11).

Several physiological parameters have been used as performance predictors in top-level cyclists that include: a) maximum oxygen uptake (VO_{2MAX}), maximum (W_{MAX}) and relative (WR_{MAX}) power output (15, 18, 24, 49, 50, 89); b) oxygen uptake (VO_{2VT2}) and absolute (W_{VT2}) and relative (WR_{VT2}) power output at ventilatory thresholds 1 and 2 (VT1 and VT2) (24, 89, 134); and c) cycling efficiency (1, 78). It has been shown that PRO record high VO_{2MAX} (70-80 mL·kg⁻¹·min⁻¹) and W_{MAX} (>500 W or 6-7.5 W/kg) in a maximal incremental test, as well as an optimized VT2 (~90% VO_{2MAX}), however, lower VO_{2MAX} (70-75 mL·kg⁻¹·min⁻¹), W_{MAX} (300-450 W or 5.0-6.0 W·kg⁻¹) have been shown in AMA compared to PRO (11, 18). The values of VO_2 and power output at VT1 (PRO: 3.20 L·min; 262 W vs AMA: 2.40 L·min; 186 W) and VT2 (PRO: 4.30 L·min; 386 W vs AMA: 3.56 L·min; 299 W) are also higher in PRO than in AMA (VT1 = 25%; 29% and VT2 = 17.2%; 22.5%, respectively) (24, 90).

In addition, PRO develop a high level of cycling efficiency compared to AMA (~24.5% vs 21.7% at 80% VO_{2MAX} , respectively; gross efficiency) that enables them to maintain extremely high workloads for long durations (1, 78, 585). Furthermore, it has been reported that PRO have lower percentage (8-10%) of body fat (BF) (14), an average fat-free mass (FFM) of 62.5 kg (58.3-68.3 kg) and bone mineral density (BMD) of 1.145 g/cm² (586). Average values of 11% in BF and 60 kg in FFM have been found in AMA, with a BMD of 1.187 g/cm² (16). Therefore, PRO have lower BF (- 22.2%) and BMD (- 3.7%) but higher 69 FFM (4.0%) compared to AMA.

In relation to bone metabolism, dietary behavior can modulate both acute bone turnover and long-term bone health, moreover nutrition can have an impact on the diurnal rhythm of markers of bone turnover at rest (587). It has been shown that eating a mixed diet with a meal of mixed nutrients (glucose, fat, protein and calcium) suppresses all markers of bone turnover (588). Previous research has studied the effects

of nutrient intake on sedentary and resting subjects. However, it has been reported that after an exhaustive running (performed treadmill running at 75% VO_{2MAX}), the immediate intake of carbohydrates + protein increases the bone formation markers and decreases the concentrations of bone resorption markers, establishing a positive balance in bone turnover (589). On the contrary, prolonged and intense exercise has been seen to produce an increase in mass resorption, with an increase in the C-terminal telopeptide of type I collagen (590), although the markers of bone formation, such as N-terminal pro-peptides of type 1 procollagen, have less response to acute exercise (591).

Cycling is a sport where some low carbohydrate and energy dietary strategies are used (592) which, combined with the high volumes and intensity in training and competition and influenced by non-impact physical activity (593), can promote bone resorption mechanisms that exceed those of synthesis. Due to these factors that influence cycling, we believe that research should be carried out into whether any bone pathology (osteopenia or osteoporosis) really exists in different cohorts of cyclists (PRO and AMA). Therefore, the main objective of this research was to determine the differences in bone and muscle mass markers (body composition) between PRO and AMA, and whether these might be related to differences in performance (PO at VT1, VT2 and VO_{2MAX}) in an incremental test.

7.2. METHODOLOGY

7.2.1. Selection of participants

Twenty-six cyclists (11 PRO; 15 AMA) completed this study. The AMA selected for this study were from southeast Spain and had to meet the following criteria: i) 20 and 40 years of age, ii) had at least 3 years of cycling experience and iii) cycling training of 6–12 h per week. PRO competed in Union Cycliste Internationale (UCI) PRO TOUR races and participated in UCI major stage races (Vuelta a España, Giro d'Italia, Tour de France). PRO riders were selected based on the following criteria: i) 20 to 40 years of age, ii) enrolled in a licensed professional team, and iii) competed in at least one major 3-week stage races in the last years. All subjects signed their informed consent before their participation. The study was performed following the guidelines of the Helsinki Declaration for Human Research (548) and was approved by the Ethics Committee of Catholic University of Murcia (CE091802).

7.2.2. Study protocol

The experimental design of the study required each cyclist to visit the laboratory twice during the post-season period. Visit 1 consisted of a medical exam, blood analysis and dual-energy X-ray absorptiometry (DXA). After 48 h, cyclists returned to

the lab (visit 2) to perform a maximal incremental test. Two hours before performing this latter test, cyclists consumed a breakfast relative to their body weight (557.7 kcal), which was composed of 95.2 g of carbohydrates (68%), 19.0 g of protein (14%) and 11.3 g of lipids (18%), established by a sports nutritionist. All subjects were previously instructed to refrain from high-intensity training for 48 h before each visit.

7.2.3. Incremental test

An incremental step test with final ramp until exhaustion was performed on a cycle ergometer (Cyclus 2TM, RBM elektronik- automation GmbH, Germany) using a metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine VT1, VT2 and VO_{2MAX} as well as the associated levels of power output. The testing protocol started with 35 W and increased by 35 W every 2 min until $RER > 1.05$ was reached, from which the final ramp ($+35 \text{ W}\cdot\text{min}^{-1}$) until exhaustion was initiated (549). To ensure VO_{2MAX} was achieved, at least 2 of the following criteria had to be met: plateau in the final VO_2 values (increase $\leq 2.0 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in the two last loads), reaching maximal theoretical HR $(220 - \text{age})\cdot 0.95$ (550), $RER \geq 1.15$ and lactate $\geq 8.0 \text{ mmol}\cdot\text{L}^{-1}$ (551, 552). Ventilatory thresholds (VT1 and VT2) were obtained using the ventilatory equivalents method described by Wasserman (553). The variables analyzed in this test were: VO_2 , VO_2 relative to weight in VO_{2MAX} (VO_2/R_{MAX}), absolute power output (W), power output relative to weight (WR), heart rate (HR) and respiratory exchange ratio (RER).

7.2.4. Blood analysis

From an antecubital vein for general analyses, was withdrawn one 3.0 mL tube with ethylenediaminetetraacetic acid (EDTA) for hemogram and another 3.5 mL tube with polyethylene terephthalate (PET) for biochemical parameters, was immediately centrifuged at 3500 rpm at 4 °C for 10 min and sent to the reference laboratory for analysis. Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls.

7.2.5. Dual-energy X-ray absorptiometry (DXA)

Body composition was evaluated by DXA of the whole body (XR-46; Norland Corp., Fort Atkinson, WI). BMD (g/cm^2), mineral content (BMC) (g), bone area (BA) (cm^2) and FFM (g) were assessed in the morning in fasted conditions. In the prior 5 years, there were no deviations detected in the calibration and there were no firmware or software updates. The measuring device had been checked by the manufacturer. During the measurements, all patients wore underwear with no metal accessories.

The scans and analysis were performed by an experienced and certified technician.

7.2.6. Statistical analyses

Levene and Shapiro-Wilks tests were performed to check for homogeneity and normality of the data, respectively. A Student's t-test for unpaired data was used to evaluate differences between groups. Analysis of covariance (ANCOVA) was used to test for mean differences in BMD, BMC and bone area between groups, adjusting for the effect of components with lean mass. The different correlations between the parameters were evaluated using Pearson's correlation (r). Linear regression analysis was performed to assess the association within body composition variables and the association between performance and body composition variables. Significance level was set at $p \leq 0.05$. The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 21.0, International Business Machines Chicago, IL, USA). Descriptive statistics are presented as mean \pm standard deviation (SD). Additionally, the standardized mean differences were calculated using Cohen's effect size (ES) with a 95% confidence interval (CI) for all comparisons. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large (558).

7.3. RESULTS

7.3.1. Subject characteristics

The general characteristics, DXA and hemogram results are presented in Table 1. Age, body mass and height were not significantly different between PRO and AMA groups. In addition, PRO had higher mean corpuscular hemoglobin (MCH) (4.8%, $p \leq 0.001$) and mean corpuscular hemoglobin concentration (MCHC) (3.6%, $p \leq 0.001$) compared to AMA (Table 14).

Table 14. Baseline general characteristics, densitometry (DXA) and hemogram variables of professional and amateur cyclists.

	PRO	AMA	p-value	Cohen's d	Effect Size
Age (years)	28.3 (4.65)	29.3 (6.54)	0.671	0.17	Trivial
Body mass (kg)	68.5 (4.43)	69.9 (5.50)	0.488	0.28	Small
Height (cm)	178.0 (6.93)	175.0 (6.71)	0.274	0.44	Small
HEMOGRAM					

RBC ($10^6 \cdot \mu\text{l}^{-1}$)	5.06 (0.281)	5.15 (0.260)	0.441	0.08	Trivial
Hb ($\text{g} \cdot \text{dl}^{-1}$)	15.6 (0.827)	15.1 (0.676)	0.107	0.49	Small
HCT (%)	44.5 (2.28)	44.6 (1.57)	0.866	0.13	Trivial
MCV (fl)	87.9 (2.19)	86.8 (2.92)	0.305	1.10	Large
MCH (pg)	30.8 (0.35)	29.4 (1.03)	<0.001	1.44	Large
MCHC (%)	35.0 (0.74)	33.8 (0.60)	<0.001	1.19	Large

Values are expressed as mean (SD). Abbreviations: BMD = bone mineral density; BMC = bone mineral content; BA = bone area; FFM = fat-free mass; RBC = red blood cell; Hb = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration and SD = standard deviation.

7.3.2. Bone and muscle mass parameters

Interestingly, PRO had significantly lower BMD (0.911 vs 1.052 g/cm^2 , $p \leq 0.001$, $ES = 1.97$), BMC (1709 vs 2993 g , $p \leq 0.001$, $ES = 4.74$), BA (1877 vs 2842 cm^2 , $p \leq 0.001$, $ES = 6.05$) and FFM ($43,758$ vs $55,943$ g , $p \leq 0.001$, $ES = 3.17$) compared to AMA (Fig. 30). All of the PRO and seven AMA had a BMD value below 1033 g/cm^2 , which is the normal BMD cutoff value for men that was established by the North American Health Survey (NHANES III) (Fig. 31). However, when we performed ANCOVA to analyse the differences in the bone variables using lean mass as a covariate, we found significant differences between groups in BMC ($p = 0.002$; $\eta^2 = 0.334$) and bone area ($p \leq 0.001$; $\eta^2 = 0.448$), with no differences between groups in BMD but with a moderate effect size ($p = 0.159$; $\eta^2 = 0.084$).

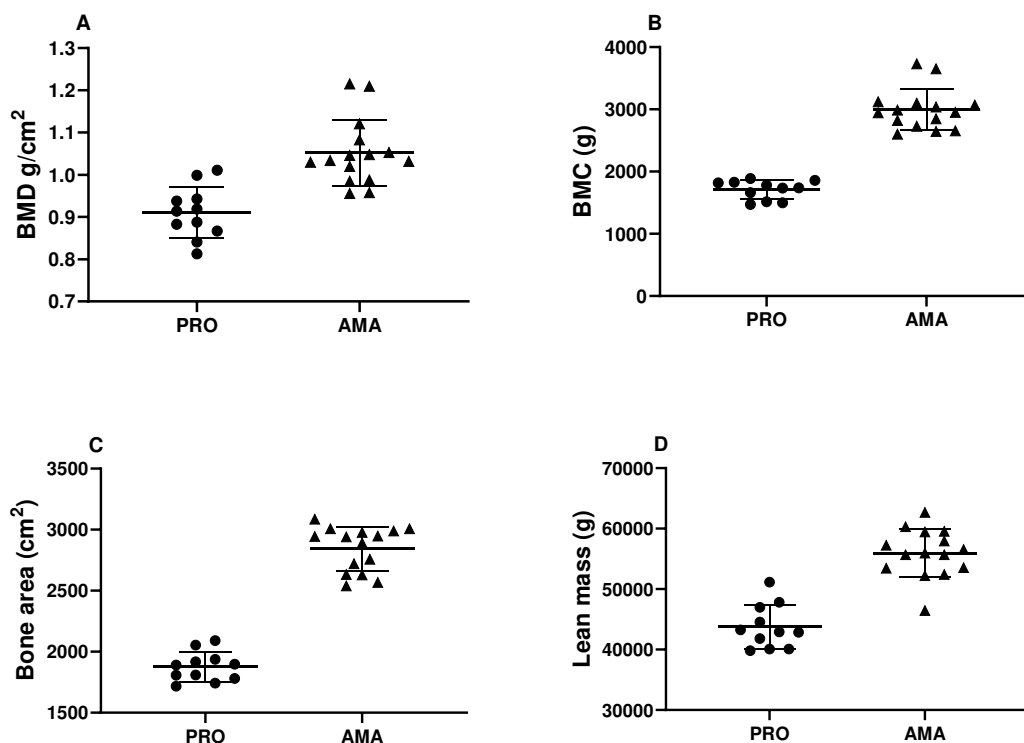


Figure. 30. Bone and muscle differences in PRO compared to AMA, measured by densitometry.

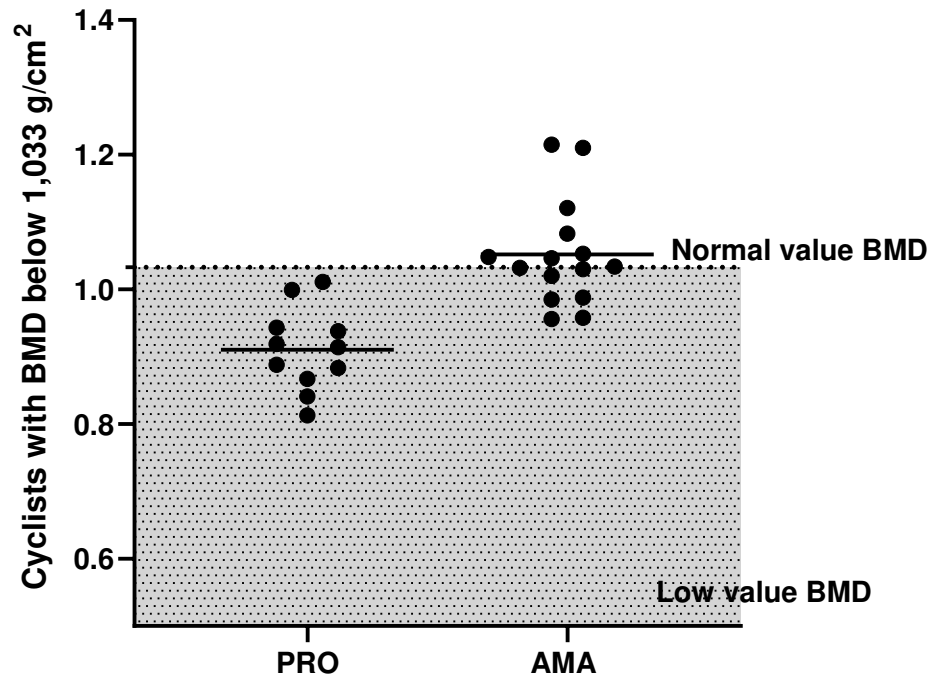


Figure. 31. Cyclists with BMD values below 1033 g/cm², NHANES III has established that BMD values above this threshold are normal.

In the lower limbs, when performing the independent sample T-test, no significant differences were observed between PRO vs AMA in BMD (1140 vs 1200 g/cm², $p=0.574$, $ES=0.49$), BMC (1038 vs 1125 g, $p=0.190$, $ES=0.54$), BA (911 vs 935 cm², $p=0.359$, $ES=0.37$) and FFM (21530 vs 20846 g, $p=0.553$, $ES=0.24$) (Figure 32). But when we performed ANCOVA using FFM as a covariate, significant differences were found in BMD ($p<0.001$; $\eta^2=0.450$), BMC ($p<0.001$; $\eta^2=0.657$) and bone area ($p<0.001$; $\eta^2=0.461$), in PRO compared to AMA.

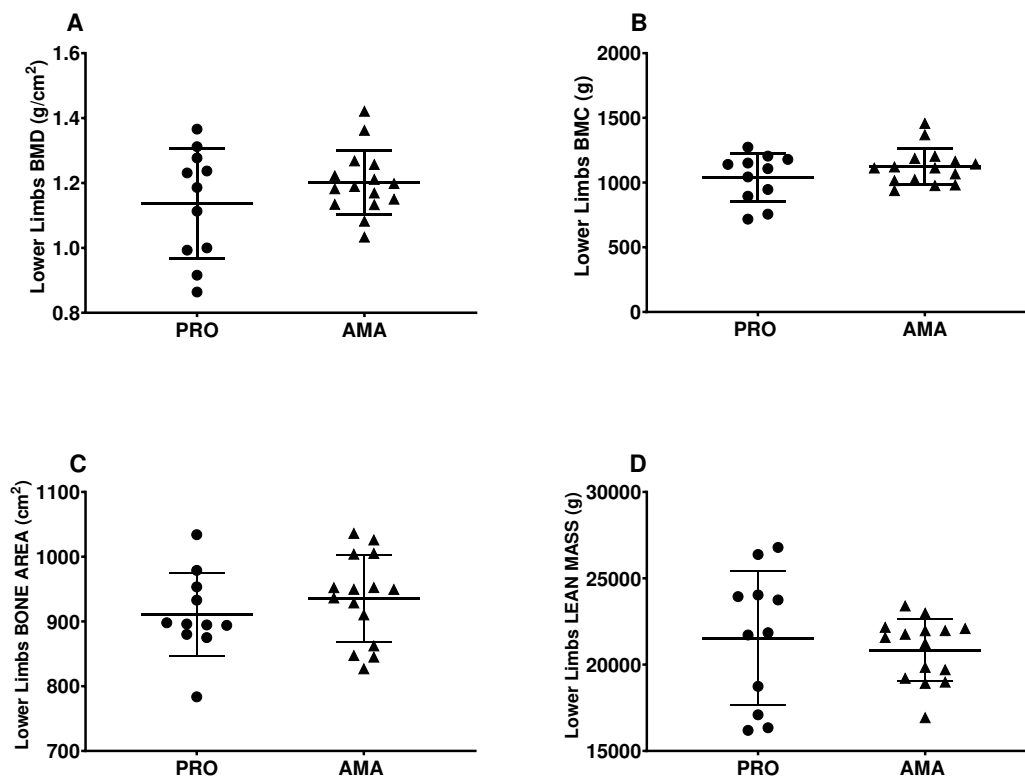


Figure. 32. Differences in bone and muscle mass in the lower limbs in PRO compared to AMA, measured by densitometry.

After the analysis of the correlations between body composition variables, PRO and AMA showed a positive correlation between BMC and FFM ($r \geq 0.562$, $p < 0.05$) (Figure 33), where linear regression showed that 55% of the variance in BMC could be due to FFM in PRO and 32% in AMA. Similar correlations were also observed in PRO and AMA between BMC and BMD and BA ($r \geq 0.686$, $p = < 0.05$) (Table 15). However, the linear regression model only attributed 27% of the variance in BMD to FFM in PRO and 3% in AMA. When correlation analysis was performed for lower limbs, in PRO a significant positive correlation was observed between BMD, BMC, BA and FFM of the legs ($r \geq 0.630$, $p < 0.05$) (Table 16). Although, in AMA, significant positive correlations were seen between BMC with BMD, BA and FFM ($r \geq 0.652$, $p < 0.01$) of the lower limbs, it was not seen between FFM and BMD in the legs. In both PRO and AMA, the linear regression model was improved by comparing the body composition variables measured between the whole body and the legs (Table 16).

Table 15. Correlation and linear regression between body composition variables (whole body) from professional and amateur cyclists.

		BMD	BMC	BONE AREA	FFM
PRO (n=11)					
BMD	<i>r</i>	Perfect line	0.690	-0.052	0.516
	<i>p-value</i>		0.02	0.88	0.10
	<i>R</i> ²		-	0.477	0.003
BMC	<i>r</i>	0.690	Perfect line	0.686	0.738
	<i>p-value</i>	0.02		0.02	0.01
	<i>R</i> ²	0.477		-	0.471
FFM	<i>r</i>	0.516	0.738	0.505	Perfect
	<i>p-value</i>	0.10	0.01	0.11	line
	<i>R</i> ²	0.266	0.545	0.113	-
AMA (n=15)					
BMD	<i>r</i>	Perfect line	0.818	0.205	0.179
	<i>p-value</i>		<0.001	0.46	0.52
	<i>R</i> ²		-	0.669	0.042
BMC	<i>r</i>	0.818	Perfect line	0.730	0.562
	<i>p-value</i>	<0.001		<0.01	0.03
	<i>R</i> ²	0.669		-	0.532
FFM	<i>r</i>	0.179	0.562	0.748	Perfect line
	<i>p-value</i>	0.52	0.029	0.001	
	<i>R</i> ²	0.032	0.316	0.560	

Values are expressed as mean (SD). Abbreviations: BMD = bone mineral density; BMC = bone mineral content; BA = bone area; FFM = fat-free mass. Values $p \leq 0.05$ are in bold.

Table 16. Correlation and linear regression between body composition variables in the legs of professional and amateur cyclists.

		BMD Legs	BMC Legs	BONE AREA Legs	FFM Legs
PRO (n=11)					
BMD Legs	<i>r</i>	Perfect line	0.937	0.320	0.807
	<i>R</i> ²	-	0.877	0.102	0.651
BMC Legs	<i>r</i>	0.937	Perfect line	0.630	0.925
	<i>R</i> ²	0.877	-	0.397	0.855
FFM Legs	<i>r</i>	0.807	0.925	0.698	Perfect
	<i>R</i> ²	0.651	0.855	0.488	-
AMA (n=15)					
BMD Legs	<i>r</i>	Perfect line	0.848	0.288	0.329
	<i>R</i> ²	-	0.719	0.083	0.108
BMC Legs	<i>r</i>	0.848	Perfect line	0.750	0.652
	<i>R</i> ²	0.719	-	0.563	0.426
FFM Legs	<i>r</i>	0.329	0.652	0.782	Perfect
	<i>R</i> ²	0.108	0.426	0.611	-

Values are expressed as mean (SD). Abbreviations: BMD = bone mineral density; BMC = bone mineral content; BA = bone area; FFM = fat-free mass. Values $p < 0.05$ are in bold.

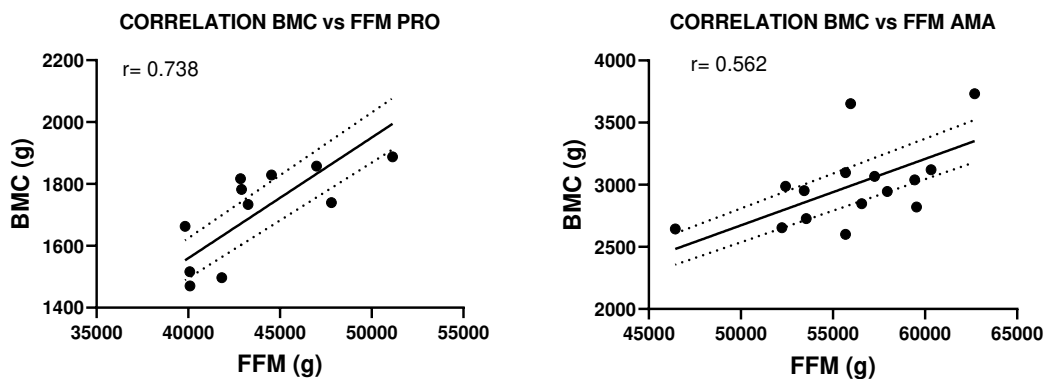


Figure 33. Correlations between bone mineral content and fat-free mass in PRO and AMA.

7.3.3. Physiological and metabolic parameters at VT1

Significant group differences were observed in VO_{2VT1} (3593 vs 2041 mL·min⁻¹, $p < 0.001$, ES= 4.40), W_{VT1} (299 vs 157, $p < 0.001$, 4.07), WR_{VT1} (4.37 vs 2.27 W·kg⁻¹, $p < 0.001$, ES= 4.14), % $VO_{2MAXVT1}$ (76.2 vs 49.7, $p < 0.001$, ES= 5.36) and HR_{VT1} (149 vs 132 beats·min⁻¹, $p = 0.004$, ES= 1.25), but not for RER_{VT1} (0.906 vs 0.899, $p = 0.707$, ES= 0.15) in PRO compared to AMA. Correlations between performance and body composition variables were also evaluated. In the PRO group, significant positive correlations were found between W_{VT1} and FFM ($r = 0.611$, $p < 0.05$) (Table 17) (Figure 34). The linear regression model showed that 11% of the variance of BMC could be explained by the power generated in VT1 (W_{VT1}), and when including FFM as a covariate, the percentage of the variance increased to 46% in PRO. The linear regression model also improved in AMA when comparing W_{VT1} and BA and adjusting for FFM (6% to 55%).

7.3.4. Physiological and metabolic parameters at VT2

Significant group differences were observed in VO_{2VT2} (4259 vs 3389 mL·min⁻¹, $p < 0.001$, ES= 2.10), W_{VT2} (379 vs 286, $p < 0.001$, ES= 2.28), WR_{VT2} (5.54 vs 4.13 W·kg⁻¹, $p < 0.001$, ES= 2.28) and % $VO_{2MAXVT2}$ (90.3 vs 84.7, $p = 0.005$, ES= 1.24), however, no differences were found in HR_{VT2} (168 vs 171 beats·min⁻¹, $p = 0.467$, ES= 0.29) y RER_{VT2} (1.01 vs 1.03, $p = 0.323$, ES= 0.40) in PRO compared to AMA. In the PRO group, significant positive correlations were found between W_{VT2} and BMC and FFM ($r \geq 0.603$, $p < 0.05$) (Table 17) (Figure 34). The linear regression model showed that 36% of the variance in BMC could be explained by the power generated in VT2 (W_{VT2}), but when adjusting for FFM (covariate) the model improved to 43% in PRO. In AMA, the model also improved when comparing W_{VT2} and BA and adjusting for FFM (3% to 50%).

7.3.5. Physiological and metabolic parameters at VO_{2MAX}

Significant group differences were found in VO_{2MAX} (4714 vs 4066 mL·min⁻¹, $p = 0.002$, ES= 1.38), VO_2/R_{MAX} (69.0 vs 58.7 mL·kg⁻¹·min⁻¹, $p = 0.003$, ES= 1.34), W_{MAX} (474 vs 383, $p < 0.001$, ES= 2.13), WR_{MAX} (6.93 vs 5.51 W·kg⁻¹, $p < 0.001$, ES= 2.09), and RER_{MAX} (1.22 vs 1.14, $p = 0.001$, ES= 1.49), but not for HR_{MAX} (186 vs 186 beats·min⁻¹, $p = 0.966$, ES= 0.02). In the PRO group, significant positive correlations were found between W_{MAX} and BMD ($r = 0.684$, $p < 0.05$) (Figure 34). We also observed a moderate correlation with trend between W_{MAX} levels and BMC ($r = 0.585$, $p = 0.059$) and FFM ($r = 0.586$, $p = 0.058$) in PRO (Table 17). The linear regression model results showed that 34% of the variance could be due to W_{MAX} , and when covarying with FFM, the variance increased to 48% in

PRO. However, in PRO, a worsening of the model was found when comparing BMD and W_{MAX} and covarying FFM (47% to 36%). In AMA, the model improved when comparing W_{VT2} and BA and adjusting for FFM (11% to 51%).

Table 17. Correlation and linear regression between of body composition and performance variables from professional and amateur cyclists.

		BMD	BMC	BONE AREA	FFM
PRO (n=11)					
W_{VT1}	r	0.441	0.326	0.011	0.611
	R^2	0.194	0.106	<0.001	0.373
	R^2a	0.114	0.462	0.244	-
W_{VT2}	r	0.563	0.603	0.268	0.775
	R^2	0.317	0.364	0.072	0.601
	R^2a	0.166	0.434	0.116	-
W_{MAX}	r	0.684	0.585	0.123	0.586
	R^2	0.467	0.343	0.015	0.343
	R^2a	0.359	0.475	0.125	-
AMA (n=15)					
W_{VT1}	r	0.027	0.149	0.235	0.010
	R^2	<0.001	0.022	0.055	<0.001
	R^2a	-0.129	0.226	0.547	-
W_{VT2}	r	0.119	0.174	0.168	0.107
	R^2	0.141	0.030	0.028	0.012
	R^2a	-0.118	0.217	0.496	-
W_{MAX}	r	0.216	0.338	0.336	0.251
	R^2	0.047	0.114	0.113	0.063
	R^2a	-0.093	0.250	0.514	-

Values are expressed as mean (SD). Abbreviations: BMD = bone mineral density; BMC = bone mineral content; BA = bone area; FFM = fat-free mass. R^2a = Linear regression for BMD, BMC and BA for each of the exercise zones (W_{VT1} , W_{VT2} and W_{MAX}) was fitted with FFM from each group. For the linear regression of FFM with the different exercise zones no covariates were used. Values $p = <0.05$, and trend ($p=0.06$) with $r>0.500$ are in bold.

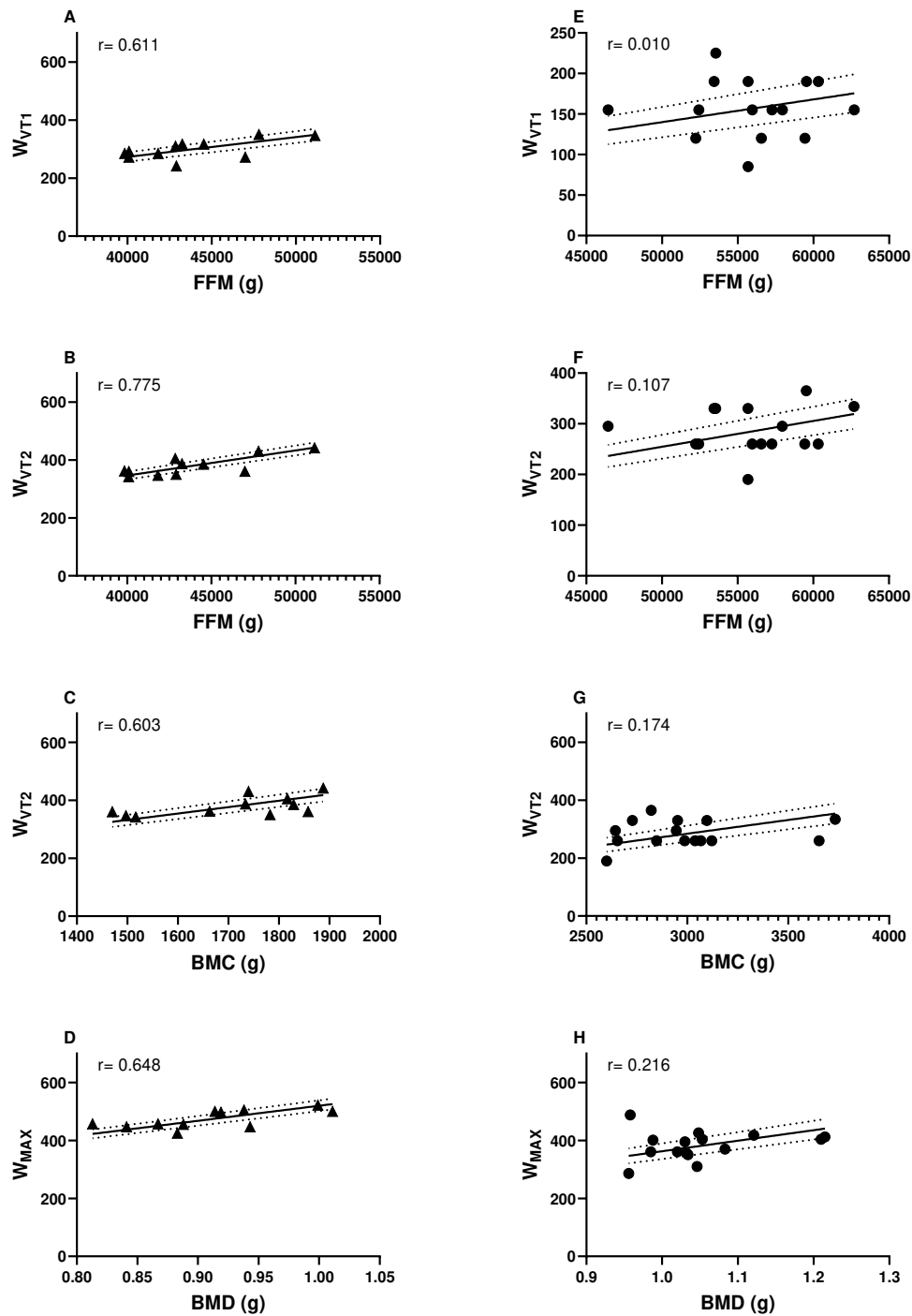


Figure 34. A-D correlations between body composition markers (bone and muscle mass) and performance in PRO. E-H correlations between body composition markers (bone and muscle mass) and performance in AMA.

7.4. DISCUSSION

This research was aimed at finding out differences in body composition and their relationship to performance. Our results demonstrate that: i) lower levels of bone (BMD, BMC and BA) and muscle mass (FFM) variables in PRO vs AMA; ii) positive correlations between BMD, BMC and FFM in PRO; iii) linear regression models were better at comparing body composition and performance variables in the legs than in the whole body in both groups; iv) improvement of the linear regression model when comparing FFM-adjusted performance and body composition variables in both groups, except for W_{MAX} and BMD relationship and v) PRO have higher levels of absolute and relative power output and VO_2 in all intensity zones (VT1, VT2 and VO_{2MAX}) than in AMA, with the largest differences found at VT1.

7.4.1. Differences in subject characteristics and body composition

Overall, the data shown in this study report lower values of bone health markers (BMD, BMC, BA) in PRO compared to AMA. According to the NHANES III, the normal BMD total values in men is 1.033 g/cm² (594). We found that all our PRO cyclists were below this value (0.911±0.061 g/cm², -2.1 to -21.3% with respect to normal values), and in AMA, there were 7 out of the 15 cyclists below the normal value for BMD (1.052±0.079 g/cm², -9.1 to +15.2% with respect to normal values). These inferior values concur with the low BMD values observed in elite and master cyclists (highly trained males) (593, 595) and are most likely explained by the nature of cycling which elicits low impact forces on the bone. Many authors have studied the relationship between cycling and bone health and have found lower BMD of the lumbar spine in PRO compared to a control group (596), as well as lower BMD of the femoral neck and lumbar spine compared to healthy males (595). González-Aguero et al. (597) found that young cyclists had lower levels of BMC and volumetric BMD in some sites of the tibia and radius than sedentary young people. This indicates that cycling induces modifications in bone status from an early age, and this is aggravated when cyclists reach higher levels of competition (professional cyclists). Previously, Olmedillas et al. (598) also described a negative impact on bone health in adolescent cyclists, BMD for pelvis, where hip, leg and whole body and leg bone area were lower but higher in the hip area (all, $P \leq 0.05$) after adjusting for lean mass and height (adolescent cyclists vs sedentary).

Peak BMC is an important parameter in monitoring bone health, particularly during aging (586). However, anthropometric characteristics (fat and muscle mass) have also been shown to have an effect on bone mass (586). Medelli et al. (586) showed 2.8 kg of BMC in PRO, which are higher than those obtained in our PRO cohort (1.7

kg). The variations in BMC data between the two studies may be due to differences in the instrumentation used or FFM.

In amateur competitive cyclists it has been seen that a reduction in energy availability leads to a significant reduction of 2.3% in lumbar BMD over a 6 month interval, however a significant increase of 2.2% in lumbar BMD was detected for those who had improved energy availability (599). On the other hand, cyclists who increased skeletal load showed a significant increase of 1.4% in lumbar BMD, compared to a significant decrease of 2.5% in lumbar BMD for those who had reduced skeletal load (599). An increase in calcium lost through sweat during 2 hours of moderate cycling has also been observed and is associated with a decrease in serum calcium accompanied by an increase in plasma parathyroid hormone concentration, which can promote bone resorption (600). Although this response was mitigated, when a calcium supplement was taken immediately before or during cycling, the parathyroid response to exercise (601). Cortisol is another factor influencing bone metabolism, as it has been seen to increase after completing an ultra-marathon (endurance sport) and is negatively correlated with bone formation markers (602). However, a recent study reported that serum cortisol concentration increased steadily post-exercise in relation to pre-exercise after 4 days of cycling training (3 h daily day), conversely, a decrease in pre-exercise cortisol was found on days 2 to 4 compared to pre-exercise on day 1 (592).

Osteocalcin is another bone marker that responds to metabolic stress and intervenes secondarily in the regulation of energy metabolism. Osteocalcin is a marker of bone formation (in its carboxylated form) and when there is low energy or high energy demand it is excreted in its non-carboxylated form, acting as a secondary messenger (603). It has been shown that a professional cycling competition (Giro d'Italia, duration 3 weeks) increased the carboxylic form of osteocalcin in the mornings (before the competition) on the 12th and 22nd (604), indicating an increase in metabolic stress and not bone synthesis. Therefore, this continuous state of a decrease in bone synthesis processes together with an increase in bone resorption processes supported by situations of low energy availability that would lead to hormonal modulation, would predispose the professional cyclist to a low BMD. But this increased response of bone turnover can be positive in non-professional athletes, however in professional athletes who train more than once a day with a short recovery time between training sessions and rest between days are more likely to have a continuous increase in bone remodeling (accumulation of micro-damage in the bones), which can lead to negative effects on bone health with an increased risk of stress fractures (592, 605, 606). This hypothesis could be in line with our results where we found a low BMD in all PRO but only in around half of the AMA. Future studies should clarify this pathological situation in the PRO and AMA, suggesting new prevention strategies in this sense.

Interestingly, Medelli et al. (586) showed abnormally low BMD values in two-thirds of PRO and observed positive correlations between FFM, BMC and BMD. In contrast to our findings where we found a moderate but non-significant correlation between FFM and BMD in PRO only ($r=0.516$) but significant between BMC and FFM ($r=0.738$) in AMA. In addition, the linear regression model showed that 55% in PRO and 32% in AMA of the variation in BMC could be due to FFM. Previous studies have detected a significant positive correlation between FFM and whole-body BMD in AMA ($r=0.634$) and in PRO ($r=0.420$) (16, 586). In contrast, we found lower correlations in AMA ($r=0.179$) and higher correlations in PRO ($r=0.516$) in whole body measurements. However, Medelli et al. (586) found no significant correlation between total BMC and whole-body FFM in PRO, although, right leg and left leg BMC were positively correlated with FFM ($r=0.44$; $p<0.05$ and $r=0.83$; $p<0.001$), respectively. In line with these previous results, we observed a significant positive correlation between BMC and FFM in PRO ($r=0.925$; $p<0.01$; $R^2 0.855$) and AMA ($r=0.652$; $p<0.01$; $R^2 0.652$) in the legs, suggesting that there might be some effect of FFM on the lower limbs. In fact, our PRO showed -21.8% in FFM compared to AMA, together with decreased BMD, BMC and BA. An effect of FFM on bone tissue has been suggested to be exert specific loading on the bones, which involve loading force, frequency and location (607). This was confirmed by our linear regression models both when assessing whole body and lower limb body composition, with the PRO model being superior to AMA. Overall, PRO have lower values in all bone health markers, which indicate a higher risk of fractures compared to AMA. These differences are not found when comparing the lower limbs. However, the correlations found between BMC and FFM in both PRO and AMA suggest that a larger muscle mass may have a protective effect on bone tissue.

7.4.2. Performance and metabolic differences

7.4.2.1. Differences in VT1

At VT1, PRO had higher values in VO_2 (76.0%), W_{VT1} (90.4%), WR_{VT1} (92.5%), $\%VO_{2MAXVT1}$ (53.3%) and HR_{VT1} (12.9%) compared to AMA. Compared to our study, Lucia et al. (24) found lower values in VO_{2VT1} (3190 mL), W_{VT1} (262 W), WR_{VT1} (3.8 $W \cdot kg^{-1}$), $\%VO_{2MAXVT1}$ (65%), HR_{VT1} (138 $beats \cdot min^{-1}$) and RER_{VT1} (0.86) in PRO at VT1. Interestingly, another study in PRO by the same authors observed higher values than ours in W_{VT1} (321 W) and WR_{VT1} (4.55 $W \cdot kg^{-1}$) and HR_{VT1} (154 $beats \cdot min^{-1}$) (134). These tests were carried out using a ramp test (continuous workload increases of $5W \cdot 12 s^{-1}$ or $25 W \cdot min^{-1}$) (134), which may explain the discrepancy between studies.

A novelty of the present study is that the differences between PRO and AMA in power produced and VO_2 were larger at VT1 (90.4% and 76.0%; respectively) than at VT2 (32.5% and 25.6%) or VO_{2MAX} (23.8% and 15.9%). However, Lucia et al (24) found

better values for power output (11.5%) and VO_2 (5.6%) at VT1 in PRO compared to elite riders, these lower differences may be mainly due to the fact that elite riders had a higher competitive level than our AMA sample. These differences could be explained by the fact that PRO cyclists have a high amount of type I muscle fibers, which is associated with a lower sub-maximum oxygen cost (greater gross efficiency) (78), possibly due to a lower turnover of ATP during the contraction (608). Moreover, the number of years spent performing endurance training is also related to the percentage of type I fibers in skeletal muscle (i.e., higher utilization of fat metabolism in PRO) (14).

We also found significant correlations between W_{VT1} and $\text{VO}_{2\text{VT1}}$, $\% \text{VO}_{2\text{MAXVT1}}$, $\text{VO}_{2\text{VT2}}$ and $\text{VO}_{2\text{MAX}}$ in both groups ($r \leq 0.683$). These findings suggest that cyclists who generated more power in VT1 had higher VO_2 in the measured metabolic zones. These correlations found in both groups indicate that cyclists who are able to generate more power at VT1 have a higher VO_2 at VT1, VT2 and $\text{VO}_{2\text{MAX}}$. There is no literature that studied similar parameters in VT1 to compare with our study. Moreover, we did not find any significant correlation in W_{VT1} between performance and bone variables but with FFM in PRO. Overall, we can establish that an essential requirement for being PRO is the ability to generate high levels of PO at low-moderate intensities (VT1), coupled with high levels of VO_2 and $\% \text{VO}_{2\text{MAX}}$.

7.4.2.2. Differences in VT2

VT2 represents high work intensity where there is a considerable accumulation of lactate in blood (production exceeds clearance), associated to a marked increase in ventilation in an effort to buffer acidosis (116, 609). At VT2, we have found higher VO_2 , W_{VT2} , WR_{VT2} and $\% \text{VO}_{2\text{MAX}}$ in PRO vs AMA. Lucia et al. (24) observed similar findings at VT2 in PRO cyclists during an incremental test (VO_2 : 5100 mL, oxygen uptake relative to weight (VO_2/R): $62.2 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, W: 385 W, WR: $5.5 \text{ W}\cdot\text{kg}^{-1}$, $\% \text{VO}_{2\text{MAX}}$: 87%, HR: $172 \text{ beats}\cdot\text{min}^{-1}$ and RER: 0.99).

Some studies have found higher $\% \text{VO}_{2\text{MAX}}$ in VT2 or lactate threshold in AMA compared to lower fitness level participants (14, 610). In PRO, high values of VT2 (~90% of $\text{VO}_{2\text{MAX}}$) have been documented, similar to those found in our study (24). Therefore, these findings indicate that high VT2 values may provide an advantage to cyclists, particularly during climbing stages where cyclists find themselves working close to VT2 for prolonged durations (~30-60 min) (15). The ability to work for long periods and at high $\% \text{VO}_{2\text{MAX}}$ at VT2 seems to be an important factor for success in professional competitions that are of long duration (> 4 hours).

On the other hand, previous studies have found a strong correlation between average power output (approximately generated in VT2) and flat cycle TT of 40 km ($r = -0.99$) (611) and during a mountain climb (10-km up-hill cycling) (-0.61 to -0.85), the latter being positively affected by lower weight (612). It has been reported that

absolute power output at VT2 correlates with the 40 km cycle TT ($r=-0.81$) (611) and that sub-maximum power output with various methods to identify ventilatory thresholds was significantly associated with 40 km cycle TT ($r=0.73$ to 0.81) in competitive cyclists (613). We have found significant correlations between W_{VT2} and the different VO_2 levels (VT1, VT2 and VO_{2MAX}) in both groups. Therefore, high power production at VT2 might be linked to higher oxygen uptake in PRO and AMA. Also, in PRO, we found a significant positive correlation between W_{VT2} with BMC and FFM ($r \leq 0.603$), which had not been described before. Furthermore, the linear regression model showed that 36% of the changes in BMC could be due to W_{VT2} in PRO, and when adjusted for FFM, the model improved to 43%. This could originate from different mechanisms. For example, W_{VT2} might correlate with BMC because of the link between W_{VT2} and FFM, due to the fact that the generation of higher tension forces at the muscle-tendon-bone level, plays a key role in the remodeling and/or prevention of bone loss. This indicates a link between muscle mass, bone mass and VT2 performance.

Therefore, we can say that there are great differences between PRO and AMA at the performance and metabolic level in VT2, with an important relationship between muscle, bone, and VT2 power in PRO. Although there is no study in the current literature that has found a cause-effect relationship between higher FFM and BMD and VT2 performance. It should also be mentioned that a greater power generated at VT2 in absolute values does not always guarantee success in a competition, because in a mountain stage the power to weight ratio is very important. Hence, cyclists, who have VT2 values close to VO_{2MAX} , would have greater performance capability in longer races.

7.4.2.3. Differences in VO_{2MAX} and maximum power output

VO_{2MAX} is one of the best predictor of performance in endurance sports (1). We found higher values in VO_{2MAX} , VO_2/R_{MAX} , W_{MAX} , WR_{MAX} and RER_{MAX} in PRO compared to AMA. Other studies have also reported higher VO_{2MAX} values ($73.9-84.0 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) in PRO compared to the data found in our PRO cohort (24, 134, 614, 615). Besides, we showed that the PRO reached average W_{MAX} of 474 W and WR of $6.9 \text{ W}\cdot\text{kg}^{-1}$.

Padilla et al. (15) found W_{MAX} ranging from 400 to 500 W (6.5 to $7.5 \text{ W}\cdot\text{kg}^{-1}$) in a 4-minute incremental test in PRO, which is consistent with our results (474W). Interestingly, other studies have shown that PRO can reach these power outputs using shorter increments (1-minute increments of 25W) (24, 89, 134, 616). In the same line, Chicharro et al. (617) found significantly higher VO_{2MAX} (12.7%; 72.0 vs $63.9 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and W_{MAX} (30.3%; 499.8 vs 383.7 W) in PRO vs AMA (respectively). The greater difference for W_{MAX} than for VO_{2MAX} suggests that the O_2 cost of cycling is likely better in PRO vs AMA. However, the same authors showed similar RER (-0.9%; PRO: 1.16 vs AMA: 1.17) and HR (1.6%; PRO: 194 vs AMA: 191 $\text{beats}\cdot\text{min}^{-1}$) at exhaustion in both groups after an incremental test (increments of $25 \text{ W}\cdot\text{min}^{-1}$).

Although high levels of VO_{2MAX} are needed to be competitive in road cycling races at world level, it has been proposed that this parameter is not a good predictor of performance for PRO (24), whereas W_{MAX} is (49). Hawley and Noakes (49) reported a significant correlation ($r = -0.91$) between W_{MAX} in a graded exercise test (25 W every 150 s) and 20km TT in trained cyclists. We found significant correlations between W_{MAX} and VO_{2VT1} , VO_{2VT2} and VO_{2MAX} in PRO and AMA. This suggests that cyclists who generate more power have a higher VO_2 , and this likely contributes to higher performance in competition. Additionally, we showed significant positive correlations between W_{MAX} and BMD ($r = 0.684$, $p = 0.02$), and a trend with BMC ($r = 0.585$, $p = 0.06$) and FFM ($r = 0.586$, $p = 0.06$), only in PRO, which have not been previously demonstrated before. Furthermore, the linear regression model showed that 47% and 34% of the changes in BMD and BMC, respectively, could be due to W_{MAX} in PRO, and when adjusting for FFM, the model improved to 48% for BMC and worsened to 36% for BMD. Based on our results and taking into account the relationship between lower W_{MAX} and BMD in PROs, special attention should be paid to bone health in PROs with lower sports performance (W_{MAX}), in order to establish some type of treatment. Overall, all of the PROs had low BMD values (NHANES), so a system for monitoring bone status in professional cycling teams should be put into place. On the other hand, Izquierdo et al. (618) observed a significant correlation between muscle cross-sectional area (at the lower third portion between the greater trochanter and lateral joint line of the knee) and W_{MAX} in amateur cyclists during an incremental test. Previous studies and the present findings question the link between muscle mass/strength and road cycling performance. González-Aguero et al. (597) that cycling may negatively impact bone health of cyclists at an early age, which could transcend into further complications later when they become professional cyclists. Furthermore, a literature review concluded that professional road cycling may be more detrimental to bone mass compared to recreational cycling, or worse than other disciplines (e.g., cross-country cycling or cycling/running combinations), hypothesizing that differences in BMD between cyclists and controls or other sport practitioners become greater from the age of 17 years and on (619). Thus, achieving high power output for long durations is important in cycling performance and the role of muscle strength in cycling performance has been repeatedly reported, especially with studies investigating the effects of concurrent aerobic and heavy strength training (69, 620-623).

7.4.3. Limitations

Our study had limitations with regards to the sample, since it is more difficult to recruit PRO athletes than lower-level athletes (AMA).

Differences in body composition markers between this study and previous works may be influenced by the instrumentation and methodology used, the timing of the season at which the measurements were made, and the training status of the cyclists.

7.5. CONCLUSIONS

In our study, 100% of PRO had BMD values below the normal values (1,033 g/cm²) proposed by NHANES III in men (594). In addition, in the physiological-metabolic profile, PRO had lower values of BMD, BMC and FFM, but superior W and VO₂ at VT1, VT2 and VO_{2MAX} compared to AMA. Interestingly, we found positive correlations in PRO between FFM with BMC and BMD, which indicates that muscle mass can promote higher levels of bone markers. Future studies should explore the physiological-metabolic profile at different times of the season in both PRO and AMA.

CAPÍTULO VIII.

ESTUDIO N° 3

CAPÍTULO VIII. ESTUDIO Nº 3: EFECTOS AGUDOS DE LA INGESTA DE HESPERIDINA EN MARCADORES DEL ESTADO OXIDANTE/ANTIOXIDANTE Y EL RENDIMIENTO EN CICLISTAS AFICIONADOS.

8.1 INTRODUCTION

The use of ergogenic aids in sports has increased considerably in recent years (624). This growing interest is driven by the emergence of studies that have shown how ergogenic aid intake can contribute to improvement in athletic performance, post-exercise recovery or antioxidant capacity enhancement, as well as changes in body composition (e.g. body fat loss or increase in lean muscle mass), by a stimulation of fatty acid mobilization (625-629). Within the ergogenic aids there are several categories, which represent the different degrees of evidence shown in a large amount of research, the category A is the highest level of evidence and in it they are found proteins, amino acids, creatine, beta-alanine, carbohydrates, etc (630).

One potentially promising group that can serve as an ergogenic aid is polyphenols (27). Polyphenols are bioactive compounds which are widely distributed in plant and plant-based foods, such as vegetables, fruit, cocoa, tea, coffee and wine (631). Polyphenols are a very diverse group of compounds, with over 500 different molecules identified in foods, which can be divided into four main categories, according to their chemical structure: flavonoids, phenolic acids, stilbenes and lignans (632). In addition, polyphenols are of the most studied compounds for their positive effects on human health (633). Specifically, these products are often used for chronic disease, delaying the ageing process, improving body composition, and increasing life expectancy. Moreover, polyphenols have been proposed to be beneficial in exercise and exercise performance (634-637). In fact, some polyphenols, such as quercetin (27, 638-640) or cocoa flavanols (641-643) have been extensively used for this purpose.

For example, polyphenols have been proposed to improve performance by increasing mitochondrial biogenesis in two different ways. Firstly, they stimulate stress-related cell signalling pathways that increase the expression of genes encoding cytoprotective proteins, such as nuclear respiratory factor 2 (NRF2) (644). Secondly, some polyphenols have been reported to modulate muscle function and mitochondrial biogenesis by activating sirtuins (SIRT1) and increasing PGC-1 α activity (645-648). Moreover, polyphenols have been shown to work effectively against exercise induced oxidative stress (649, 650), since as seen in many investigations, the exercise increases reactive oxygen species (ROS) production, which may result in oxidative stress, and

lead to muscle fibre damage, which eventually results in muscle fatigue (256, 651-653). Within the large family of biomolecules that are polyphenols, the most studied in the field of sports performance is quercetin (638, 639), although new molecules of this family such as luteolin, mangiferin (654) and hesperidin (32) are being investigated.

Hesperidin is a polyphenol, specifically a flavonoid, that is mostly found in citrus fruits (460). Hesperidin is the most relevant flavonoid in some citrus species, such as sweet orange (*Citrus sinensis*), finding high concentration of hesperidin in orange juice (up to 513 mg/L) (655). Hesperidin is a chiral molecule, and can be found in two isomeric forms, as 2S- and 2R-Hesperidin. However, the 2S-Hesperidin form is naturally predominant in citrus fruits (656), being present in fresh sweet orange juice with an 2S isomer:2R isomer ratio of 15.4:1 in favour of the 2S-epimer (92% 2S-Hesperidin) (657). However, during the industrial extraction and isolation of Hesperidin, part of this 2S-epimer naturally present in the citrus fruits is transformed into the 2R-epimer. In commercial hesperidin samples, the 2S isomer:2R isomer ratio is close to 1.5:1 in favour of the 2S-epimer (about 60% 2S-Hesperidin). Cardiose® is a natural orange extract, produced by HealthTech BioActives (Murcia, Spain), that due to its unique manufacturing process maintains most of the natural hesperidin isomeric form (NLT 85% 2S-Hesperidin).

Hesperidin antioxidant (454), anti-inflammatory (30) and health promoting (658) properties have been extensively described. Moreover, the intake of hesperidin improves the nitric oxide (NO) synthesis through the activation of phosphorylation of proto-oncogene tyrosine-protein kinase (Src), protein kinase B (Akt), adenosine monophosphate-activated protein kinase (AMPK) and endothelial NO synthase; leading to an increased flow-mediated dilation (31). An increased NO production leads to an improved endothelial function, allowing an enhanced O₂ transport to working muscles during acute exercise and prolonged exercise (659). In humans, the intake of flavanone-rich foods (a type of flavonoids including Hesperidin) has been linked to an increase in NO production, increased endothelium-dependent microvascular reactivity as well as a reduction in diastolic blood pressure (494, 660-663). Endothelial function and different cardiovascular parameters were also improved after hesperidin supplementation in individuals with metabolic syndrome (31) and in overweight individuals (664).

Hesperetin, hesperidin aglycone and its main metabolite, has shown to boost mitochondrial energy production (spare capacity by 25%), an increased intracellular ATP by 33%, reduced oxidative stress in a human skeletal muscle cell model (35). Moreover, in this same study also observed that the intake of hesperidin in aged mice (50 mg/kg/d) reverted the age-related muscle loss, improving its running performance. In the same direction, the effects of hesperidin on biochemical parameters and physical performance have been also studied in humans. Pittaluga et al. (2013) investigated the

effect of supplementation with a self-administrated amount of 250 mL of fresh red orange juice (ROJ), which is a natural source of hesperidin, thrice a day and 1 h before each meal for 4 weeks, after a single bout of exhaustive exercise in healthy trained elderly women. The working capacity expressed as metabolic equivalents (METs) was significantly higher after ROJ supplementation (9.0%) than in placebo (-1.5%), while there was no significant increase VO_{2MAX} in the ROJ group (532).

Previous studies, have observed effects in biochemical markers after the intake of hesperidin, De Oliveira et al. (2013), after supplementation with hesperidin (glucosyl hesperidin; 100 mg/kg body mass) led to a decline of serum glucose with combined beneficial effect on swimming. Continuous or intermittent swimming with hesperidin supplementation lowered total cholesterol (-16%), LDL-C (-50%) and triglycerides (-19%), and increased HDL-C (48%) (530), in rats. Previous research has shown that daily consumption of 500 mL of orange juice, which is a natural source of hesperidin, ingested for 3 months during three months decrease lactic acidosis generated by the incremental exercise. The decrease in plasma lactate concentration was higher in the trained and hesperidin supplemented group (-27%) than in the control trained group (-17%), overweight middle-aged women subjected to aerobic training (665).

Another ability of hesperidin, is its capability to modulate the antioxidant state. De Oliveira et al. (2013), found that the consumption hesperidin enhanced the antioxidant capacity on the continuous swimming group (183%) and lowered the lipid peroxidation (TBARS) on the interval swimming group (-45%), in rats (530). Similarly, Estruel-Amades et al. (2019) observed an impact of hesperidin on the oxidant/antioxidant status of lymphoid tissues after an intensive training program was evaluated on rats. Supplementation with hesperidin, enriched in its 2S-isomer, led to a prevention of the increased ROS production and the decrease in SOD and catalase activities after an exhaustion test. These antioxidant effects of hesperidin were associated with a higher performance in the assessed training model (33).

In trained male athletes, supplementation for 4 weeks with 2S-Hesperidin (500 mg/day) improved cycling time-trial performance with a significant increase in power output during the exercise test (32). Gelabert-Relato et al. (2019) compared acute and chronic effect (48 h and 15 days of supplementation) with high- and low-dose intake of polyphenols (luteolin and mangiferin), on sprint test and endurance exercise in physically active men (654). The results showed a significant improvement in the sprint test (sprint 15 s after ischemia) in peak power output (PPO) and mean power output, after the polyphenol supplementation. Also, in the Wingate test, the experimental group improved by 4% in. Also, Davis et al. (2010) conducted a crossover study examining quercetin's ability to increase endurance capacity and maximal oxygen uptake (VO_{2MAX}) in healthy untrained volunteers. They showed that a 7-day quercetin

supplementation (1000 mg/day) produced improvement in time-to-fatigue and $\text{VO}_{2\text{MAX}}$ by 13.2% and 3.9%, respectively, during a cycling test (53).

Previous studies, which have used different types of polyphenols and dosages, have observed enhancements in performance. Although the exercise protocols used were different, all of them showed improvements in exercise performance with high aerobic component (32, 53, 431, 666-668). However, to our knowledge, only one study reported improvements in the anaerobic component of exercise (654). Thus, it is not clear if there is a beneficial effect of polyphenols on anaerobic exercise. Furthermore, only one study has examined the effect of hesperidin on exercise performance, showing an increase of absolute power output by 5% (32). Moreover, given the importance of anaerobic component on performance in endurance sport and the limited evidence found related to the intake of hesperidin in anaerobic performance and its mechanisms, it seems necessary to conduct more research in order to clarify this relationship.

Therefore, the aims of the present study were (1) to examine the effects of acute intake of Cardiose® (500 mg of 2S-hesperidin) on anaerobic performance (peak power, power average, time to peak power, max speed and total energy ($\Sigma 4$ sprint test), (2) to determine the metabolic markers during exercise in ventilatory threshold 1 (VT1), and (3) to compare oxidative/antioxidant state during a rectangular test and after 24 h recovery in amateur cyclists. The recommendations from this experimental study will have the potential to inform about the optimal supplementation guidelines to optimise the performance and the recovery practices in athletes and provide sports nutritionist key information regarding the effects of polyphenol intake on sports performance and markers of oxidative stress.

8.2. METHODOLOGY

Fifteen healthy male amateur cyclists (Table 18) completed the study. All participants had to meet the following inclusion criteria: aged 18–50 years, normal BMI (19–25 $\text{kg}\cdot\text{m}^{-2}$), at least 1-year of cycling experience, undergoing 6–12 h/week of training and being regular citrus consumers. The exclusion criteria were: (a) smokers or alcohol drinkers, (b) metabolic or cardiorespiratory pathologies or anomalies, (c) acute or chronic digestive pathologies that may interfere with the capacity to absorb nutrients, (d) injury in the last 6 months, (e) intake any type of supplementation or drug in the last 2 weeks, (f) no normal values in some parameter of the previous blood analysis and regular consumer of citrus fruits (≥ 5 oranges and derivatives/week). All subjects signed their informed consent before participating in the study. The study was performed in accordance with the guidelines of the Helsinki Declaration for Human

Research (669) and was approved by the Ethics Committee of the Catholic University of Murcia.

Table 18. Baseline general characteristics of the study participants.

Age (years)	Height (cm)	Weight (kg)	BMI (kg/m ²)	BF (%)	VO _{2max} (mL·kg ⁻¹ ·min ⁻¹)	VT1 (%)	VT2 (%)
33.3 ± 7.9	174.9 ± 4.2	69.4 ± 4.5	22.7 ± 1.2	11.2 ± 2.2	61.6 ± 7.4	53.0 ± 6.1	86.0 ± 4.7

BMI = Body mass index; BF = Body fat; VO_{2max} = Maximum oxygen consumption; VT1 = Ventilatory threshold 1 (aerobic); VT2 = Ventilatory threshold 2 (anaerobic).

8.2.1. Experimental Design

A randomized, single-blinded cross-over design was performed (Figure 35). Participants completed a total of 2 exercise sessions. Five hours before the exercise sessions, they ingested Cardiose® (500 mg of 2S-hesperidin) or placebo (500 mg of microcellulose), supplied by HealthTech BioActives (Murcia, Spain). Cardiose® contains standardized hesperidin (90% hesperidin, being at least 85% as 2S-hesperidin isomer) from sweet orange (*Citrus Sinensis*).

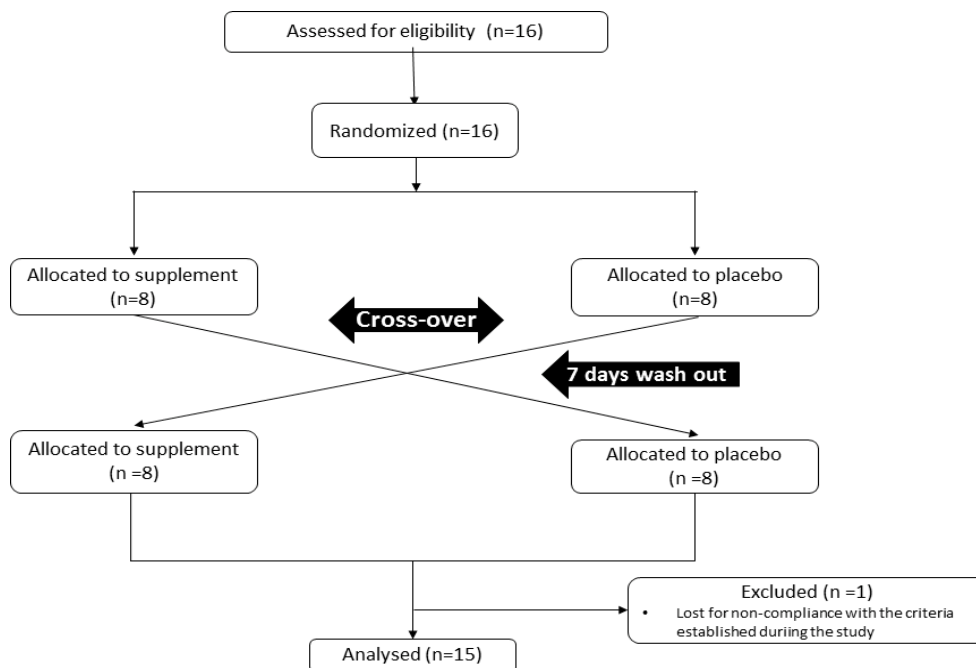


Figure 35. Consolidated Standards of Reporting Trials flow chart of participants during the study intervention.

8.2.2. Procedures

Participants visited the Research Center for High Performance Sport at the Catholic University of Murcia at six different times. Visit 1 consisted of a medical examination, blood extraction, and anthropometry. Visit 2 consisted of a 24 h diet questionnaire before testing and an incremental test until exhaustion on a bike. Five hours prior to visit 3, participants were supplemented with Cardiose® or placebo, according to the treatment arm. During Visit 3, participants underwent another 24 h diet questionnaire before testing, a 20 min test at ventilatory threshold 1 (VT1) intensity on a bike before and after a repeated all-out sprints test on a cycle ergometer, and four blood extractions. Visit 4 (24 h following visit 3) consisted of blood extraction and obtaining 24 h urine collection from the participant. Visits 5, 6, and 7 involved the same procedures performed as in visit 2, 3 and 4, respectively, but five hours prior to visit 6, participants were supplemented the other ingredient (Cardiose® or placebo). There were no significant differences between the 24-h diet questionnaire made by the subjects. A standardized breakfast was consumed 2.5 h prior to each testing session (visits 2 and 3). The breakfast contained 95.16 gr of carbohydrates (68%), 18.86 g of protein (14%) and 11.30 g of lipids (18%), prescribed by a sports nutritionist.

8.2.3. Tests

8.2.3.1. Medical Exam

The medical exam included a medical history, resting electrocardiogram, and medical examination (auscultation, blood pressure reading, etc.), so as to confirm that the participant was healthy and without any risk to be enrolled in the study.

8.2.3.2. Anthropometry

The same researcher (International Society for the advancement of the Kinanthropometry Level-1 certified) performed the anthropometric measurements in both, pre- and post-test. Height and body weight were measured using a digital scale for clinical use with a stadiometer (SECA 780; Vogel & Halke GmbH & Co. Hamburg, Germany). The skinfold thickness was assessed in accordance with ISAK guidelines (670), using Holtain Skinfold Calipers (Holtain, Ltd. Crymych Pembrokeshire, UK). Percentage of body fat was determined with the Faulkner Equation (671) and the percentage of muscle mass with the modified Matiegka equation (672). The sum of the eight skinfolds was also calculated.

8.2.3.3. Maximal Test

An incremental step test was performed with the metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine the ventilator threshold 1 and 2 (VT1 and VT2) and VO_{2MAX} . The test started at 35 W and increased 35 W every 2 minutes until exhaustion. To verify VO_{2MAX} , the following criteria were assumed: plateau in the final VO_2 values (increase $\leq 2.0 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in the 2 last loads), maximal theoretical HR ($220 - \text{age}$), $RER \geq 1.15$ and a lactate value $\geq 8.0 \text{ mmol}\cdot\text{l}^{-1}$ (551, 552). The ventilator threshold was obtained using the ventilator equivalents method described by Wasserman (553).

8.2.3.4. Rectangular Test

A constant effort was carried out on the bike at VT1 intensity during 20 min before and after the repeated all-out sprints test. The main objective of this test was to determine cardiorespiratory variables (VO_2 , CO_2 , RER, HR, and exercise economy) during a steady effort at low-intensity (Figure 36). This test was conducted before and after the repeated sprints test.

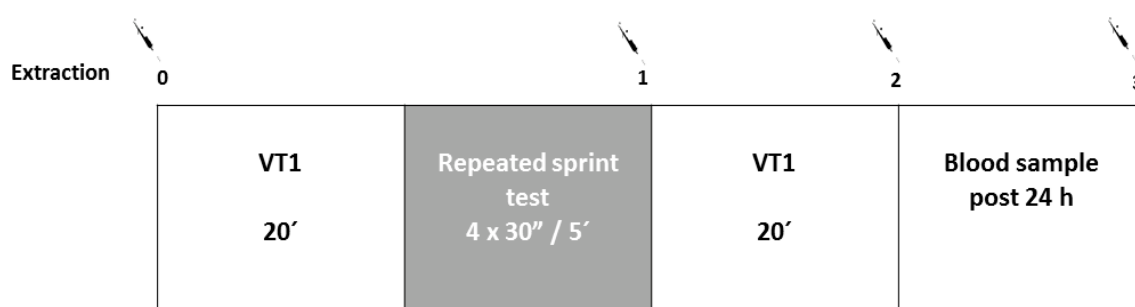


Figure 36. Exercise protocol and blood sampling plan during Visit 3/6. VT1 = ventilatory threshold 1; syringe = blood sample.

8.2.3.5. Repeated Sprints Test

The exercise protocol consisted of 4×30 sec all-out sprints (Wingate test; WAnT) performed on a cycle ergometer (Monark Ergomedic 894E Peak Bike, Vansbro, Sweden) with 5 min of rest between sprints. For every sprint, the breaking resistance was constant (7.5% of body mass) and individualized for each participant (Figure 36). All subjects were verbally encouraged to continue to pedal as fast as they could for the entire 30 s. Peak power and anaerobic capacity were calculated and recorded in watts (W) and watts per kilogram body weight (W/kg^{-1}). The total energy was calculated as the energy produced during the four test sprints in joules (J).

8.2.3.6. Blood and Urine Analysis

Venous blood samples were taken for general analytics, in one tube of 3 mL ethylenediaminetetraacetic acid (EDTA) for hemogram, and in another tube of 3.5 mL with polyethene terephthalate (PET) for biochemical parameters. Red blood cells count (RBC) was carried out in an automated Cell-Dyn 3700 analyzer (Abbott Diagnostics, Lake Forest IL, USA), using internal (Cell-Dyn 22, Abbott Diagnostics, IL, USA) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, hemoglobin, haematocrit, and hematimetric indexes (mean cell volume, MCV; mean cell haemoglobin, MCH; mean corpuscular hemoglobin concentration, MCHC; and red cell distribution width, RDW) were estimated.

Additionally, venous blood samples were collected pre VT1 test, post repeated sprint test, post second VT1 test, and 24 h after the end of the testing session for the measurement of antioxidant parameters (Figure 36). At each of the extraction points, 6 tubes of 3 mL of EDTA were obtained and one of them was centrifuged at 3500 rpm at 4 °C during 10 min and sent to the laboratory for later analysis. Urine samples, corresponding to 24 h urine collection from each participant after the supplementation, were frozen in liquid nitrogen after collection and thawed for its analysis.

8.2.3.7. Hesperidin Metabolites Urine

Fifty μL of urine were mixed with 100 μL of water with 1% formic acid containing the internal standard (rac-Hesperetin-d3). Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series Agilent Technologies, Sta. Clara, CA, USA). The method was validated using a pool of samples by determining the limit of detection (MDL) and quantification (MQL), repeatability (expressed as relative standard deviation RSD), and accuracy (%). Metabolites were quantified by external standard calibration using rac-Hesperetin-d3 as the internal standard.

8.2.4. Markers of Oxidative Stress and Antioxidant Status

8.2.4.1 TBARS

Thiobarbituric acid reactive substances (TBARS) are a by-product of the oxidative degradation of lipids by reactive oxygen species (lipid peroxidation), a commonly used marker of oxidative stress (673). The principle of the method consists of isolating the lipid fraction of the plasma by precipitation of the lipids with phosphotungstic acid, followed by a reaction with thiobarbituric acid (TBA) that forms an adduct that allows detection by UV-VIS spectrophotometer at a wavelength of 532 nm. The assay involves the reaction of malondialdehyde (MDA), a product of lipid peroxidation, with

thiobarbituric acid (TBA) under high temperature and acidic conditions to form an MDA-TBA complex that can be measured colourimetrically (674)

8.2.4.2. *Catalase*

Catalase (CAT) activity was determined using a UV-VIS spectrophotometer. The principle of the method is that the absorbance of H₂O₂ decreases at 240 nm proportional to its decomposition, so that the concentration of H₂O₂ is critical in this determination. The decrease in absorbance per unit time is the measure of catalase activity. This is expressed in sec⁻¹ per gram of hemoglobin.(675). The coefficient of variation between replicas must be less than or equal to 4.9%.

8.2.4.3. *SOD*

Superoxide dismutase (SOD) activity was measured using a SD125 Ransod kit (Randox Ltd. Crumlin, Reino Unido). This method consists of the use of xanthine and xanthine oxidase to produce superoxide anion (O₂⁻), which responds with the 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) reactive and forms a red complex detectable at 420 nm. The activity of SOD was measured through the inhibition of this reaction. The SOD activity is then quantified by measuring the degree of inhibition of this reaction (555). The coefficient of variation between replicas must be less than or equal to 5.1%.

8.2.4.4. *Glutathion*

Glutathion (GSH) was analyzed by the glutathione-S-transferase assay described by Akerboom and Sies (556). The GSH was determined from whole blood, which was treated with perchloric acid to a final concentration of 6%, obtaining the supernatant after vortexing and centrifuging at 10.000 rpm for 10 minutes. After collecting the supernatants in vials, it was quantified by high performance liquid chromatography (HPLC) using a Waters ODS S5 NH₂ Column (0.052, 25 cm) for separation purposes. Glutathion oxidized form, glutathione disulphide (GSSG) was determined in a similar way to GSH as shown above, as described by Asensi (557).

8.2.5. Statistical Analyses

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 21.0, International Business Machines Chicago, IL, USA). Descriptive statistics are presented as mean ± SD. the assumption of normality was verified using the Shapiro–Wilks test. Paired sample t-test was used to evaluate differences between groups (Cardiose® or placebo supplement). Moreover, a General Linear Model (repeated measures, analysis of variance, ANOVA) was performed for analyzing the

within-group effects of the intake of the Cardioise® or placebo supplement (4 time points). Additionally, the standardized mean differences (Cohen's d (ES)) between groups was calculated together with the 95% confidence intervals and η^2 to analyze the size between groups. Finally, the relationships between levels of excreted hesperidin metabolites in urine and total energy and catalase activity were analyzed using Spearman correlation analysis (r). For all procedures, a level of $p \leq 0.05$ was selected to indicate statistical significance.

8.3. RESULTS

8.3.1. Repeated Sprint Test

Results for each variable were analyzed in two different ways: taking the best data of each of the four sprint test included in the series, and considering all the sprints as a unique exercise, using for each variable the average of all sprints results.

On one hand, taking into account the data corresponding to the best sprint, significant positive changes were observed in Cardioise® compared to placebo in average power, maximum speed, and total energy (Σ 4 sprint test). However, no significant changes were found in peak power and time-to-peak power comparing Cardioise® versus placebo group using these data (Table 19) (Figure 37).

Considering the average values of the four sprints trials, positive changes were observed in the peak power, time-to-peak, and total energy in the Cardioise® group compared to placebo, but not reaching the statistically significance (Table 19) (Figure 37). In addition, there was a positive significant correlation ($r = 0.547$; $p = 0.043$) between the levels of excreted hesperidin metabolites in urine and the difference in total energy (Σ 4 sprint test) between the placebo and supplemented group.

Table 19. Repeated sprint test outcomes.

Parameters	Best sprint data		Average (all sprints)	
	Cardioise® Mean \pm SD	Placebo Mean \pm SD	Cardioise® Mean \pm SD	Placebo Mean \pm SD
Peak Power (w)	835.50 \pm 96.08	803.79 \pm 110.43	740.16 \pm 74.52	729.55 \pm 91.36
Power average (w)	567.84 \pm 55.44*	555.25 \pm 51.81*	511.71 \pm 52.68	510.78 \pm 52.99
Time to peak power (ms)	2840.69 \pm 715.99	3235.85 \pm 1516.06	3003.13 \pm 950.28	3476.14 \pm 1546.57
Max speed (rpm)	132.86 \pm 9.59*	128.70 \pm 9.24*	120.83 \pm 7.79	119.92 \pm 9.79

Total energy (J)	16246.29 ± 1600.37*	15827.79 ± 1505.86*	14874.79 ± 1570.83	14818.36 ± 1608.24
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Δ = percentage of pre-post change; W = watts; ms = millisecond; J = joules; Max speed = maximum speed; rpm = revolutions per minute SD = standard deviation. * = between-group significant changes (p < 0.05).

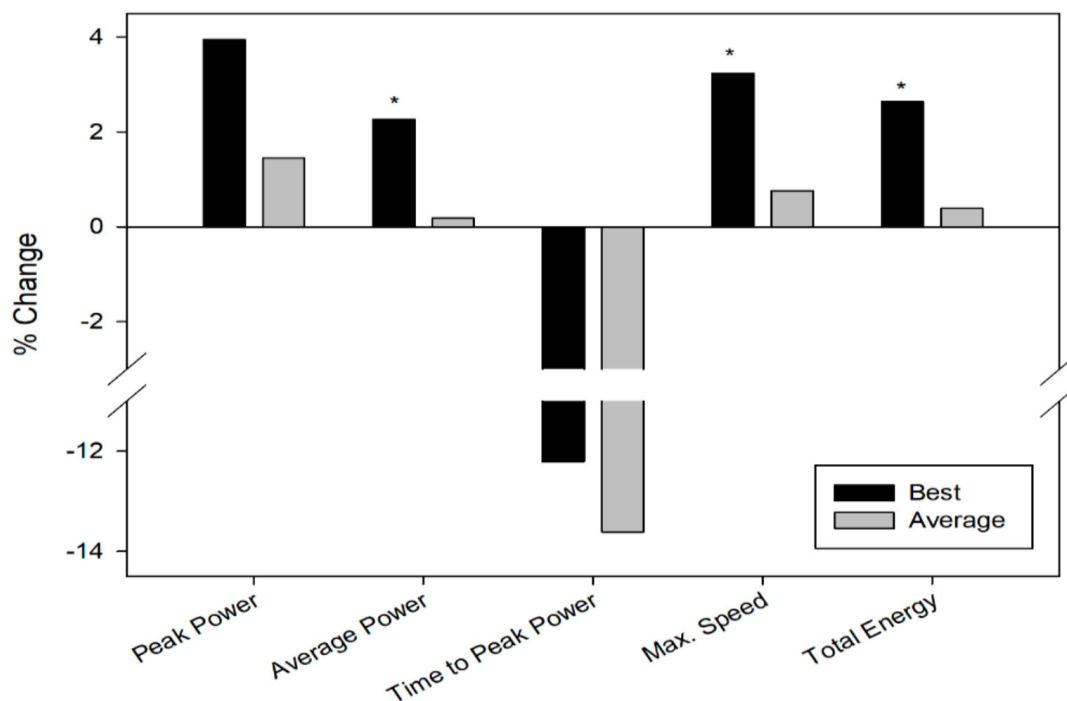


Figure 37. Changes in the repeated sprint test results after supplementation with Cardioise® using best data of each of the four sprint tests and the average of all sprints. * = between-group significant changes (p < 0.05).

8.3.2. Metabolic Parameters

Metabolic parameters were evaluated and compared for the rectangular tests (20 min at VT1) carried out before and after the repeated all-out sprints test. During the VT1 testing period, no significant differences were observed in pre-post changes between Cardioise® and placebo group (Table 20).

Table 20. Metabolic parameters in VT1 stage before and after the repeated sprint test.

Metabolic parameters		Cardioise®	Placebo
		Mean ± SD	Mean ± SD
VO ₂ (L/min)	Pre	2.11 ± 0.39	2.06 ± 0.39

	Post	2.15 ± 0.45	2.13 ± 0.51
VCO ₂ (L/min)	Pre	1.93 ± 0.41	1.86 ± 0.35
	Post	1.83 ± 0.43	1.77 ± 0.43
RER	Pre	0.91 ± 0.03	0.90 ± 0.02
	Post	0.85 ± 0.03	0.83 ± 0.02
Efficiency (mL/Kg/W)	Pre	3.97 ± 0.48	3.85 ± 0.39
	Post	4.02 ± 0.49	3.94 ± 0.51
HR (pul/min)	Pre	128.55 ± 9.53	128.04 ± 8.95
	Post	144.15 ± 12.50	144.74 ± 11.31
Carbohydrates	Pre	105.5 ± 33.3	97.6 ± 19.7
	Post	72.9 ± 28.5	63.6 ± 17.7
Fat	Pre	14.1 ± 6.0	15.9 ± 6.1
	Post	28.5 ± 4.0	31.8 ± 8.7

Δ = percentage of pre-post change; heart rate (beats·min⁻¹); SD = standard deviation; VO₂ = oxygen uptake (L·min⁻¹); VCO₂ = carbon dioxide production (L·min⁻¹); W = watts; HR = Heart rate; RER = Respiratory exchange ratio.

In relation to the cardiorespiratory parameters, a non-significant increase in oxygen consumption and a significant decrease in CO₂ production were observed in both groups. However, no significant differences between groups were observed for these two parameters. In the same way, respiratory exchange ratio (RER), which is the ratio between the amount of CO₂ produced and oxygen used in metabolism, significantly decreased from the first to the second rectangular test in both groups, but without significant differences between them. Exercise economy is defined as the oxygen uptake relative to body mass used at the VT1 workload. After the repeated sprint test, there was a non-significant increase in exercise economy in both groups. Also, there were no significant differences between groups. Moreover, the high-intensity repeated sprint test caused a significant increase in heart rate during the second VT1 stage of rectangular test in both groups. However, there were no relevant differences between supplements (Table 20).

Regarding energy substrates, a significant decrease in carbohydrates consumption and a significant increase in fat energy contribution from the first to the second rectangular test was observed in both groups, as expected after an intense physical activity such as repeated sprint test. Despite no significant differences observed between the Cardiose® and placebo groups, this modulation of the energy substrates was slightly different in both supplements. Cardiose® led to a higher increase (15.1%) in the fat energy contribution (Figure 38).

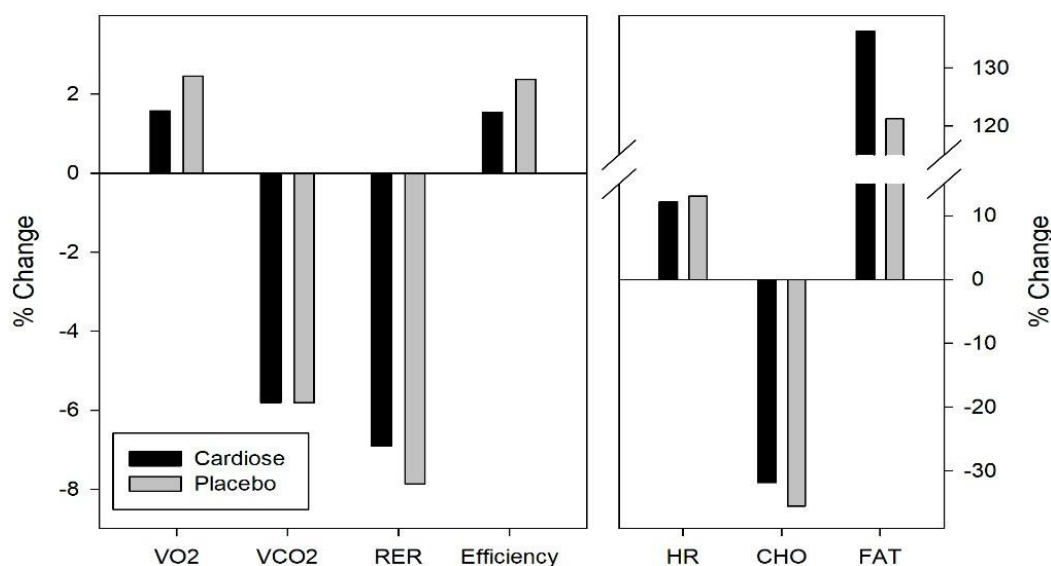


Figure 38. Changes in cardiorespiratory and metabolic parameters between the rectangular test (VT1 intensity during 20 min) carried out before and after the repeated all-out sprints test.

8.3.3 Antioxidant Parameters

Effects of each treatment on the modulation of oxidative status were evaluated by the activity of the endogenous antioxidant system, such as SOD, CAT, GSH, GSSG, and TBARS, as biomarker of lipid peroxidation. These markers were evaluated prior to the testing session (E1: pre VT1 test, and 5 h after supplementation), post repeated sprint test (E2), post second VT1 test (E3), and 24 h after the end of the testing session (E4).

Regarding the activity of the CAT enzyme, the exercise protocol included in this study led to significant differences in CAT activity at the different evaluated points in both groups. For instance, a significant decrease was observed from the end of the repeated sprint test to the end of rectangular test in both groups. Regarding differences between the Cardioise® and placebo groups, catalase activity was slightly increased in the Cardioise® group after supplementation with this product prior to the testing session, after the repeated sprint test, and at the end of the testing session despite catalase activity being almost the same in both groups 24 h after the end of the testing session. However, these differences between groups were not statistically significant (Table 21). An inverse significant correlation between the levels of hesperidin metabolites excreted in urine and the percentage variations in the catalase activity at points E1 and E3 ($r = -0.625$; $p = 0.013$) between the placebo and supplemented group was observed.

Regarding the activity of SOD, repeated sprint test increased SOD activity both in Cardioise® and placebo groups. However, a completely different trend was observed

after this strenuous exercise in SOD activity of both groups. At the end of the rectangular test, SOD activity decreased in the Cardioise® group, while it slightly increased in the placebo group. In contrast, both groups experienced a decrease in SOD activity from the end of the physical test to 24 h after exercise. However, these differences were not statistically significant (Table 21) (Figure 39).

Physical activity included in the exercise protocol increased TBARS levels in both groups. However, a greater attenuation of lipid peroxidation, identified by a decrease in TBARS, was observed in the Cardioise® group from after repeated sprint test to the end of rectangular test. However, no significant changes were observed between interventions and between blood extraction points (Table 21) (Figure 39).

Table 21. Antioxidants and TBARS measured in repeated sprint test. Antioxidant markers were evaluated prior to the testing session (pre VT1 test, and 5 h after supplementation) (E0), post repeated sprint test (E1), post second VT1 test (E2), and 24 h after the end of the testing session (E3).

Antioxidant/oxidant status markers	Cardiose®						Placebo		
	Mean ± SD						Mean ± SD		
	E0	E1	E2	E3	E0	E1	E2	E3	
CAT (U/g Hb)	25.66 ± 4.74	53.93 ± 13.41*	27.53 ± 6.54*	24.66 ± 4.27	24.02 ± 3.13	51.41 ± 16.41*	27.07 ± 4.63*	24.53 ± 4.18	
SOD (U/g Hb)	1298.00 ± 261.75	1349.13 ± 225.31	1269.27 ± 271.13	1228.33 ± 229.77	1319.00 ± 145.54	1352.13 ± 201.31	1364.80 ± 272.74	1337.67 ± 193.97	
GSH (nmol/mg protein)	25.02 ± 2.80	24.89 ± 2.90	23.73 ± 2.10	24.36 ± 2.75	24.36 ± 2.24	23.59 ± 3.37	23.62 ± 3.19	24.60 ± 1.72	
GSSG (nmol/mg protein)	0.351 ± 0.073	0.334 ± 0.075	0.315 ± 0.067	0.378 ± 0.152	0.325 ± 0.073	0.316 ± 0.078	0.336 ± 0.068	0.388 ± 0.130	
% GSSG/GSH	1.42 ± 0.32	1.35 ± 0.31	1.34 ± 0.31	1.54 ± 0.54	1.34 ± 0.28	1.37 ± 0.41	1.45 ± 0.37	1.57 ± 0.48	
TBARS (nmol/mg protein)	2.49 ± 0.34	2.71 ± 0.45	2.56 ± 0.44	2.63 ± 0.26	2.43 ± 0.22	2.63 ± 0.36	2.57 ± 0.38	2.58 ± 0.32	

Abbreviations: CAT = catalase; SOD = superoxide dismutase; GSH = Reduced glutathione; GSSG = oxidized glutathione; % GSSG/GSH = % oxidized glutathione/ Reduced glutathione; TBARS = Thiobarbituric acid reactive substances; SD = standard deviation. * = intra-group significant changes ($p < 0.05$)

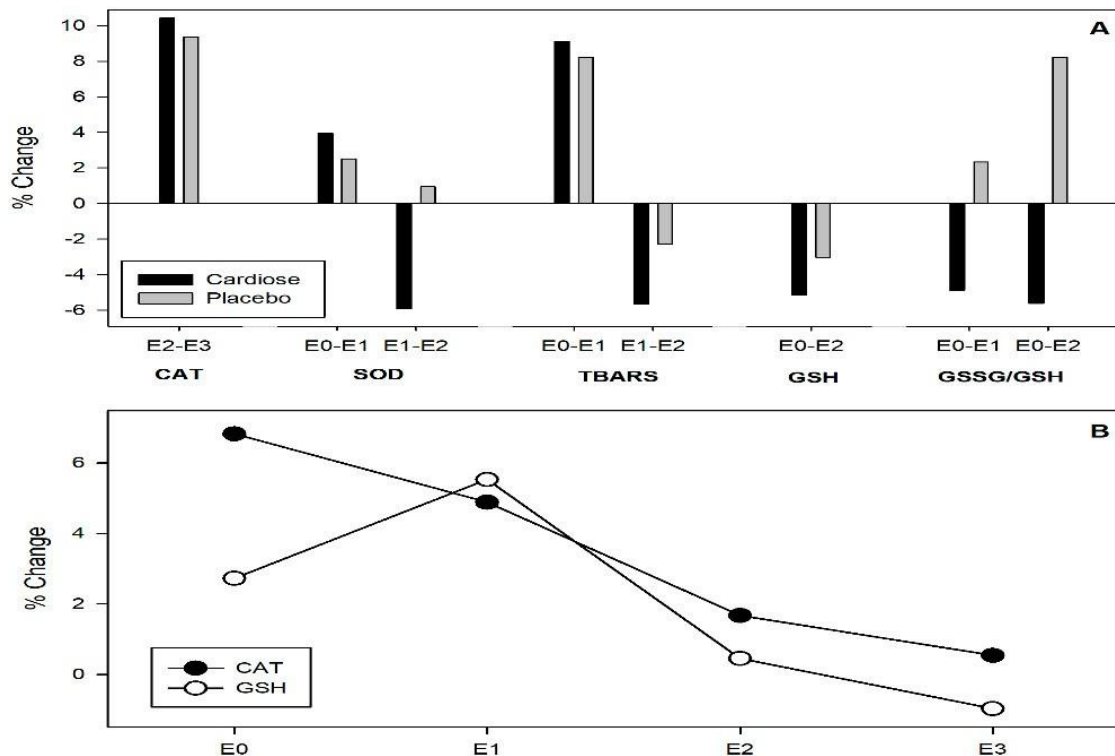


Figure 39. Changes in antioxidants and TBARS measured in repeated sprint test. (A) Changes between different time points. (B) Changes between treatments. Antioxidant markers were evaluated prior to the testing session (pre VT1 test, and 5 h after supplementation) (E0), post repeated sprint test (E1), post second VT1 test (E2), and 24 h after the end of the testing session (E3).

No significant decrease in GSH levels during the exercise protocol was observed in both groups. Despite levels of this antioxidant peptide being higher after Cardiose® supplementation at baseline and after the repeated sprint test, differences between treatments were not significant (Figure 39). A positive significant correlation between the levels of hesperidin metabolites in urine and the percentage variations in the levels GSH $\% \Delta$ 01-02 ($r = 0.551$; $p = 0.033$) between the placebo and supplemented group was observed.

A completely different trend in GSSG/GSH ratio was observed in both groups during the testing session. In the Cardiose® group, GSSG/GSH ratio decreased during the testing session: after the repeated sprint test, and after the end of rectangular test. In the placebo group, GSSG/GSH ratio increased during the physical exercise: after the repeated sprint test and after the end of the testing session. Despite this different behavior in the GSSG/GSH ratio during the test, differences in the GSSG/GSH ratio were not statistically significant (Table 21) (Figure 39).

8.3.4. Hesperidin Metabolites Urine

Different hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analyzed in urine of the participants after the intake of Cardiose®. The main metabolite was Hesperetin-3- glucuronide, representing $78.9 \pm 5.0\%$ ($n = 15$) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate were $6.9 \pm 2.9\%$ ($n = 15$) and $14.7 \pm 4.1\%$ ($n = 15$) of the excreted metabolites, respectively. Despite the similarities in the excreted metabolites profile, a large interindividual variability was observed in the amount of hesperidin metabolites excreted, ranging from 2.3 to 37.5 μmol . These wide differences between subjects in the absorption and excretion of hesperidin have been already reported (676).

8.4. DISCUSSION

The main objective of this study was to assess the acute effects of 500 mg of 2S-hesperitin on physical performance, specifically in exercises with high anaerobic component (677), and secondary in metabolism and antioxidant status in amateur cyclists. Exercise. The results showed that a single supplementation with 500 mg amount of 2S-Hesperidin may improve anaerobic parameters in a repeated sprint test for the Cardiose® group. In addition, an improvement in antioxidant capacity and energy metabolism were observed after Cardiose® supplementation during exercise protocol.

A significant improvement in average power (2.27%; $p = 0.023$), maximum speed (3.23%; $p=0.043$) and total energy (2.64%; $p=0.028$), but without significant changes in peak power (3.94%) and time to peak (-12.21%), were observed after Cardiose® supplementation when the best data of the sprint test series were considered. However, no significant improvements in anaerobic performance parameters were found for Cardiose® group when average values of the repeated sprint test were evaluated. In addition, a positive correlation between excreted hesperidin metabolites in urine and the differences in total energy ($\Sigma 4$ test) between placebo and Cardiose® was also found. Therefore, these data suggest that supplementation with Cardiose® improves physical performance in an anaerobic trial such as the repeated sprint test. These results are in line with the improvement in physical performance observed in trained rats after 2S-Hesperidin supplementation (33) or in cycling time-trial performance in trained male athletes after supplementation with 2S-Hesperidin (500 mg/day) for 4 weeks (32). These studies reported improvements of 58% in the time the exhaustion test and 5% in absolute power output a 10 min time trial, respectively. Since anaerobic power is a key factor in sport performance (678), but sometimes difficult to improve, achieving small improvements in anaerobic performance as those described in this study may be very important for athletes, especially in high sports performance.

Antioxidant status, and endogenous antioxidant capacity, are key factors for the athlete's performance (679). Especially during high-intensity and short-duration or low-intensity and high-duration exercises which provoke high production of free radicals (ROS), these may be mediated through a variety of pathways (680). Our study showed small changes in different antioxidant enzymes (CAT and SOD), peptides with antioxidant activity (GSSG/GSH) and oxidation markers (MDA-TBARS) between the Cardioise® and placebo groups. Exercise-induced ROS production causes lipid peroxidation (256), superoxide anion generation through xanthine oxidase (XO) activation, and the increase in oxidized/reduced glutathione (GSSG/GSH) ratio (652, 653). Enzymes like SOD and glutathione are important antioxidant defences that protect cells from ROS-induced oxidative stress (681). Oxidative stress may cause cellular damage through modifications to macromolecules, including proteins, lipids, and nucleic acids, and can occur as a result of high-intensity or moderate- to long-duration exercise (651). In our study, the intense physical exercise causes an increase in CAT activity, which was observed in both experimental groups. An increase in the activity of CAT, versus placebo, was observed following the acute supplementation with Cardioise®. Also, an inverse correlation between the excreted levels of hesperidin metabolites in urine, and the percentage variations in the activity of CAT $\% \Delta$ 01-03 ($p = 0.013$) was observed. These results suggest that the acute intake of Cardioise® might promote the activity of this antioxidant enzyme. In rats submitted to intense exercise, 2S-hesperidin supplementation contributed to maintain catalase activity, and avoid changes induced by physical activity (33). An increase in catalase activity during exercise may offer an advantage in high intensity efforts (e.g., sprint), where there is a high rate of ROS production, decreasing damage to the muscle cell.

Hesperidin has been also described to increase the activity of this antioxidant enzyme during senescence (682) or modulate its activity when it is impaired by different conditions (683, 684). In general, intense physical activity increases SOD activity (685). However, a decrease in SOD activity after repeated sprint test to the end of rectangular test was observed in Cardioise® (-5.9%) but not in placebo (0.9%). This decrease was maintained 24 h after the end of exercise session. Cardioise® seems to reduce the overexpression of SOD induced by physical exercise. In previous studies, supplementation with 2S-Hesperidin decreased SOD activity in trained rats (33). Due to its scavenging activity hesperidin neutralizes reactive oxygen species, such as superoxide anion, generated during conditions of oxidative stress, as intense physical exercise. The decrease in SOD activity may be related to the reduced need for this endogenous enzyme when an exogenous antioxidant, such as hesperidin or other flavonoids, is provided (686, 687). Therefore, this decrease in SOD activation would indicate a lower production of free radicals, which leads to less damage to muscle cell structures and a better post-exercise recovery.

As we have already mentioned, intense physical activity increases ROS production and consequently lipid peroxidation, producing an increase of malondialdehyde and TBARS (688). ROS produced during physical activity may react with unsaturated fatty acids comprising cellular membrane, leading to lipid peroxidation, a chain reaction that oxidizes fatty acids and produce more ROS (689). In our exercise protocol, high-intensity exercise increases TBARS in both groups. However, a greater attenuation of lipid peroxidation (TBARS) was observed in Cardiose® (-5.7 %) from after repeated sprint test to the end of rectangular test versus placebo (-2.3 %). In previous studies, rats submitted to interval swimming, the intake of hesperidin lowered (-45%) the lipid peroxidation (530). Flavonoids such as hesperidin play a key role as free radical scavengers *in vivo*, preventing the increase in lipid peroxidation associated with high-intensity exercise. Furthermore, the antioxidant activity of citrus flavanones is not only related to their radical scavenging activity, but also to their ability to increase cellular defences via the NRF2-ARE pathway, which regulates the expression of antioxidant genes including SOD, CAT, HO-1, GPX, and TXN, decreases intracellular pro-oxidants and enhances antioxidant enzymes (682).

On the other hand, glutathione is a widespread peptide with antioxidant properties that may be found in plasma either as glutathion (GSH), or as glutathione disulfide (GSSG), its oxidized form (690). Cardiose® supplementation led to no significant increase in glutathione (GHS) levels, and additionally a significant correlation (GSH Δ 01-02, $r = 0.551$; $p = 0.033$) between the levels of hesperidin metabolites in urine and the percentage variations in the GSH levels was observed. The ration between the oxidized (GSSG) and reduced (GSH) glutathione form is also evaluated as an antioxidant status marker (691). A different trend in this ratio was observed according to the supplementation. Placebo led to an increase in the GSSG/GSH ratio during the exercise protocol, while Cardiose® supplementation decreased the GSSG/GSH ratio. These results suggest that Cardiose® promotes glutathione antioxidant role, indicating a better antioxidant status in the experimental group. The reason of these modifications in GSH and GSSG could be mediated by lower concentrations in LPOs and hydrogen peroxide, which are metabolized by glutathione peroxidase (GPX), can generate an increase in GSSG (692). This increase is neutralized by the increase in the activity of glutathione reductase (GR) (693). This effect can explain the finding from our study with GSH and GSSG/GHS ratio. In previous works (694, 695) hesperidin supplementation has showed to minimize the impairment of glutathione antioxidant system induced by different alterations, restoring the usual levels of this body's antioxidant peptide. All these changes in the endogenous antioxidant system (CAT, SOD, GSH and GSSG) generate an ideal muscular environment to improve performance and recovery. Taken together, these

results suggest that the intake of Cardiose® affects the body's own antioxidant capacity, even after an acute single intake.

The improvements in performance in endurance sports, it may be due to adaptations at metabolic level may be explained due to the activation of PGC-1 α , a key regulator of energy metabolism that increases biogenesis and mitochondrial working capacity. Finally, in terms of the cardiorespiratory parameters analyzed, no significant differences were found between the Cardiose® group and placebo. Furthermore, no significant differences between treatments were found regarding the consumption of energy substrates, carbohydrates and fats, but Cardiose® supplementation promoted the use of fats as energy substrates (+15.1% versus placebo). Polyphenols induce changes in PGC-1 α activity via increased activation of the intracellular signalling pathways AMP-activated protein kinase (AMPK). Another factor that promotes metabolic adaptations induced by exercise is NRF2, a member of the Cap-N-Collar family of transcription factors, plays an important role in mitochondrial biogenesis, and variants of the NRF2 gene have been associated with endurance performance (696, 697). In vitro studies have shown that hesperidin also activates AMPK stimulating its phosphorylation (31), besides, in animal tissue an increase in NRF2 expression was also observed (682). These metabolic changes generate muscular level adaptations that prioritize the oxidation of fatty acids versus glucose, leading to higher to energy efficiency (698-702), and therefore may predispose the athlete to a better sports performance. However, the small differences in oxygen consumption might indicate a best athlete's energy management under stress situations.

As shown previously, Cardiose® supplementation seems to improve physical performance during a complete exercise protocol, modulating athlete's oxidative status during the physical activity in semi-professional cyclists. The absence of human studies with hesperidin including anaerobic power tests, make difficult any comparison. It is important to highlight that these results were obtained after an acute and single intake of 500 mg of Cardiose® and placebo. The small changes observed after this single intake may be increased after a chronic consumption of this product.

As shown previously, Cardiose® supplementation seems to improve physical performance during a complete exercise protocol, modulating athlete's oxidative status during the physical activity in semi-professional cyclists. The absence of human studies with hesperidin including anaerobic power tests, makes difficult any comparison. It is important to highlight that these results were obtained after an acute and single intake of 500 mg of Cardiose® and placebo. The small changes observed after this single intake may be increased after a chronic consumption of this product. The main limitation of this study was the size of the sample. A larger sample could improve our results' power and the lack of previous research studies on the topic. Future research should be conducted to evaluate the chronic effect of 2S-hesperidin supplementation

on sports performance and oxidative stress, as well as to clarify if hesperidin can improve physical performance during high-intensity exercise.

8.5. CONCLUSIONS

A single acute intake of Cardiose® (500 mg of 2S-hesperidin) improves performance in maximum anaerobic effort in semi-professional cyclists. In addition, oxidative status and antioxidant defenses were slightly modulated. These findings could help improve performance in high-intensity exercises for both amateur and high-performance athletes.

CAPÍTULO IX.

ESTUDIO N° 4

Figure 40. Structure of hesperidin enantiomers *S* and *R* and their metabolites hesperetin, produced by the intestinal microbiota. Modified from Li et al. (462).

Regarding performance, only one acute effects study in humans has investigated 2*S*-hesperidin (549). This study showed that after ingesting one single 500 mg dose of either 2*S*-hesperidin 5 hours before the test, trained cyclists significantly improved average power (2.3%), maximum speed (3.2%) and total energy (Σ 4 sprint test; total work) (2.6%) with 500 mg hesperidin supplementation in the best sprint out the four repeated sprint test (4 \times 30 sec all-out sprints with 5 min of rest between sprints). No significant changes were observed in any of these variables with placebo.

In humans, chronic supplementation of hesperidin has also been studied. Pittaluga et al. (532) investigated the effect of 250 mL of red-orange juice, which has a high content of hesperidin, on exercise performance (incremental test) in healthy, trained older women. Following four weeks of consumption of ROJ (3 per day), these older women significantly increased their work capacity by 9.0% compared to placebo (-1.5%). Another chronic study evaluated the effect of a four-week supplementation of 2*S*-hesperidin (500 mg/day) in trained cyclists and observed significant increases in average power output (14.9 W = 5.0%) in a 10 min time-trial test on a cycle ergometer, whereas those that consumed placebo had a non-significant increase in average power output (3.8 W = 1.3%), moreover, differences were found when comparing the groups (32). In addition, another performance-enhancing mechanism has been observed in other substances, such as menthol or capsaicin (polyphenols) through taste, but this pathway has not been explored with 2*S*-hesperidin (705).

The effect of long-term intake of hesperidin has also been investigated in animal studies. Biesemann et al. (35) observed that six weeks of hesperetin supplementation (main metabolite of hesperidin) (50 mg·kg⁻¹·d⁻¹) improved running performance by 28.8% (exercise time until exhaustion) compared to placebo in aged mice. This study also found an improvement in endogenous antioxidant enzymes, such as reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH:GSSG ratio. De Oliveira et al. (530) found that four weeks of hesperidin consumption (100 mg/kg body mass) enhanced the antioxidant capacity in the continuous swimming group (183%) and decreased the lipid peroxidation (TBARS) in the interval swimming group (-45%) compared to placebo in rats. In the same line, a recent study in trained animals reported that intake of hesperidin for four weeks improved performance and prevented immune alterations induced by exhausting exercise compared to placebo (34). Recently, one parallel-group study has shown improvements in the time until exhaustion (58%) on maximal exercise test at 3 weeks of a 5-week chronic supplementation of 2*S*-hesperidin (200 mg/kg), but not in placebo group (with differences between groups) (33). In the same study, it was observed an enhancement

of the antioxidant state (superoxide dismutase (SOD), glutathione peroxidase (GPx)) in the lymphoid and hepatic tissue after the test until exhaustion in the rats that consumed 2S-hesperidin compared to placebo.

Hesperidin strongly increases intracellular ATP compared to the AMP-activated protein kinase (AMPK) activator 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), even when AICAR concentration has been increased by 10-fold (100 μ M) (35). In addition, hesperetin (10 μ M) has been shown to increase intracellular ATP by 33% and mitochondrial spare capacity by 25%, as well as establish an antioxidant state (35). Based on the understanding behind the mechanism of hesperidin *in vitro*, as well as the evidence presented above, hesperidin is a good candidate for improving performance.

Currently, some animal trials have shown that chronic hesperidin supplementation can improve performance, but in humans the evidence is weak and more research is needed. We hypothesised that chronic intake of 2S-hesperidin would improve performance at submaximal and maximal exercise intensities. Therefore, the main aims of this study were to examine the chronic effects of 2S-hesperidin (500 mg, Cardiose®) supplementation on: 1) power production at FatMax, ventilatory threshold 1 and 2 (VT1 and VT2) and maximum power in an incremental test (high aerobic component), and 2) maximum absolute and relative power during a Wingate test (high anaerobic component). The secondary objective was to evaluate whether hesperidin supplementation modified metabolic (O_2 and CO_2) and energy substrate (carbohydrates and fats) markers during a step test that could explain a possible enhancement in performance.

9.2. METHODOLOGY

9.2.1. Participants

Forty healthy, male amateur cyclists participated and completed the study. All the participants had to meet the following inclusion criteria: 18–55 years, BMI of 19–25.5 $kg \cdot m^{-2}$, at least 3 years of cycling experience and training for 6–12 $h \cdot wk^{-1}$. Volunteers were excluded if they: (a) were smokers or regular alcohol drinkers, (b) had a metabolic, cardiorespiratory, or digestive pathology or anomaly, (c) had an injury in the prior 6 months, (d) were supplementing or medicating in the prior 2 weeks and/or (e) had non-normal values in the blood analysis parameters. First, participants were informed about the procedures, and a signed informed consent was obtained. The study was conducted according to the guidelines of the Helsinki Declaration for Human Research (548) and was approved by the Ethics Committee of the Catholic University of Murcia (CE091802).

9.2.2. Study Design

A double-blind, parallel, and randomized experimental design was performed. Randomization was performed using computer software (Randomizer) to assign codes to the groups established in this study (706). Participants were divided into two groups: experimental (2S-hesperidin; $n = 20$) and control (Placebo; $n = 20$). Total distance of usual training was balanced to make it similar between groups (Table 22). Participants consumed two capsules of 250 mg at the same time of either 2S-hesperidin (500 mg) (Cardiose®, produced by HTBA (HealthTech BioActives—Murcia, Spain)) or placebo (microcellulose) for 8 weeks. Specifically, Cardiose® is a natural orange extract that, due to its unique manufacturing process, maintains most of the natural hesperidin isomeric form (NLT 85% 2S-hesperidin). The placebo supplements were also in capsulated form and similar in appearance to the 2S-hesperidin capsule. Cyclists were instructed to take the supplement along with breakfast and to continue their usual diet and training schedule. Subjects in both groups were instructed not to consume foods high in citrus flavonoids (grapefruit, lemons, or oranges) for 5 days prior to and during the study. This was verified by diet recalls records.

Table 22. Baseline general characteristics and training variables of participants.

	2S-Hesperidin	Placebo	<i>p</i> -Value
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Body mass (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI (kg·m⁻²)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
VO₂MAX (L·min⁻¹)	3.99 (0.36)	3.98 (0.63)	0.971
VO₂MAX (mL·kg⁻¹·min⁻¹)	57.5 (6.97)	57.9 (9.53)	0.880
HR_{MAX} (bpm)	184.9 (11.11)	183.2 (8.68)	0.593
VT1 (%)	50.9 (5.63)	50.0 (4.78)	0.610
VT2 (%)	84.9 (5.85)	84.1 (5.70)	0.644
Training variables	2S-Hesperidin	Placebo	<i>p</i> -value
Total distance (km)	1121.12 (534.99)	1082.43 (810.46)	0.868
HR_{AVG} (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
W_{AVG} (W)	174.9 (15.79)	163.5 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are expressed as mean (SD). BMI = body mass index; BF = body fat; VO₂máx = maximum oxygen volume; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); Total distance = of all the training sessions carried out during the study period; HR_{avg} = average heart rate of all the training sessions carried out during the study period; W_{avg} = average power output of all training sessions during the study period.

9.2.3. Procedures

Participants visited the laboratory on seven occasions. Visit 1 consisted of a medical examination, blood extraction to determine health status and a familiarisation session with the Wingate test. When urine samples were collected on visit 2 in the fasted state, both groups consumed the supplements under the supervision of an investigator, which was followed by a standardized breakfast. On visits 2 and 5, a 24-h diet recall and a Wingate test were performed. On visits 3 and 6, another 24-h diet recall was conducted, followed by an incremental test until exhaustion on a cycle ergometer. On visits 4 and 7, the 24-h diet recall was repeated, and participants performed a step test on the cycle ergometer (Figure 41 and Table 23). Prior to each testing session (visits 2, 3, 4, 5, 6, and 7), a standardized breakfast (557.7 kcal) composed of 95.2 g of carbohydrates (68%), 18.9 g of protein (14%) and 11.3 g of lipids (18%) was prescribed by the sport nutritionist.

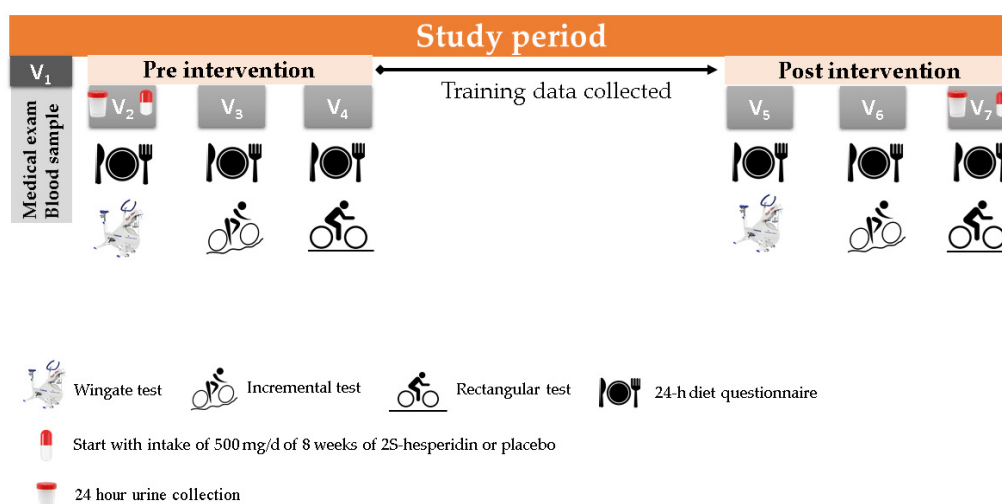


Figure 41. Study planning with explanation of the different visits (V 1–7).

Table 23. Between-group comparisons in dietary intake of cyclists.

	Pre-Intervention			Post-Intervention		
	2S-Hesperidin	Placebo	<i>p</i> -Value	2S-Hesperidin	Placebo	<i>p</i> -Value
Kcal	2163.6 (519.02)	2100.2 (515.77)	0.708	1974.1 (377.97)	2133.5 (437.98)	0.237
Kcal/BM	31.1 (9.34)	30.2 (8.71)	0.768	27.9 (6.53)	30.3 (6.46)	0.249
CHO (g)	245.7 (73.46)	222.0 (69.68)	0.312	216.6 (63.47)	248.3 (58.15)	0.117
CHO/BM	3.5 (1.31)	3.2 (1.14)	0.416	3.1 (1.08)	3.5 (0.94)	0.173

PRO (g)	113.5 (25.21)	115.2 (25.37)	0.837	109.0 (23.05)	101.5 (23.67)	0.332
PRO/BM	1.6 (0.41)	1.7 (0.48)	0.778	1.5 (0.35)	1.5 (0.42)	0.596
LP (g)	80.8 (27.24)	83.5 (23.65)	0.739	71.5 (17.61)	71.6 (18.89)	0.985
LP/BM	1.2 (0.45)	1.2 (0.37)	0.758	1.0 (0.27)	1.0 (0.29)	0.823

Values are expressed as mean (SD). Kcal = kilocalories; CHO = carbohydrates; PRO = protein; LP = lipids; BM = body mass. The mean values correspond to the average of all 24-h diet recall data collected at pre-intervention (visits 2, 3 and 4) and post-intervention (visits 5, 6 and 7).

9.2.4. Testing

9.2.4.1. Medical Exam

A medical examination, performed by the research centre's medical doctor and including health history, resting electrocardiogram and examination (auscultation, blood pressure, etc.), was used to confirm that the volunteer was healthy enough to be enrolled in the study.

9.2.4.2. Incremental Test

An incremental step with a final ramp test was performed on a cycle ergometer (Cyclus 2, RBM Elektronik-Automation GmbH, Leipzig, Alemania) using a metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine maximal fat oxidation zone (FatMax), VT1 and VT2 and maximal oxygen consumption (VO_{2MAX}). Participants began cycling at 35W for 2 min, increasing then by 35W every 2 min upon attainment of $RER > 1.05$, participants completed a final ramp 35W/min until volitional exhaustion. To ensure VO_{2MAX} , at least 2 of the following criteria had to be achieved: plateau in the final VO_2 values (increase ≤ 2.0 mL·kg⁻¹·min⁻¹ in the 2 last loads), reaching maximal theoretical HR $(220 - \text{age}) \cdot 0.95$, $RER \geq 1.15$ and lactate ≥ 8.0 mmol·l⁻¹. VT1 was determined using the criteria of an increase in $VE \cdot VO_2^{-1}$ (VE = pulmonary ventilation) without further increase in $VE \cdot VCO_2^{-1}$ and departure from the linearity of VE , whereas VT2 corresponded to an increase in both $VE \cdot VO_2^{-1}$ and VE/VCO_2^{-1} (7, 707). All VT1 and VT2 assessments were made by visual inspection of graphs in which were time-plotted against each relevant respiratory variable measured during testing. Ventilatory thresholds were obtained using the ventilatory equivalents method described by Wasserman (553). FTP was defined as the highest average power output (PO) that can be maintained for 1 hour (708). The estimated functional threshold power (FTP) was calculated using the following equation (54):

$$\text{FTP (W)} = \text{Pmax (W)} \times 0.865 - 56.484$$

9.2.4.3. Step Test

Step test was performed on a cycle ergometer (Cyclus 2, RBM Elektronik-Automation GmbH, Leipzig, Alemania) using a metabolic cart (Metalyzer 3B, Leipzig, Germany) (maximal error: 2%; in power values <100 W) and applying the power output values resulting from the incremental test (FatMax, VT1 and VT2). Participants exercised continuously from FatMax (W) to VT1 (W) and to VT2 (W) for 10 min at each step without rest between them. Cardiorespiratory variables (oxygen consumption (VO_2), relative oxygen consumption to body mass (VO_2R), carbohydrate oxidation (CHO), fat oxidation (FAT) and cycling efficiency = (work/energy expenditure) \times 100) were determined for each metabolic zones.

9.2.4.4. Wingate Test

In visit 1 a familiarisation session was performed for this test. Prior to the Wingate test (WAnT), participants warmed up on a cycle ergometer for 10 min at 50 W. The WAnT consisted of an all-out, 30-s sprint on a cycloergometer (Monark Ergomedic 894E Peak Bike, Vansbro, Sweden). Breaking resistance was held constant at 7.5% of each individual's body mass (59). All participants were verbally encouraged to pedal as fast as possible during the entire sprint. The anaerobic capacity (non-oxidative) was determined by obtaining the absolute and relative (i.e., to body mass) peak power, initial absolute and relative power, power at maximum speed, time at peak power and time at maximum speed. Participants were familiarized with the WAnT on the same day as the medical exam.

9.2.4.5. Blood Samples

For blood analytics, two samples were taken, namely one in a 3-mL tube with ethylenediaminetetraacetic acid (EDTA) and another in a 3.5-mL tube with polyethylene terephthalate (PET). Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, haematocrit, and haematimetry indexes were determined. These data were used to verify the health status of the subjects and were not included in the study.

9.2.4.6. Urine Samples

Main hesperidin metabolites were analysed in participants' urine. Urine samples, corresponding to the collection of urine 24 h before (V2) and after (V7) the

supplementation in both groups for each participant, were frozen in liquid nitrogen after collection and thawed for its analysis. For analysis, 50 μL of urine were mixed with 100 μL of water with 1% formic acid containing the internal standard. Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series Agilent Technologies, Sta. Clara, CA, USA). Metabolites were quantified by external standard calibration, using rac-Hesperetin-d3 as the internal standard

9.2.5. Statistical Analysis

Statistical analysis was carried out using IBM Social Sciences software (SPSS, v.21.0, Chicago, IL, USA). Data are presented as mean \pm SD. Levene and Shapiro–Wilk tests were performed in order to check for homogeneity and normality of the data, respectively. Depending on the normality and homogeneity outcomes obtained, paired T-test or Wilcoxon signed-rank test were carried out to examine within-group pre-post differences. Likewise, between-group comparison was calculated using ANCOVA test or Mann–Whitney U test, using pre-test values as covariates (to eliminate any possible bias caused by the initial level of each group in the different dependent variables). Partial eta squared (η^2) was calculated as effect size for between-group comparisons. Partial eta square thresholds were used as follow: <0.01 , irrelevant; ≥ 0.01 , small; ≥ 0.059 , moderate; ≥ 0.138 , large (710). Furthermore, the step test data analysis was done using repeated measures T-test to obtain within-group differences when comparing the different time points. Relationships between levels of excreted hesperidin metabolites in urine and other evaluated parameters were analysed using Pearson correlation analysis (r). Significance level was set at $p \leq 0.05$. Cohen's d effect sizes (ES) (95% confidence interval) were calculated for comparisons between groups. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large (710).

9.3. RESULTS

9.3.1. Hesperidin Metabolites Urine

Different hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analyzed in the urine of the participants after 2S-hesperidin intake. The main metabolite detected was hesperetin-3-glucuronide, representing $78.9 \pm 5.0\%$ ($n = 20$) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate made up $6.9 \pm 2.9\%$ ($n = 20$) and $14.7 \pm 4.1\%$ ($n = 20$) of the excreted metabolites. Despite the similarities in the excreted metabolites profile, a large interindividual variability was observed in the excreted amount, with hesperidin metabolites ranging from 2.3 to 37.5 μmol . These

differences between subjects indicate differences in the absorption and excretion of hesperidin, which have been previously reported (676).

9.3.2. Incremental Test

Figure 42 shows the pre- and post-intervention values and changes in VT1 and VT2 power, estimated FTP and maximum power achieved during the incremental test. At VT1, there was no differences in pre-post power neither in 2S-hesperidin group (-3.7% = -6.0 W; $p = 0.437$) nor in Placebo group (3.4% = 5.3 W; $p = 0.453$), without differences in VT1 power changes between groups ($p = 0.423$; $\eta^2 = 0.017$; ES = 0.35). At VT2, there was no differences pre-post in power output in Placebo (-3.1% = -8.9 W; $p = 0.264$), and no changes were observed in 2S-hesperidin group (1.0% = 2.9 W; $p = 0.642$). Comparison between groups showed no effect ($p = 0.299$; $\eta^2 = 0.029$; ES = 0.38).

Interestingly, there were pre-post increases in maximum power (1.9% = 7.4 W; $p = 0.049$) and estimated FTP (2.3% = 6.4 W; $p = 0.049$) in 2S-hesperidin group. However, there were no changes in estimated FTP (-0.9 % = -2.51 W; $p = 0.387$) and maximum power (-0.8% = -2.9 W; $p = 0.388$) after the intervention in the placebo group. Between-group comparisons revealed an effect with the increase in estimated FTP (3.2% = 8.9 W; $p = 0.042$; $\eta^2 = 0.107$; ES = 0.68) and maximum power (2.7% = 10.3 W; $p = 0.042$; $\eta^2 = 0.107$; ES = 0.68) in 2S-hesperidin group versus placebo.

Additionally, there was a positive correlation between the levels of excreted hesperidin metabolites in urine and the difference in maximum power ($r = 0.701$; $p < 0.001$) and estimated FTP ($r = 0.725$; $p < 0.001$) in the supplemented group.

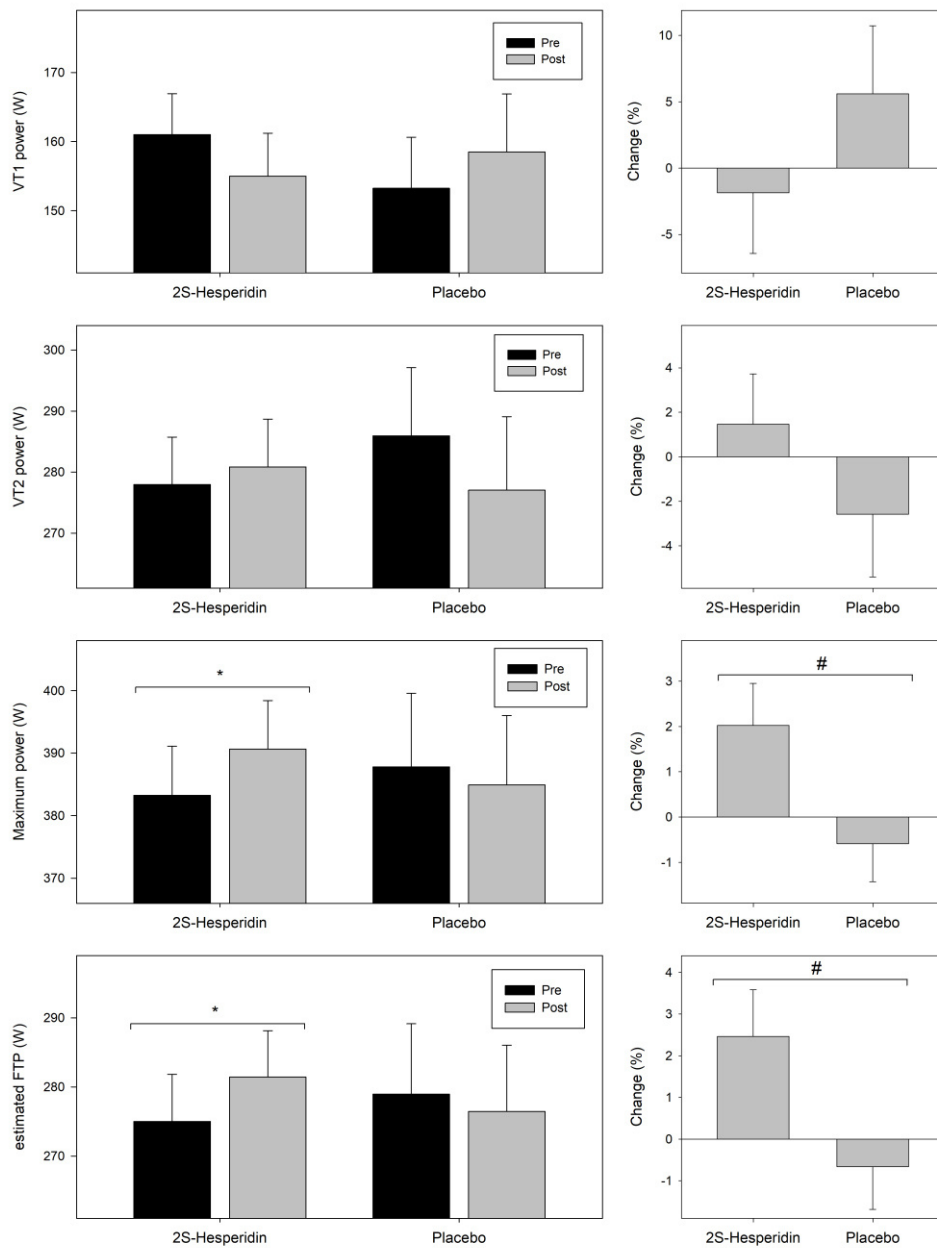


Figure 42. Changes in ventilatory 1 (VT1) power, ventilatory threshold 2 (VT2) power, estimated functional threshold power (FTP) and maximum power during the incremental test. Values are mean \pm SE. * Within-group significant changes ($p \leq 0.05$). # Between group significant changes ($p \leq 0.05$).

9.3.3. Step Test

At FatMax, there was a pre-post decrease in fat oxidation (FAT) ($p = 0.007$) and efficiency ($p = 0.010$) in the Placebo group, whereas the 2S-hesperidin supplemented group showed no changes in these parameters (Table 24). No differences were found for between-group comparisons in FAT ($p = 0.125$; $\eta p^2 = 0.084$; ES = 0.59).

At VT1, there was a pre-post increase in carbohydrate oxidation (CHO) ($p = 0.020$) and a decrease pre-post in fat oxidation ($p = 0.003$) in Placebo group, but no changes were observed in 2S-hesperidin (Table 24). No changes were found between groups in CHO ($p = 0.314$; $\eta p^2 = 0.028$; ES = 0.57) and FAT ($p = 0.205$; $\eta p^2 = 0.044$; ES = 0.53).

After the supplementation period, there was a decrease in VO_2 (L/min) (-8.3%; $p = 0.002$) and VO_2R (mL/kg/min) (-8.9%; $p = 0.002$) at VT2 in Placebo group, in contrast to 2S-hesperidin, which showed no changes (Table 24). Between-group comparison showed a trend towards a decrease ($p = 0.074$; $\eta p^2 = 0.084$; ES = 0.67) in VO_2R (mL/kg/min) for placebo versus 2S-hesperidin group.

Table 24. Changes in metabolism, energy substrate, energy and energy efficiency in FatMax, ventilatory threshold 1 (VT1) and ventilatory threshold 2 (VT2) during the step test.

	2S-Hesperidin			Placebo			ηp^2	ES
	Pre-Intervention	Post-Intervention	p -Value	Pre-Intervention	Post-Intervention	p -Value		
FatMax								
VO_2 (L·min ⁻¹)	2.23 (0.50)	2.02 (0.37)	0.063	2.27 (0.48)	2.10 (0.57)	0.151	0.005	0.08
VO_2R (mL·kg ⁻¹ ·min ⁻¹)	31.45 (6.17)	28.54 (5.43)	0.060	32.40 (6.82)	29.51 (6.99)	0.100	0.003	0.00
CHO (g·min ⁻¹)	2.20 (0.58)	2.01 (0.37)	0.169	2.20 (0.50)	2.27 (0.56)	0.521	0.090	0.47
FAT (g·min ⁻¹)	0.29 (0.90)	0.26 (0.14)	0.247	0.32 (0.14)	0.21 (0.14)	0.007	0.064	0.59
Efficiency (%)	26.68 (2.95)	26.05 (3.90)	0.411	26.94 (2.79)	24.62 (2.27)	0.010	0.064	0.49
VT1								
VO_2 (L·min ⁻¹)	2.19 (0.39)	2.10 (0.35)	0.396	2.10 (0.41)	2.09 (0.47)	0.961	0.001	0.17
VO_2R (mL·kg ⁻¹ ·min ⁻¹)	31.05 (5.34)	29.62 (5.20)	0.357	29.96 (5.84)	29.64 (6.37)	0.824	0.001	0.17
CHO (g·min ⁻¹)	2.08 (0.47)	2.07 (0.30)	0.974	1.86 (0.47)	2.19 (0.49)	0.020	0.028	0.57
FAT (g·min ⁻¹)	0.31 (0.10)	0.27 (0.15)	0.184	0.35 (0.12)	0.23 (0.14)	0.003	0.044	0.53
Efficiency	26.55 (2.62)	25.25 (5.38)	0.250	27.49 (3.25)	25.86 (5.85)	0.282	<0.001	0.77

(%)	VT2							
VO ₂ (L·min ⁻¹)	3.49 (0.43)	3.36 (0.41)	0.135	3.63 (0.52)	3.33 (0.54)	0.002	0.039	0.49
VO ₂ R (mL·kg ⁻¹ ·m in ⁻¹)	49.48 (6.83)	48.25 (6.84)	0.211	51.90 (8.17)	47.29 (7.76)	0.002 [†]	0.084	0.67
CHO (g·min ⁻¹)	5.11 (1.18)	5.42 (1.37)	0.349	5.53 (1.45)	5.25 (1.13)	0.369	0.022	0.43
FAT (g·min ⁻¹)	0.04 (0.08)	0.04 (0.09)	1.000	0.02 (0.06)	0.01 (0.03)	0.334	0.048	0.03
Efficiency (%)	20.58 (3.09)	19.65 (3.37)	0.272	20.15 (2.25)	20.20 (4.30)	0.965	0.009	0.24

Values are mean (SE). VO₂ = volume of oxygen uptake; VO₂R = body mass oxygen consumption; FatMax = intensity at which maximum fat oxidation is given; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); CHO = carbohydrate oxidation; FAT = fat oxidation; efficiency = percentage. The *p*-values refer to intra-group comparisons. There were no significant changes when comparing the groups. The trend towards significance between groups is indicated by a †.

9.3.4. Wingate Test

Table 25 shows the results of the parameters evaluated during the Wingate test prior and after supplementation, which are also summarized in Figure 43. In the 2S-hesperidin group, there were increases in absolute (4.9% = 35.5 W; *p* = 0.001) and relative (4.3% = 0.44 W·kg⁻¹; *p* = 0.004) initial power (first five seconds of the test), but no differences between groups. In the experimental group, there was an increase in absolute (6.1% = 49.8 W; *p* < 0.001) and relative (5.6% = 0.64 W·kg⁻¹; *p* = 0.001) peak power. Also, there was a trend towards an increase in power at maximum speed (4.4% = 34.0 W; *p* = 0.051) and a descending trend in time at peak power (-18.1% = -641.2 ms; *p* = 0.052) after the supplementation with 2S-hesperidin. No changes were observed in time at maximum speed.

Placebo group showed an increase in absolute (6.1% = 47.2 W; *p* = 0.016) and relative peak power (5.6% = 0.64 W·kg⁻¹; *p* = 0.014), and a decrease in time at maximum speed (-13.2% = -929.2 ms; *p* = 0.001). No changes were observed in absolute and relative initial power, power at maximum speed and time at peak power for placebo.

Between-group comparison only reported a trend to decrease in time at maximum speed (-12.5% = -878.4 ms; *p* = 0.059) in Placebo compared with 2S-hesperidin.

Table 25. Changes in performance parameters in the Wingate test.

	2S-Hesperidin			Placebo			ηp^2	ES
	Pre- Intervention	Post- Intervention	<i>p</i> - Value	Pre- Intervention	Post- Intervention	<i>p</i> - Value		
Initial power absolute (W)	718.8 (143.05)	754.3 (143.09)	0.001 *	712.5 (103.46)	743.0 (101.78)	0.084	0.003	0.08
Initial power relative (W)	10.2 (1.82)	10.6 (1.78)	0.004 *	10.1 (1.38)	10.6 (1.29)	0.078	<0.001	0.01
Absolute peak power (W)	810.8 (160.26)	860.6 (170.37)	<0.001 *	792.0 (100.96)	840.2 (118.93)	0.016*	<0.001	0.02
Relative peak power (W)	11.5 (2.04)	12.1 (2.27)	0.001 *	11.3 (1.37)	11.9 (1.49)	0.014*	<0.001	0.02
Power at maximum speed (W)	760.0 (156.45)	793.5 (132.23)	0.051 †	746.3 (110.30)	754.3 (96.14)	0.709	0.044	0.30
Time at peak power (ms)	3541.4 (1722.52)	2900.2 (923.99)	0.052 †	3193.4 (1218.48)	2816.9 (1013.54)	0.138	0.001	0.82
Time at maximum speed (ms)	7208.7 (1098.24)	7157.9 (2005.11)	0.888	7024.4 (1347.65)	6095.2 (957.33)	0.001*	0.119	0.73

Values are mean (SE). * Within-group significant changes ($p \leq 0.05$). † Within-group trend to significant changes ($p = 0.05-0.010$).

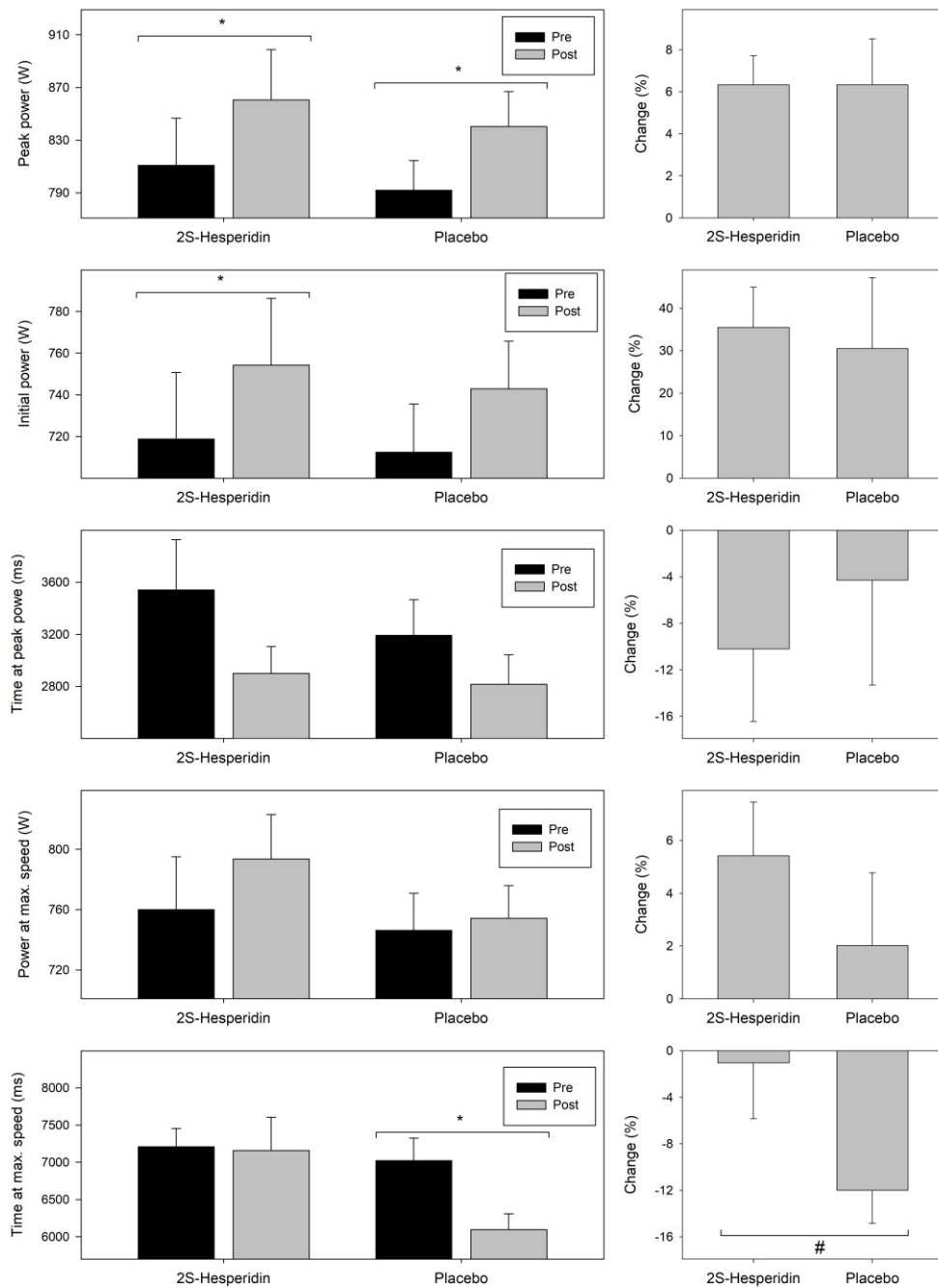


Figure 43. Changes in parameters evaluated during the Wingate test prior and after supplementation. Values are mean \pm SE. * Within-group significant changes ($p \leq 0.05$). # Between group trend t significant changes ($p = 0.05$ – 0.010).

9.4. DISCUSSION

The main objective of this study was to evaluate the effects of chronic intake of 2S-hesperidin on non-oxidative/glycolytic and oxidative metabolism and performance markers in amateur cyclists. For this purpose, participants were supplemented for eight-weeks with 500 mg 2S-hesperidin, a natural extract of sweet orange (*Citrus sinensis*) which contains hesperidin in its natural 2S form (NLT 85% 2S-hesperidin). Following the eight-week intervention, 2S-hesperidin supplementation led to significant improvements in submaximal and maximal intensity exercise performance in the incremental tests versus placebo. There was a significant decrease in VO_2R (mL/kg/min) at VT2 in placebo compared with 2S-hesperidin, in the step test.

The bioavailability of hesperidin is a factor that must be taken into account when examining its effectiveness, since the average maximum peak blood plasma concentration occurs after 5-7 hours of its ingestion and is almost eliminated post-24h (467). However, the excreted metabolites in urine has been shown to reach at maximum levels at post-24 h with continued remnants after 48 h (467). It is interesting to mention that the area under the curve was more than doubled (0.5L orange juice; 4.19 $\mu\text{mol h/L}$ vs 1L orange juice; 9.28 $\mu\text{mol h/L}$) at 24 h when high doses of hesperidin were consumed (1L orange juice = 444 mg hesperidin) (467). This indicates that high doses increase exposure to the body of 2S-hesperidin metabolites than low doses (222 mg/L). The dose that the cyclists in our study consumed was equivalent to more than one liter of orange juice, with the high carbohydrate load that it entails. The metabolites of hesperidin that appear mainly in the blood are glucuronides (87%) and sulfoglucuronides (13%) (467). These results are very similar to those found in this study.

Another key factor in the metabolism and absorption of 2S-hesperidin is the intestinal microbiota. In particular, Amaretti et al. (457) established that the species *Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum* had the ability to hydrolyze hesperidin, because in their genome they have the gene encoding for the enzyme α -L-rhamnose (limiting enzyme), which contributes to the release of aglycone from certain routine-conjugated polyphenols, such as hesperidin. A recent study suggests that the contradictory finding regarding the intake of hesperidin in humans may be due, in part, to the interindividual variability in its bioavailability, which highly depends on the α -rhamnosidase activity and the composition of the gut microbiota (470). On the other hand, hesperidin has shown to have a probiotic effect by promoting the growth of some beneficial bacterial species in the colon, the key role being the production of short-chain fatty acids (SCFA) (*Bifidobacterium spp.*, *Lactobacillus spp.*, or *Akkermansia muciniphila*) (470).

9.4.1. Incremental test

The results of this study showed an improved performance in eFTP and maximum power (\uparrow generated power) after chronic intake of 2S-hesperidin compared to placebo in incremental test. These changes are supported by a significant positive correlation between excretion of urinary 2S-hesperidin metabolites and maximum power ($r = 0.701$) and estimated FTP ($r = 0.725$). Regarding flavonoid supplementation, a previous study reported a 5% increase in absolute power output in a 10-min time trial (TT) after four weeks of 2S-hesperidin intake (500 mg) in cyclists (32). Other authors have also reported performance improvements (time until exhaustion $\sim 58\%$) in animals (33, 34). Currently there are no other studies that analyzing the effects of chronic hesperidin intake on performance. Several authors have reported that hesperidin exerts an antioxidative effect and promotes nitric oxide synthesis in different pathological study models (33, 531, 711-714). In a rat model with pleurisy, the antioxidant activity of hesperidin reduced the production of ROS in the liver and increased the liver activities of CAT and SOD (711). Estruel-Amades et al. (33) observed that five weeks of supplementation with 2S-hesperidin (200 mg/kg three days per week) prevented an increase in ROS and decline in SOD and CAT activity after a test until exhaustion in the thymus and spleen of mice with an intensive training plan. This scavenging activity hesperidin neutralizes reactive oxygen species, such as superoxide anion, generated during conditions of oxidative stress, like intense physical exercise (714). In particular, citrus flavanones (such as hesperidin and hesperetin) have the ability to modulate cellular antioxidant defenses through the NRF2-ARE pathway, which regulates gene expression of antioxidant enzymes, such as SOD, CAT, HO-1 and GPx, decreasing intracellular pro-oxidants (682). In addition, several authors have described a stimulating effect of nitric oxide production after hesperidin supplementation (31, 531, 712, 713), by an increase in endothelial activity NO synthase and gene expression of endothelium nitric oxide synthase. NO can relax human vascular cells (vasodilatation), which leads to improved blood flow during rest and exercise. Vasodilation is a physiological mechanism used not only for the supply of oxygenated blood, but also for the delivery of glucose, lipids and other nutrients to a variety of tissues (715). Theoretically, increased blood flow would increase the delivery of O₂ and nutrients (e.g. amino acids and glucose) to exercising skeletal muscle, thus aiding exercise performance during high intensity (conditions of hypoxia) (716). These mechanisms may be responsible for performance improvement in eFTP and maximum power in the incremental test in the group that consumed 2S-hesperidin.

Other flavonoids such as quercetin, has also demonstrated to improve the 5 km running performance time (-11.3% quercetin group; -3.9% control group) after its 14 days supplementation (250 mg/d) by trained triathletes (717). However, a systematic review that included 13 randomized controlled trials found that cocoa-derived

flavonoid (epicatechin and catechin, and oligomeric procyanidin) supplementation did not affect performance (642). Thus, there may be some specificity regarding the type of flavonoid that affects physical performance.

It should be noted that this study was carried out during a period when cyclists are reducing their training and competitions (late September-mid December) which involves training misadaptations (physiological and metabolic changes) (718, 719). These changes may justify the drop in the performance at sub-maximal and maximum intensities for placebo in our study. However, the intake of 2S-hesperidin was not able to prevent the loss of performance at VT1, although it was not significant, but it did maintain performance at VT2 and improve it at eFTP and maximum power. This supports our hypothesis, that the chronic intake of 2S-hesperidin could help generate or maintain adaptations at the mitochondrial level and of the endogenous antioxidant system in a period where the volume and intensity of training is decreasing, as in the conducted study (late September-mid December), maintaining performance levels in high-intensity exercise in amateur cyclists. The fact that 2S-hesperidin has an effect on different physiological mechanisms (454, 684, 703, 704) may be the reason why it cannot maintain performance at low but in high exercise intensities. In line with our hypothesis, the intake of hesperetin in elderly rats (hesperidin metabolite) has been shown to prevent loss of performance by improving mitochondrial and endogenous antioxidant status (35). The improvements in training adaptations of cyclists who ingested 2S-hesperidin may be due to the ability of this molecule to increase gene expression of the peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1 α) and nuclear factor respiratory 2 (NRF2), also, it increased the level of proteins of PGC-1 α and of complexes I, III and IV of the electron transport chain in the mitochondria, in muscle cells (*in vitro*) (35). In addition, hesperetin has shown increased activation of AMPK in liver cells (720) and fibroblasts (721). AMPK is a sensor of cellular energy status that plays a central role in skeletal muscle metabolism, regulating muscle exercise capacity, mitochondrial function and contraction-stimulated glucose uptake (722). PGC-1 α and AMPK are an important transcriptional masters regulators of mitochondrial biogenesis (\uparrow biogenesis mitochondrial and oxidative capacity) (722, 723) and NRF2 which is an essential regulator in the control of cellular redox homeostasis y controls glutathione synthesis (reactive oxygen species (ROS) scavenging) (724). Modifications in these transcription factors have shown performance improvements in endurance athletes (725). Therefore, 2S-hesperidin has the ability to promote muscle-level adaptations of endurance athletes, which could improve their performance in competitions.

It has been hypothesized that some molecules with anti-inflammatory and antioxidant activity may interfere with exercise-generated adaptations causing a decline in performance when ingested chronically (534). Although, there is controversy

on this issue, since supplementation of polyphenols, such as quercetin, has been shown to improve performance (429). With the results obtained in the incremental test, we can say that the chronic intake of 2S-hesperidin improves the power generated in eFTP and maximum power that would enhance the performance of endurance athletes for competition, avoiding the loss of performance (eFTP and maximum power) observed in the placebo group due to the loss of adaptations achieved during the cycling post-season. In addition, our results were strengthened by the positive correlations found between performance improvements at eFTP and maximum power with the excretion of metabolites in urine after 2S-hesperidin intake. Therefore, an increase in power production at high intensity is a key factor in cycling performance, which can increase your success in endurance competitions. However, at low intensity exercise levels there were no differences between groups. This could be because, at high intensities, the antioxidant action of 2S-hesperidin could improve performance (33, 530), but this capacity does not influence exercises at low intensities where oxidative stress is lower.

9.4.2. Step test

In the step test, the differences found between 2S-hesperidin and placebo indicate mismatches mediated by the reduction in training volume and intensity over the period of the study (718, 719), identified as a decrease in FAT (FatMax and VT1) and a decrease in VO_2R (mL/kg/min) (VT2) in placebo. These findings were in line with those found in the incremental test, where mismatches to training (\downarrow generated power at eFTP and maximum power) were also found. In endurance athletes, a 7% ($p < 0.05$) and 16% ($p < 0.05$) decrease in $\text{VO}_{2\text{MAX}}$ after 21 and 56 days of inactivity respectively has already been described in scientific literature, related to a decrease in systolic volume and decrease in citrate synthase and succinate dehydrogenase in muscle activities (726). Besides, a decrease in oxygen consumption values in the ventilatory thresholds and in maximum exercise has been associated with a decrease in power outputs in professional cyclists after three weeks of cycling competition (727). In the detraining process could also be involved the loss of oxidative capacity mediated by the reduction of PGC-1 α (\downarrow mitochondrial content) (35). Therefore, it is normal that after a period of detraining there are changes in different physiological-biochemical markers that lead to a loss of performance in athletes.

However, the 2S-hesperidin group maintained the oxidation of fats at FatMax and VT1, without decreasing the oxygen consumption in VT2. Similarly, a treatment with low doses of (-)-epicatechin (flavonoid) has shown an attenuation of training losses (14 d of detraining) in skeletal muscle capillarity and bioenergetics achieved after five weeks of resistance training (719). This suggests a similar effect of both molecules in preventing the physiological changes produced by detraining. In addition, hesperidin ($0.5 \text{ mmol}\cdot\text{kg}^{-1}$ of body mass) intake has been shown to be

effective in reducing the accumulation of body fat mass, glucose levels and blood lipids in rats fed a high-fat diet (728). The possible pathways used by chronic intake of 2S-hesperidin to decrease physiological changes derived from detraining would be related to the modulating gene components, such as AMPK and PGC-1 α (35, 721, 729), which control energy production, utilization of metabolic substrates (fats and carbohydrates), mitochondrial biogenesis and oxidative capacity (722, 723). Our results suggest that chronic intake of 2S-hesperidin may prevent the decrease in VO_2R (mL/kg/min) (VT2) that is associated with a decrease in the ability to produce power in cyclists, and a drop in FAT (FatMax and VT1), increasing carbohydrate utilization at moderately low intensities, which could anticipate fatigue in subsequent high-intensity work, such as in a cycling competition.

9.4.3. Wingate test

The results obtained in Wingate test (high anaerobic component) after intake 2S-hesperidin showed a improvement in both initial power absolute and relative when compared to placebo. On the other hand, both groups improved both power variables for a 30s sprint (Wingate test), without differences when comparing the groups. Currently, there are no other studies that have evaluated the chronic intake of 2S-hesperidin using a Wingate test. Martínez *et al.* (549) observed improvements in average power (2.3%) and maximum speed (3.2%) during a repeated 30-s sprint test in amateur cyclists following an acute intake of 2S-hesperidin. However, there are no previous studies that have evaluated the effect of chronic hesperidin intake on maximum anaerobic capacity (non-oxidative). In addition, combined intake of mangiferin and luteolin (polyphenols) for 15 days has also displayed improvements in average power (5.0%) during a Wingate (654).

In the short maximum effort tests, some of the changes can be explained by an initial learning effect, followed by a typical variation within the test(s) (730). Considering that the significant differences between the 2 experimental conditions have been small in the measurements evaluated in the Wingate test, it should be taken into account that in this type of trial they may be susceptible to the effects of placebo, nocebo or Hawthorne (731, 732). Intra-individual variability and therefore the probability of committing a type one error was further reduced by assessing study subjects at approximately the same time of day, thus avoiding effects of the circadian system about physiological, psychological, and molecular mechanisms in the body, resulting in varying physical performance over the day (733). We consider that in this type of test (Wingate) familiarisation can have an important effect on the final results, therefore, for future research we will introduce a comparison between the values obtained in familiarisation and the placebo, in order to observe variations that can

affect the final result or when comparing experimental groups taking into account the variability of the test (734).

One limitation of our study is the lack of having muscle biopsies to examine the possible mechanisms that could explain these improvements due to financial restrictions. They could have provided valuable.

9.4.4 Practical applications

The data found in this research shows how chronic intake of 2S-hesperidin enhances performance in FTP and maximum power. Advances in these areas of intensity are crucial for improving results in cycling competitions. Furthermore, as observed in the step test, 2S-hesperidin has the ability to maintain oxygen consumption in VT2 and fatty acid oxidation levels in FatMax and VT1, in periods with a decrease in training exercise volume and intensity (i.e., this study was conducted in the off-season). Given the effects reported by 2S-hesperidin, sports nutritionists would have other ergogenic aids available to improve the performance of their athletes. In this period, cyclists had decreased the volume and intensity of training with respect to other periods of the year. This is an important aspect to consider when comparing our results with other studies, as the outcomes could be different due to the volume and intensity of usual training during the testing time period.

9.5. CONCLUSIONS

Supplementation with 2S-hesperidin for eight weeks promotes an improvement in estimated FTP and maximum power in amateur cyclists during an incremental test. Furthermore, the supplementation with 2S-hesperidin can prevent a drop in VO_2R (VT2) and FAT (FatMax and VT1) in step test on training periods with less volume and load. These findings support the use of 2S-hesperidin as a natural new ergogenic aid, which can help cyclists improve both their aerobic performance.

CAPÍTULO X.
ESTUDIO N° 5

CAPÍTULO X. ESTUDIO Nº 5: LA SUPLEMENTACIÓN DE 8 SEMANAS CON 2S-HESPERIDINA MODULA EL ESTADO ANTIOXIDANTE E INFLAMATORIO DESPUÉS DEL EJERCICIO HASTA EL AGOTAMIENTO EN CICLISTAS AFICIONADOS

10.1 INTRODUCTION

Flavonoids are bioactive substances found mainly in fruits and vegetables, being more than 15.000 molecules identified within this family (735). However, one of the most well-known is hesperidin, that is a flavonoid present at high concentrations in citrus fruits, being the main one in sweet orange (*Citrus sinensis*). Hesperidin may be found in two isomeric forms, 2S- and 2R-, where the 2S isomer is predominant in nature (736). When hesperidin reaches the intestine, bacterial flora converts it into hesperetin (aglycon), which is effectively absorbed, being the main metabolite of hesperidin (470). Previous studies have shown the positive effects of hesperidin on some diseases (neurological, cardiovascular, insulin sensitization) due to its antioxidant and anti-inflammatory properties (454, 737). Moreover, the intake of hesperidin (in orange juice) has shown to modulate leukocyte gene expression, boosting its antioxidant and inflammatory profile, showing therefore a nutrigenomic effect (495). On the other hand, the ability of 2S-hesperidin to improve performance has been observed (738). It should be noted that there are other important factors that can modulate the effect of flavonoids like hesperidin, such as intestinal flora transformations, absorption and bioavailability (739).

The antioxidant effect of hesperidin is mainly related to its radical scavenging capabilities, as well as the increase in antioxidant cellular defense catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and oxidized glutathione (GSSG)) via the nuclear respiratory factor 2 (NRF2) signalling pathway (454). On the other hand, the hesperidin anti-inflammatory effect is produced by a decrease in inflammatory markers, such as nuclear factor kappa B (NF- κ B), interleukin 6 (IL6), tumour necrosis α (TNF α) and inducible nitric oxide synthase (iNOS) (454).

Regarding the potential of hesperidin on physical performance, a recent study reported that the acute intake of 500 mg of 2S-hesperidin significantly improved anaerobic performance (549). In the same study, they also found small non-significant changes in CAT, SOD, GSH and GSSG/GSH ratio compared to placebo during a rectangular test (with different intensities) in amateur cyclists. Similarly, a study performed in rats observed that 2S-hesperidin (200 mg/kg, three days per week during five weeks) showed a protective effect on the oxidative stress induced by an exhausting

exercise (33). Hesperidin supplementation prevented the increase in ROS production and avoided a decrease in SOD and catalase activities, while leading to a higher physical performance. In the same way, 6-weeks of hesperetin (main metabolite of hesperidin) supplementation ($50 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) significantly increased GSH/GSSG ratio and improved running performance (exercise time) in aged mice (35). In addition, a recent study found that eight weeks' intake of 2S-hesperidin improved performance at the threshold of estimated functional power and maximum power in an incremental test until exhaustion compared to placebo, in amateur cyclists (738). Other polyphenols have shown hesperidin-like effects. For example, the intake of 100 mL day for six weeks of acai berry-based juice (\uparrow anthocyanins) increased the levels of GSH and CAT post-exercise and after 1h of recovery, without changes in SOD and exercise performance (300 m running times) in junior athletes (740).

With regards to exercise, it is known that almost 0.15% of the oxygen consumed is converted into ROS, which can be detrimental to muscle and mitochondrial function (741). In sports physiology, it is hypothesized that rapid increases in ROS during intensive exercise may be a contributor to fatigue (742). Based on recent findings, a new theory proposes that antioxidant supplementation (vitamins A, C, E, thiols, ubiquinones and flavonoids) may delay fatigue (534). However, this mitigation of ROS generation may disrupt cellular signalling involved in training adaptations (743). ROS are intracellular messenger and activator of transcription factors that promote expression of genes related to training adaptations and performance improvement (743). Thus, antioxidant supplementation could decrease ROS production and delay fatigue, but in turn it may slow down the physiological adaptations of training (744, 745). Due to the current controversy on this topic, further investigations are required to evaluate if the intake of antioxidant polyphenols, such as hesperidin, could improve endogenous antioxidant status without negatively affecting performance.

Currently, studies have shown that acute (549) and chronic (738) intake of 2S-hesperidin in amateur cyclists improves anaerobic and aerobic performance, respectively. However, no research has explained the metabolic, biochemical and molecular mechanisms by which 2S-hesperidin intake improves performance. We hypothesized that the chronic intake of 2S-hesperidin would improve amateur cyclist's antioxidant status, evaluated through markers such as CAT, SOD, GSSG, GSH and hemoxygenase 1(HO1), but decrease inflammatory markers, such as IL6, TNF α , monocyte chemoattractant protein-1 (MCP1) and C reactive protein (CRP). However, the implications of long-term or prolonged use are unknown. Therefore, this study aimed to evaluate the effect of eight weeks of 2S-hesperidin supplementation (500 mg/day) on the antioxidant-oxidant (CAT, SOD, GSH, GSSG, HO1 and TBARS) and anti-inflammatory (IL6, TNF α , MCP1 and CRP) state in amateur cyclists before, at the end of the rectangular test and after the resting phase.

10.2. METHODOLOGY

10.2.1. Study Design

A randomized, double-blind, parallel clinical trial was conducted. Forty subjects were divided into 2 groups: 2S-hesperidin (n = 20) and placebo (n = 20). Subjects were randomized into groups using the Randomizer software. Participants consumed two 250 mg capsules of either Placebo (microcellulose, 500 mg) or 2S-hesperidin (500 mg Cardiose®, produced by HealthTech BioActives (HTBA), Murcia, Spain) at breakfast for 8 weeks. The Cardiose® supplement consisted of a natural orange extract that, due to its unique manufacturing process, retains most of the natural isomeric form of hesperidin (NLT 85% 2S-hesperidin). The placebo supplements were similar in appearance to the 2S-hesperidin capsule. Cyclists were instructed to continue their usual diet and training program. The usual total training distance was balanced between the groups (Table 26).

Table 26. Baseline general characteristics and training variables of the cyclists.

	2S-Hesperidin	Placebo	p-value
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Body mass (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI (kg·m⁻²)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
VO₂MAX (L·min⁻¹)	3.99 (0.36)	3.98 (0.63)	0.971
VO₂MAX (mL·kg⁻¹·min⁻¹)	57.5 (6.97)	57.9 (9.53)	0.880
HR_{MAX} (bpm)	184.9 (11.11)	183.2 (8.68)	0.593
VT1 (%)	50.9 (5.63)	50.0 (4.78)	0.610
VT2 (%)	84.9 (5.85)	84.1 (5.70)	0.644
Training variables	2S-Hesperidin	Placebo	p-value
Total distance (km)	1121.12 (534.99)	1082.43 (810.46)	0.868
HR_{AVG} (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
W_{AVG} (W)	174.86 (15.79)	163.47 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are expressed as mean (SD). BMI = body mass index; BF = body fat; VO₂max = maximum oxygen volume; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); Total distance = of all the training sessions carried out during the study period; HR_{avg} = average heart rate of all the training sessions carried out during the study period; W_{avg} = average power output of all training sessions during the study period and RPE = rating of perceived exertion of all training sessions during the study.

10.2.2. Participants

Forty healthy male, amateur cyclists completed the study (Table 1). Subjects met the following inclusion criteria: 18–55 years old, BMI of 19–25.5 kg m⁻², at least 3 years of cycling experience, and training for 6–12 h wk⁻¹. Amateur cyclists were excluded if: (a) regular smoking or alcohol drinking, (b) metabolic, cardiorespiratory or digestive pathology or abnormality, (c) injury in the previous 6 months, (d) supplements or medication in the previous 2 weeks and (e) abnormal values in blood test parameters. Before the start of the study, participants were informed about the procedures, and signed informed consent was obtained. The study was conducted following the Declaration of Helsinki guidelines for research on human subjects (548) and was approved by the Ethics Committee of the Catholic University of Murcia (CE091802), registered in ClinicalTrials.gov (Identifier: NCT04597983).

10.2.3. Procedures

Participants visited the laboratory on five different occasions. Visit 1 consisted of a medical examination and blood extraction to determine health status. On visits 2 and 4, a 24-h diet recall was conducted, followed by an incremental test until exhaustion on a cycle ergometer to estimate the rectangular test zones. On visits 3 and 5, the 24-h diet recall was repeated, and participants performed a rectangular test on the cycle ergometer (Figure 44) (Table 27). Before each testing session (visits 2, 3, 4 and 5), a standardized breakfast composed of 95.2 g of carbohydrates (68%), 18.9 g of protein (14%) and 11.3 g of lipids (18%) was prescribed by a sports nutritionist. Intake of both treatments began at visit 1 under the supervision of an investigator and finished at visit 5. Subjects in both groups were instructed not to consume foods with a high content of citrus flavonoids (grapefruit, lemons, or oranges) for 5 days prior to and during the study. This was verified by diet recall records.

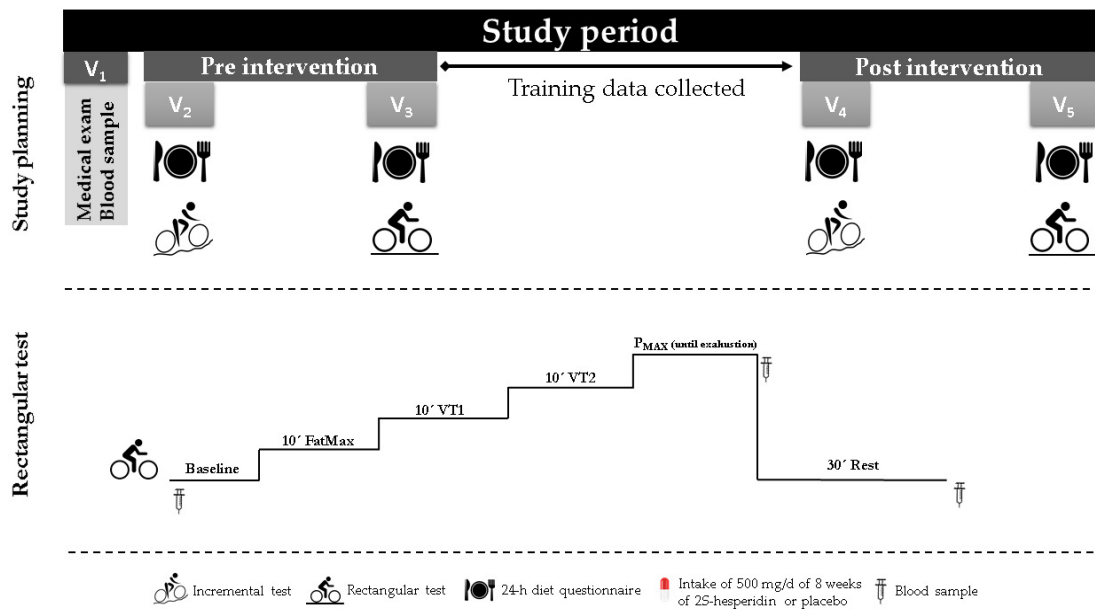


Figure 44. Study planning and rectangular test protocol.

Table 27. Between-group comparisons of dietary intake of cyclists.

	Pre-intervention			Post-intervention		
	2S-Hesperidin	Placebo	p-value	2S-Hesperidin	Placebo	p-value
Kcal	2163.6 (519.02)	2100.2 (515.77)	0.708	1974.1 (377.97)	2133.5 (437.98)	0.237
Kcal/BM	31.1 (9.34)	30.2 (8.71)	0.768	27.9 (6.53)	30.3 (6.46)	0.249
CHO (g)	245.7 (73.46)	222.0 (69.68)	0.312	216.6 (63.47)	248.3 (58.15)	0.117
CHO/BM	3.5 (1.31)	3.2 (1.14)	0.416	3.1 (1.08)	3.5 (0.94)	0.173
PRO (g)	113.5 (25.21)	115.2 (25.37)	0.837	109.0 (23.05)	101.5 (23.67)	0.332
PRO/BM	1.6 (0.41)	1.7 (0.48)	0.778	1.5 (0.35)	1.5 (0.42)	0.596
LP (g)	80.8 (27.24)	83.5 (23.65)	0.739	71.5 (17.61)	71.6 (18.89)	0.985
LP/BM	1.2 (0.45)	1.2 (0.37)	0.758	1.0 (0.27)	1.0 (0.29)	0.823

Values are expressed as mean (SD). Kcal = kilocalories; CHO = carbohydrates; PRO = protein; LP = lipids; BM = body mass. The mean values correspond to the average of all 24-hour diet recall data collected at pre-intervention (visits 2, 3 and 4) and post-intervention (visits 5, 6 and 7). * indicates significant differences ($p \leq 0.05$).

10.2.4. Testing

10.2.4.1. Medical exam

Medical examination was conducted by the research centre's medical doctor and consisted of medical and health history, resting electrocardiogram and examination (auscultation, blood pressure, etc.). These evaluations confirmed that the volunteer was healthy enough to be enrolled in the study.

10.2.4.2. Maximal test

Incremental step with final ramp test was performed on a cycle ergometer using a metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine maximal fat oxidation zone (FatMax), ventilatory thresholds 1 (VT1) and 2 (VT2) and maximal oxygen consumption (VO_{2MAX}). Participants started cycling at 35W for 2 min, increasing by 35W for every 2 min until $RER > 1.05$, and then initiating the final ramp ($+35W \cdot min^{-1}$) till exhaustion. To ensure reaching VO_{2MAX} , at least 2 of the following criteria had to be fulfilled: plateau in final VO_2 values (increase $\leq 2.0 mL \cdot kg^{-1} \cdot min^{-1}$ in the 2 last loads), reaching maximal theoretical HR ($220 - age \cdot 0.95$), $RER \geq 1.15$ and lactate $\geq 8.0 mmol \cdot L^{-1}$. Ventilatory thresholds were obtained using the ventilatory equivalents method described by Wasserman (553).

10.2.4.3. Rectangular test

Rectangular test procedures are shown in Figure 44. This test was performed on a cycle ergometer using power output values achieved during the maximal test at different intensity zones (FatMax, VT1, VT2 and maximum power). Participants exercised continuously as follows: 10 min at FatMax, 10 min at VT1, 10 min at VT2, at maximum power until exhaustion (post-PMAX) and 30 min rest (post-REC). There were no rest periods between phases.

10.2.4.4. Blood samples

Venous blood (arm antecubital area) was collected into one 3 mL ethylenediaminetetraacetic acid (EDTA) tube for haemogram and another 3.5 mL polyethylene terephthalate (PET) tube by a nurse for overall health analysis (visit 1). Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, haematocrit and haematimetric indexes were estimated.

Additionally, venous blood samples were collected in the baseline, after maximum power stage (post-PMAX) and during resting phase (post-REC), to measure antioxidant and anti-inflammatory parameters (visits 3 and 5) (Figure 44). During

every extraction point, 6 tubes of 3 mL of EDTA were obtained. Blood samples were centrifuged at 3.500 rpm in 4 °C for 10 min and sent to the laboratory for later analysis.

10.2.4.5. *Urine samples*

Main hesperidin metabolites were analysed in the urine of participants. Urine samples were collected for 24 h before V2 and V5 visits from each participant, before and after the supplementation, and were frozen in liquid nitrogen after collection and thawed for its analysis. For analysis, 50 µL of urine was mixed with 100 µL of water with 1% formic acid containing the internal standard. Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series Agilent Technologies, Sta. Clara, CA, USA). Metabolites were quantified by external standard calibration using rac-Hesperetin-d3 as the internal standard.

10.2.5. Antioxidant and inflammatory state markers

The following parameters were selected to measure the antioxidant and inflammatory status.

10.2.5.1. *TBARS (Lipoperoxidation biomarker)*

Thiobarbituric acid reactive substances (TBARS) are a by-product of the oxidative degradation of lipids by reactive oxygen species (lipid peroxidation), that is commonly used as oxidative stress marker (673). TBARS assay involves the reaction of malondialdehyde (MDA), a product of lipid peroxidation, with thiobarbituric acid (TBA) under high temperature and acidic conditions to form an MDA-TBA complex that can be measured colourimetrically (674). The coefficient of variation between replicas had to be less than or equal to 4.6 %. (Supplementary file 1).

10.2.5.2. *Catalase (CAT)*

CAT activity was determined using a UV-VIS spectrophotometer. This was expressed in sec-1 per gram of haemoglobin (675). The coefficient of variation between replicas had to be less than or equal to 4.9 %. (Supplementary file 1).

10.2.5.3. *Superoxide dismutase (SOD)*

SOD activity was measured using an SD125 Ransod kit (Randox Ltd. Crumlin, United Kingdom) (555). The coefficient of variation between replicas had to be less than or equal to 5.1 %. (Supplementary file 1).

10.2.5.4. *Glutathione reduced (GSH) and oxidized (GSSG)*

GSH was analysed by the glutathione-S-transferase assay described by Akerboom and Sies (556). Glutathione oxidized form and glutathione disulphide (GSSG) was determined in a similar way to GSH as shown above, as described by Asensi (557). The coefficient of variation for GSH between replicas must be less than or equal to 4.1 %. (Supplementary file 1)

10.2.5.5. *Hemoxygenase 1 (HO1)*

A commercial kit was used based on the Enzyme-Linked ImmunoSorbent Assay (ELISA) method (Shanghai BlueGeneBiotech Co., Ltd., Shanghai, China) with a detection limit of 0.1 ng/mL, according to the manufacturer's instructions. The coefficient of variation between replicas must be less than or equal to 4.9 %. (Supplementary file 1).

10.2.5.6. *Measurement of cytokines IL6, TNF α and MCP1*

These assays employed the quantitative sandwich enzyme immunoassay technique (DRG Instruments GmbH, Marburg, Germany), according to the manufacturer's instructions. A monoclonal antibody specific for IL6, TNF α and MCP1 was precoated onto a microplate. Standards and samples were placed into the wells, and any IL6, TNF α and MCP1 present were bounded by the immobilized antibody. After washing away any unbounded substances, an enzyme-linked polyclonal antibody specific for IL6, TNF α and MCP1 was added to the wells. Following a wash to remove any un-bounded antibody-enzyme reagent, a substrate solution was added to the wells, and colour developed in proportion to the amount of IL6, TNF α and MCP1 bounded in the initial step. The colour development was stopped, and the intensity of the colour was measured. The coefficient of variation for IL6, TNF α and MCP1 between replicas must be less than or equal to 4.4, 6.4 and 4.7 %, respectively. (Supplementary file 1)

10.2.5.7. *C reactive protein (CRP)*

For CRP-ultrasensitive (PCR-Turbilátex, Spinreact, Girona, Spain) detection, a turbidimetric test was used for the quantification of low serum CRP levels, according to the manufacturer's instructions. Latex particles coated with anti-human CRP antibodies were agglutinated by CRP that was present in the subject's sample. The agglutination process caused an absorbance change proportional to the CRP concentration of the sample, and by comparison with a CRP calibrator of known concentration, the CRP content in the analysed sample was determined. The coefficient of variation between replicas had to be less than or equal to 4.7 %.

10.2.6. Statistical analyses

Data analysis was conducted using IBM Social Sciences software (SPSS, version 21.0, Chicago, IL, USA). Descriptive statistics are presented as mean and standard deviation (SD). Levene's and Shapiro-Wilk tests were applied to check the homogeneity and normality of the data, respectively. A group \times time \times moment ANOVA was conducted to analyse within-group and between-group differences in all dependent variables and for every time-point of measurement (baseline, post-P_{MAX} and post-REC) and in both moments (pre-test and post-test). In addition, area under the curve (AUC), resulting from the integration of the three time-points of measurement taken during the rectangular test, was calculated for each variable. AUC was used to analyse pre-post differences both within-group and between-groups. The within-group differences in AUC were analysed by repeated-measures T-test; and between-group comparisons in AUC were conducted applying an independent samples T-test. Cohen's d effect size (ES) (95% confidence interval) was calculated for all comparisons. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large (558). Significant differences were considered for $p \leq 0.05$.

10.3. RESULTS

10.3.1. Biomarkers of Antioxidants and Oxidants Endogenous

Obtained values for CAT, SOD, GSSG, GSH, GSSG/GSH and HO1 during the rectangular test, pre- and post-intervention, are presented in Table 28. For each parameter, within group changes at each time point (baseline, Post-P_{MAX} and Post-REC) during supplementation were evaluated. A significant increase in SOD activity was found for the 2S-hesperidin group in post-P_{MAX} (15.5%) and post-REC (16.3%), while the placebo showed a significant increase in SOD at baseline (18.1%), intragroup pre-post-intervention (Figure 45). In addition, a similar increase in SOD in the AUC was found in 2S-hesperidin (14.1%) and placebo (11.9%) in the intragroup statistical analysis, without significant differences between groups (Figure 46).

Additionally, a trend towards a decrease with a moderate size effect in GSSG levels at post-P_{MAX} (-17.7%) was found in 2S-hesperidin in the post-intervention intragroup statistical analysis. In contrast, a significant decrease with a large size effect in GSSG was observed in the placebo at post-P_{MAX} (-15.1%) after the intervention (Figure 45). When comparing baseline post-intervention between groups, 2S-hesperidin had lower GSSG values (-20.1%) than the placebo (Figure 45). After the analysis of the AUC intragroup, there was a decrease in GSSG (-14.6%) only in 2S-hesperidin, without differences between groups (Figure 46).

For GSH, a decrease was reported at baseline (-9.4%) and post-P_{MAX} (-10.7%) in 2S-hesperidin after the intervention. On the other hand, a significant decline was found at baseline (-8.3%) in the placebo (Figure 45). Intragroup AUC analysis of GSH showed a decrease in 2S-hesperidin (-9.5%) and the placebo (5.5%), without differences between groups (Figure 46).

After the intragroup analysis, HO1 significantly increased at post-REC (19.7%) in the placebo, while there was a non-significant increase with a moderate size effect in HO1 at post-P_{MAX} (22.8%) in 2S-hesperidin (Figure 45). Intragroup AUC analysis showed an increase in HO1 in the placebo (20.9%) without any differences between groups (Figure 46).

When we analyzed the intragroup TBARS data, we found a trend towards an increase with a moderate size effect at baseline (9.4%) in 2S-hesperidin, without significant differences between groups.

When results for each parameter at each time point during supplementation were compared between groups, no significant changes were found in any antioxidant-oxidant parameter.

Table 28. Changes in enzymes and peptides endogenous antioxidants before, during and after rectangular test comparing pre- and post-intervention.

	2S-Hesperidin					Placebo					Between-Group Comparison				
	Baseline	Post-P _{MAX}	Post-REC	AUC	Baseline	Post-P _{MAX}	Post-REC	AUC	ΔBaseline	ΔPost-P _{MAX}	ΔPost-REC	ΔAUC			
CAT	Pre-Int	23.89	38.66	23.89	63.29	25.15	39.14	24.34	63.88						
	Post-Int	(5.14)	(11.40)	(3.75)	(14.20)	(4.54)	(8.51)	(3.85)	(11.20)						
SOD	Pre-Int	25.17	35.57	25.50	61.91	24.78	35.45	25.01	60.34						
	Post-Int	(4.95)	(9.66)	(7.65)	(12.62)	(4.02)	(11.12)	(4.41)	(12.74)						
GSSG	<i>P-value</i>	0.272	0.148	0.263	0.526	0.757	0.058	0.633	0.188	0.316	0.760	0.636	0.527		
	<i>Effect size</i>	0.24	0.26	0.41	0.09	0.08	0.42	0.17	0.30	0.32	0.07	0.15	0.20		
SOD	Pre-Int	1509	1442	1541	2971	1566	1573	1563	3138						
	Post-Int	(435.05)	(282.29)	(280.16)	(584.34)	(284.36)	(274.41)	(303.05)	(479.34)						
GSSG	Pre-Int	1698	1666	1792	3391	1849	1727	1718	3511						
	Post-Int	(238.55)	(246.11)	(231.20)	(308.32)	(364.77)	(491.32)	(412.24)	(754.07)						
GSSG	<i>P-value</i>	0.110	0.045	0.004	0.011	0.009	0.124	0.057	0.023	0.449	0.699	0.402	0.826		
	<i>Effect size</i>	0.42	0.76	0.86	0.69	0.95	0.54	0.49	0.75	0.20	0.16	0.27	0.07		
GSSG	Pre-Int	0.357	0.322	0.314	0.657	0.363	0.369	0.340	0.720						
	Post-Int	(0.19)	(0.07)	(0.06)	(0.14)	(0.13)	(0.07)	(0.10)	(0.11)						

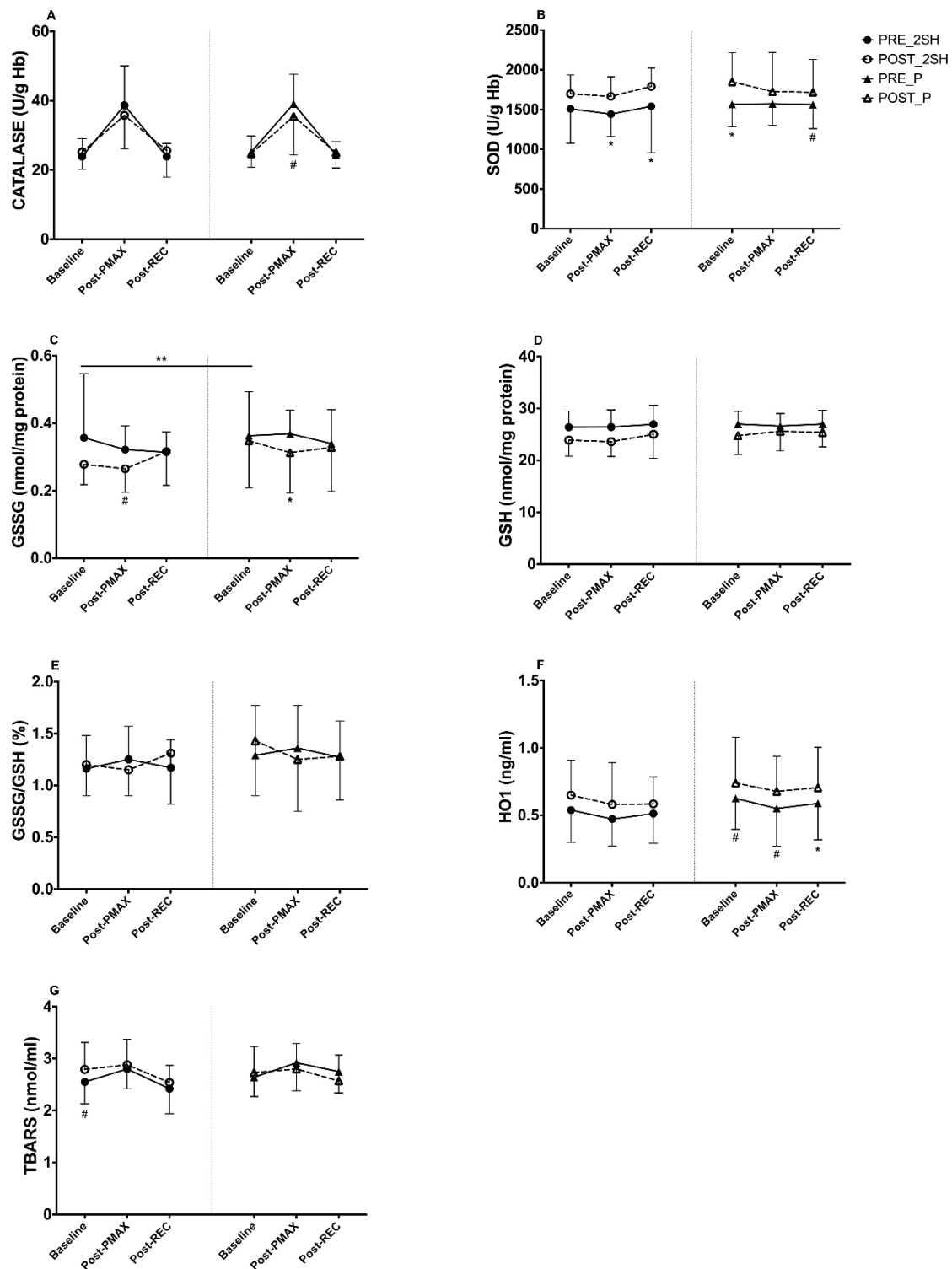


Figure 45. Differences between pre- and post-intervention intragroup in antioxidant and oxidant parameters at different points of the rectangular test (A–G). (C), a significant difference ($p = 0.04$) appears, comparing baseline of the second rectangular test between groups. * $p < 0.05$. # $p = 0.05-0.06$. ** $p < 0.05$ between post-intervention time points of rectangular test between groups (2S-hesperidin vs. placebo).

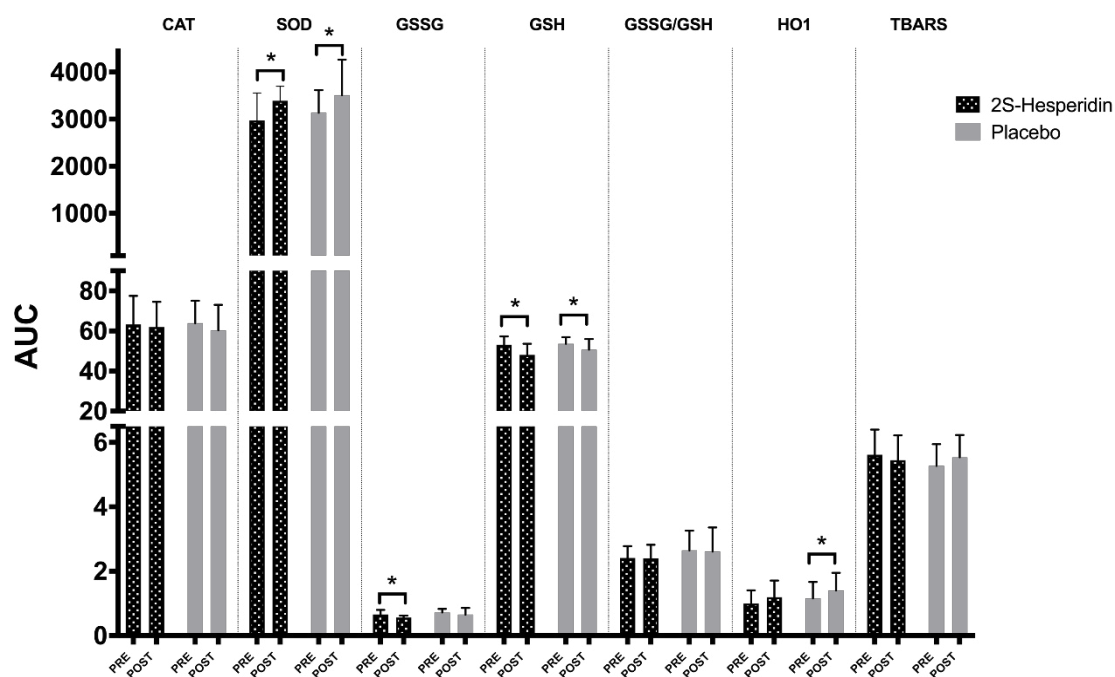


Figure 46. Intragroup differences between AUC (pre- and post-intervention) in antioxidant parameters. $*p < 0.05$. There were no significant differences between groups in AUC.

10.3.2. Inflammatory Biomarkers

Table 29 shows the obtained values for inflammatory biomarkers IL6, TNF α , MCP1 and CRP during the rectangular test, pre- and post-intervention. Within-group changes for each parameter and time point (baseline, Post-P_{MAX} and Post-REC) during supplementation have been evaluated. The placebo group showed a significant decrease in IL6 at Post-P_{MAX} (-35.7%), without significant changes in 2S-hesperidin (Figure 47), in the intra-group comparison pre- and post-intervention. However, after the intragroup analysis, we reported a decline in the AUC of IL6 (-33.0%) in the placebo after the supplementation period, without differences between groups (Figure 48).

Table 29. Changes in inflammatory status markers before, during and after rectangular test comparing pre- and post-intervention.

	2S-Hesperidin				Placebo			Between-Group Comparison				
	Pre	Post- P _{MAX}	Post- REC	AUC	Pre	Post- P _{MAX}	Post- REC	AUC	ΔBaseline	ΔPost- P _{MAX}	ΔPost-REC	ΔAUC
Pre-Int	2.46	3.05	4.85	6.71	5.41	7.17	9.44	14.59				
	(3.78)	(4.92)	(6.55)	(9.94)	(9.26)	(12.02)	(10.62)	(20.67)				
Post- Int	2.04	2.57	2.01	4.59	4.35	4.61	5.96	9.77				
	(2.32)	(2.94)	(2.61)	(5.01)	(8.65)	(7.78)	(11.98)	(17.69)				
P-value	0.537	0.695	0.128	0.255	0.129	0.045	0.065	0.021	0.514	0.243	0.807	0.310
Effect size	0.11	0.10	0.42	0.20	0.11	0.20	0.31	0.22	0.21	0.39	0.08	0.33
Pre-Int	8.06	8.43	7.97	16.44	8.71	8.90	7.94	17.22				
	(1.51)	(2.07)	(2.08)	(3.17)	(1.62)	(1.83)	(1.35)	(2.76)				
Post- Int	7.58	8.17	7.44	15.68	7.55	7.61	7.41	15.09				
	(1.70)	(1.98)	(2.00)	(3.40)	(1.89)	(2.46)	(1.94)	(3.60)				
P-value	0.271	0.466	0.148	0.338	0.015	0.038	0.127	0.021	0.252	0.210	0.999	0.239
Effect size	0.31	0.12	0.25	0.23	0.69	0.67	0.38	0.74	0.37	0.40	0.00	0.38
MCP-1 Pre-Int	568	614	631	1214	714	766	656	1452				

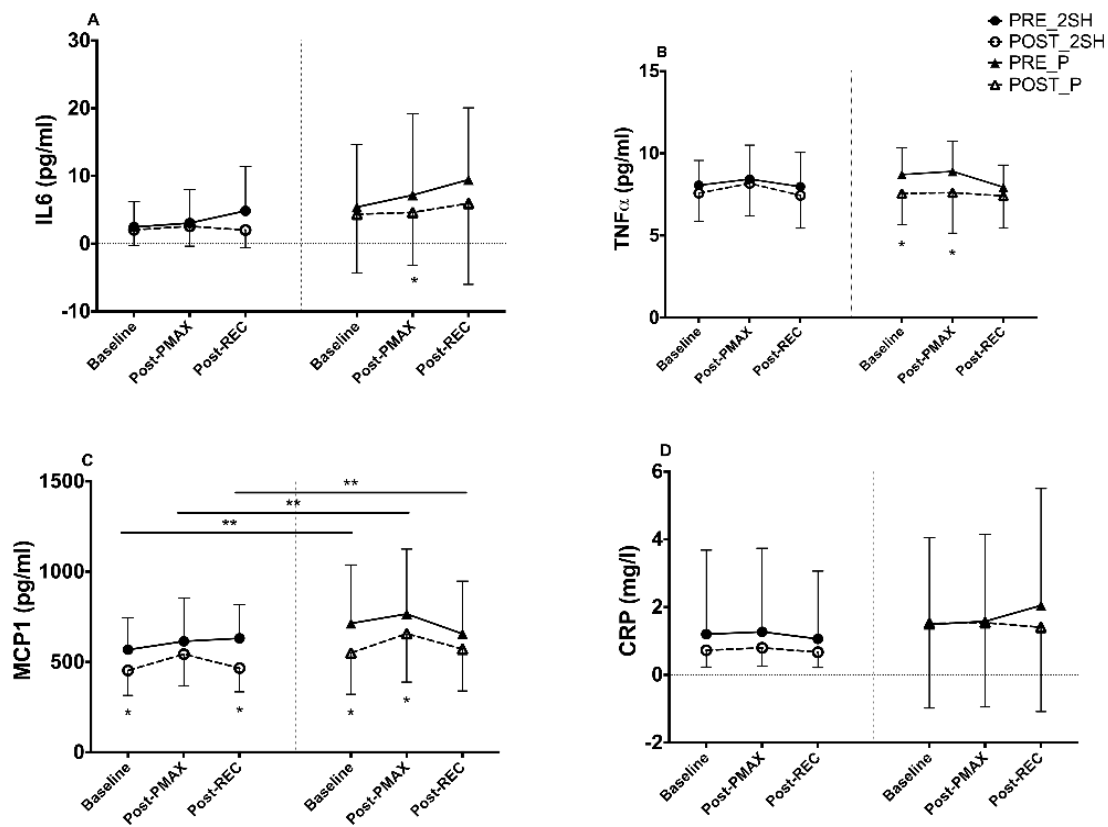


Figure 47. Differences between pre- and post-intervention intragroup and intergroup in inflammatory parameters at different points of the rectangular test (A–D). (C) a significant difference appears, comparing baseline ($p = 0.043$), Post- P_{MAX} ($p = 0.026$) and Post-REC ($p = 0.045$) of the second rectangular test between groups. * $p < 0.05$. ** $p < 0.05$ between post-intervention time points of rectangular test between groups (2S-hesperidin vs. placebo).

Regarding $TNF\alpha$, a significant drop in levels at baseline (-13.3%) and post- P_{MAX} (-14.5%) were found in placebo (Figure 47). In addition, intragroup AUC analysis of $TNF\alpha$ found a decrease (-12.4%) in placebo without differences between groups (Figure 48).

Significant decreases were observed in MCP1 at baseline (-20.2%) and post-REC (-26.1%) in 2S-hesperidin. In placebo, significant decreases were also observed in MCP1 at baseline (-23.0%), post- P_{MAX} (-14.2%) in the post-intervention intragroup statistical analysis (Figure 4). When comparing MCP1 at different times (baseline, Post- P_{MAX} and Post-REC) of the rectangular test post-intervention between groups, 2S-hesperidin had lower MCP1 values (-17.6%, -17.4% and -18.4%, respectively) than placebo (Figure 47). In addition, a similar decreased in AUC were found in 2S-hesperidin (-17.5%) and placebo (16.1%) in intragroup statistical analysis, but in the case of 2S-hesperidin a moderate size effect was observed. Without significant differences between groups (Figure 48).

No significant within-group changes were reported for CRP in any group (Figure 4). When results for each parameter at each time point during supplementation have been compared between groups, no significant changes were found in any inflammatory parameter.

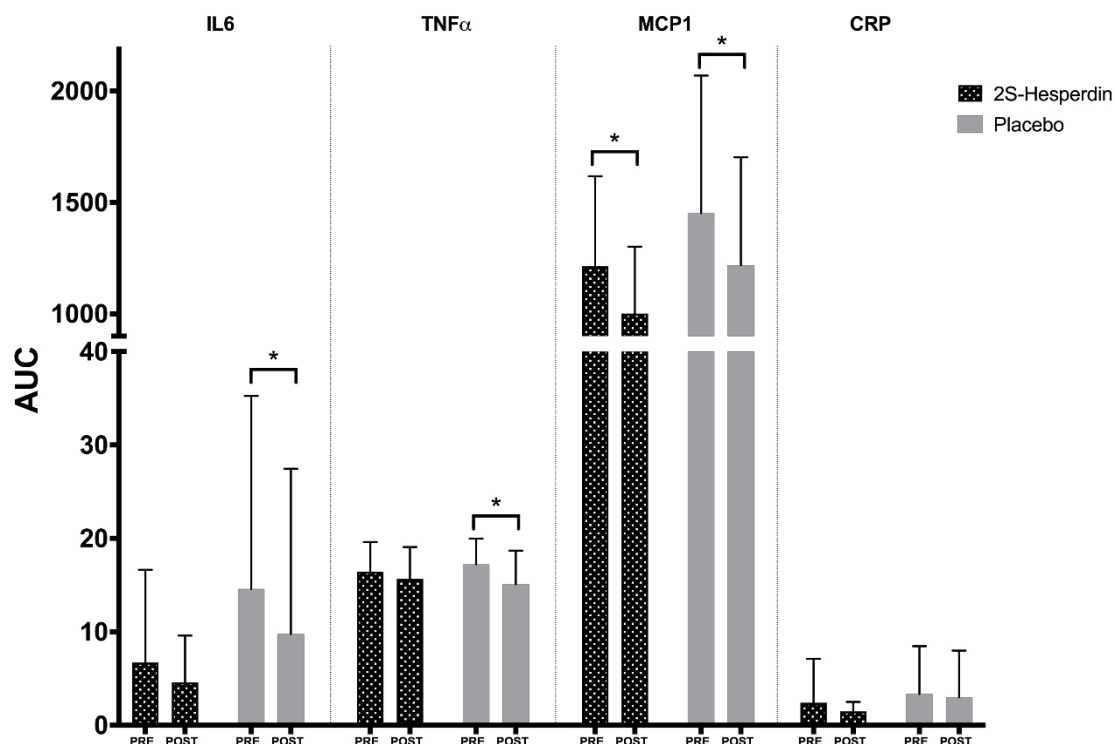


Figure 48. Intragroup differences between AUC (pre- and post-intervention) in inflammatory parameters. * $p < 0.05$. There were no significant differences between groups in AUC.

10.3.3. Hesperidin Metabolites Urine

Different hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analyzed in urine after Cardiose® intake. The main metabolite detected was hesperetin-3-glucuronide, representing $78.9 \pm 5.0\%$ ($n = 20$) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate made up $6.9 \pm 2.9\%$ ($n = 20$) and $14.7 \pm 4.1\%$ ($n = 20$) of the excreted metabolites. Despite the similarities in the excreted metabolites' profiles, a large interindividual variability was observed in the number of excreted hesperidin metabolites ranging from 2.3 to 37.5 μmol .

10.4. DISCUSSION

This study evaluated the effect of 8-week supplementation with 500 mg/d of 2S-hesperidin or placebo on antioxidant and inflammatory status in amateur cyclists during a rectangular cycle-ergometer test. To the best of our knowledge, this is the first study that examines the effect of chronic 2S-hesperidin intake on the antioxidant and inflammatory status of athletes at baseline, during and after exercise. In the rectangular test, oxidative status improved (\downarrow GSSG AUC) after the 2S-hesperidin intervention, but not with placebo. In addition, significant improvements in la antioxidant capacity (\uparrow SOD) after maximal exercise (Post-P_{MAX}), and inflammatory status after the acute recovery phase (\downarrow MCP1) were found in 2S-hesperidin group and compared to placebo (baseline and Post-REC).

10.4.1. Changes in endogenous antioxidant markers

SOD activity is usually increased after training, as an exercise-mediated adaptation (536). In contrast, a previous study showed no increases in SOD activity in untrained individuals after an 8-week moderate training program (35-min aerobic cycle, 3 times/wk) (746). Conversely, we observed a maintenance of SOD from baseline to Post-P_{MAX} and an increase to Post-REC in the post-intervention rectangular test, with significant increased in Post-P_{MAX} and Post-REC pre-post-intervention in 2S-hesperidin. However, an increase in SOD activity levels, evaluated as AUC, was observed for both groups, during intervention. In amateur cyclists, acute intake of 2S-hesperidin (single dose; 500 mg) led to a no-significant decrease in SOD at baseline (549). In animals, 2S-hesperidin supplementation (200 mg/kg for three days per week), along with a 5-week training program, led to no significant changes in SOD activity in rats after an exhaustive exercise test (33). Additionally, 2S-hesperidin also significantly increased SOD activity in heart tissues, which was attenuated by doxorubicin (induced cardiac toxicity) treatment (747). The 2S-hesperidin antioxidant capacity enhancement may be explained by the antioxidant characteristics of this molecule, related to hydroxyl groups in its B-ring (748). In addition, Parhiz *et al.* found that hesperidin had significant radical scavenging activity and prevented H₂O₂-induced oxidative damage on the cellular membranes of red blood cells, with radical scavenging activities comparable of ascorbic acid and trolox (a vitamin E derivative) (749). Furthermore, 2S-hesperidin shows neutralizing effect on non-enzymatic lipid peroxidation and superoxide, hydroxyl, peroxyxynitrite, and nitric oxide radicals (454, 748), leading to a lower depletion of antioxidant enzymes, allowing the maintenance of high antioxidant levels even after exercise-induced oxidative stress.

Another mechanism that has been reported *in vitro* and in animal models, but has yet to be confirmed in humans, is the improvement of the antioxidant status through a

nutrigenetic effect. Hesperidin has shown increased regulation of respiratory nuclear factor 2 (NRF2) (682). NRF2 is a basic leucine zipper transcription factor that binds and activates the antioxidant response element in the promoters of many antioxidant and detoxification genes encoding proteins, such as SOD, glutathione, thioredoxin and HO1, and thus it promotes the regulation of the intracellular redox environment (750). Interestingly, flavonoids have been proposed as inductors of the expression of genes related with enzymes of the endogenous antioxidant system, through the activation of the NRF2 transcription pathway (751). The higher SOD activity at the end of maximal effort and after a short recovery period after the intervention indicates that chronic intake of 2S-hesperidin improves antioxidant capacity at maximal effort and in the acute phase of recovery in cyclists amateur.

The oxidation of GSH to GSSG is a sensitive marker of oxidative stress (752). In addition, a GSH decrease and GSSG and GSSG/GSH ratio increases have been observed in professional cyclists after competition (543). When comparing both groups at baseline after the intervention, the 2S-hesperidin group had lower GSSG values than placebo, indicating lower levels of oxidative stress. This is in line with the decrease found in the AUC (GSSG) in 2S-hesperidin, indicating a decrease in oxidative stress when considering the whole rectangular test, which may be related to detraining adaptation. In fact, lower training volumes and intensities are associated with lower levels of GSH and GSSG in professional cyclists (568), which was also found Post-P_{MAX} in placebo. Therefore, this decrease in both groups is due to a lower exposure to high levels of free radicals leading to a maladaptation in the glutathione antioxidant system. The main advantage of incorporating the AUC in this study is that it allows us to precisely define the duration and magnitude of the variable being evaluated, which cannot be done in a point-by-point comparison (753). Despite there are no previous studies in humans evaluating the effects of chronic hesperidin intake on GSH and GSSG, instead, non-significant decreases in GSH, GSSH and GSSG/GSH ratio were observed after a repeated sprint test in amateur cyclists after a single-dose of 2S-hesperidin (500 mg) (549). In the same way, pathological animal models have shown the positive effect of hesperidin supplementation on these glutathione markers (\uparrow GSH and \downarrow GSSG) (694, 754).

It is known the regulation that exists between GSH and GSSG by the enzymes glutathione reductase (GR) and GPx, to maintain a balance between both molecules and avoid an increase of ROS (755). The changes observed in GSSG in the experimental group could be due to the modulation of GPx and GR activity, which were not measured in this study. In addition, another factor that may influence the GSSG/GSH ratio is the levels of nicotinamide adenine dinucleotide phosphate (NADPH), which are used with an indispensable cofactor by the GR and GPx enzymes to synthesize the GSH and GSSG forms (756). In this context, NADPH donates two electrons to reduce

GSSG to GSH by GR; the recycled GSH can then be used to reduce H₂O₂ to water by GPxs (757). In addition, increased glucose-6-phosphatase dehydrogenase (G6PD) (is a major source of cytosolic NADPH) activity by genetic or pharmacological means has been seen to raise cellular stores of NADPH and GSH, promote detoxification of ROS, and increase cell viability in primary vascular endothelial and smooth muscle cells *in vitro* (758). Increased G6PD activity is positively correlated with increased GR activity, where hesperidin was capable of restoring the activity of G6PD in rat (759). In addition, Salvemini *et al.* (760) reported that a three-fold increase in G6PD activity resulted in a two-fold increase in GSH levels, as well as a very significant increase in resistance to oxidative stress. As we can see, the GSSG/GSH ratio can be modulated by different components involved in the endogenous antioxidant system, which makes it difficult to explain its changes. Therefore, chronic intake of 2S-hesperidin could decrease GSSG levels (evidenced by ↓ AUC), indicating a better antioxidant state in the rectangular test, but specifically after immediately after exercise. This would facilitate faster post-training recovery or competition for cyclists.

In relation to HO1, in placebo there was an increase Post-REC with an increasing trend in baseline and Post-P_{MAX} post-intervention, however, in after 2S-hesperidin supplementation no significant change was seen, but there was a moderate effect in Post-P_{MAX}. The high variability in the HO1 data in 2S-hesperidin may have been the consequence of no significant changes being observed. What is clear is that amateur-level cycling for 8 weeks improves HO1 levels.

Although there is no clear pattern of improvement in antioxidant markers in 2S-hesperidin, there is an improvement in certain components (↑SOD, ↓GSSG) of the endogenous antioxidant system measured in this study and at key times during recovery. However, further studies are needed to provide clarity on this issue.

10.4.2. Changes in inflammatory markers

The production of ROS at the mitochondria of the working muscle stimulates the production of myokines or pro-inflammatory cytokines (761). IL6 (inflammatory cytokine) plasma levels can increase up to 100-fold after exercise, and circulating muscle-derived IL6 levels are closely related with the duration and intensity of exercise (762). To our knowledge, no studies have evaluated the effects of 2S-hesperidin intake on inflammatory markers in humans. In this study, IL6 levels increased during the first and second rectangular test from baseline to Post-P_{MAX} in both groups, but there were different trends from Post-P_{MAX} to Post-REC in second rectangular test (↓2S-hesperidin and ↑placebo). A significant decrease in IL6 during the recovery stage was observed in placebo, post-intervention. Other flavonoids, such as cocoa-derived flavanols, have also failed to inhibit the increase of IL6 after intense exercise (75% of peak power

output for 30 min) in cyclist (763). We believe that the high variability in the IL6 data was a factor that did not allow us to find significant intra- and intergroup differences. In addition, IL6 values in placebo were quantitatively higher than those of 2S-hesperidin, which may favour a significant decrease in IL6 after the reduction of the training load performed by cyclists from post-season to pre-season, as was the period in which the study was conducted (from the end of September to the end of December). As IL6 is known to stimulate the expression of TNF α (337), a decrease in IL6 levels in placebo would lead to a decrease in TNF α levels (baseline and Post-P_{MAX}). Since there is less training load, there is less induction of oxidative stress (568) and, consequently, less stimulation of the inflammatory system.

However, numerous *in vitro* studies (inflammatory models) have shown the ability of hesperidin to lower IL6 levels and TNF α (764-767). A recent study in trained animals showed that hesperidin intake (200 mg/kg for three days per week) during 5 weeks prevented an increase in IL6 levels in peritoneal macrophage after an exhausting exercise (34). Interestingly, in this study, a significant increase in IL6 after an exhausting exercise, from pre-training to post-intervention, was observed in placebo group. Hesperidin intake has also led to a decrease in IL6 in a rheumatoid arthritis rat model (703). In rats, the intake of alcohol to induce a gastric ulcer increasing the expression of cyclooxygenase-2 mRNA and decreased GPx, SOD, CAT but the intake of hesperidin reversed these changes, improving the antioxidant and inflammatory status (768). Besides, in a model of Alzheimer's disease in mice, treatment with hesperidin (40 mg/kg, 90 days intragastric) increased HO1 and decreased levels of TNF α , CRP, NF- κ B and MCP1, suppressing oxidative stress and inflammation (769).

A hypothesis has been generated at the nutrigenomic level of how the intake of hesperidin can improve the inflammatory state, related to the activation of the Akt/NRF2 axis and inhibition of NF- κ B (769), the latter being a transcription factor well known for its role in the innate immune response and a transcriptional activator of inflammatory mediators such as cytokines (770). On the other hand, NRF2 is not only important for redox signalling, but also for the attenuation of the inflammatory mediator synthesis (769). In this sense, the impairment of NRF2 signalling by ultraviolet B (UVB) was reversed by the topical application of hesperidin methyl chalcone that inhibited the production of the cytokines TNF α , IL-1 β , IL6, and IL-10 that had been induced by UVB irradiation in hairless mice (771). This suggests that there is a connection between the antioxidant and inflammatory status and their signaling pathways. In our case, the group ingesting 2S-hesperidin did not experience a significant decrease in TNF α .

MCP1 is another inflammatory cytokine that increases after exercise in plasma (772). In our study, lower MPC-1 levels during the whole exercise (AUC) were observed after supplementation in both groups. This decrease was statically significant

at baseline and during the recovery phase for 2S-hesperidin supplemented group. In addition, when comparing between groups at different post-intervention test times, the 2S-hesperidin group had lower levels compared to placebo. In previous studies with acute lung damage model, both in vitro and in vivo, hesperidin has shown immunomodulatory effects, down-regulating the expression of MCP1, as well as other pro-inflammatory cytokines, such as IL6 and TNF α (764, 773). Precisely, treatment with hesperetin-7-O-glucuronide (5 mg kg⁻¹) has been observed to decrease the MCP1 mRNA expression in rat aortic endothelial cells (773). And on the other hand, oral administration of 100 or 200 mg/kg of hesperidin three times a week for four weeks in rats produced a decrease in the pro-inflammatory cytokines interferon-gamma- γ and MCP1 in the lymphocyte of the mesenteric lymph node (33). Additionally, polyphenols and hesperidin can modulate gut microbial composition or functionality, which modulate the release of microbial-derived metabolites (487). In addition, hesperidin has the ability to inhibit the growth of harmful bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Prevotella* spp., *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, among others (470). In particular, hesperidin can increase the abundance of *Faecalibacterium prausnitzii*, which inhibits NF- κ B activation and consequently attenuates the inflammatory response (774). The inhibitory capacity of hesperidin in some bacteria may modify the composition of the intestinal microbiota acting as an immunomodulator and anti-inflammatory (\downarrow IL-1 β , TNF α , and IL6), with a direct relationship between the two effects (470). In contrary, the effects of quercetin (flavonoid) intake (1g/day) for 3 weeks in trained cyclists were evaluated by Nieman et al. (772). In this study, no significant changes in MCP1 plasma levels were observed after 3 week supplementation and a 3-day period in which subjects cycled for 3 h/day at ~57% maximal work rate. Muscle biopsies showed a within-group significant post-exercise increase in muscle cytokine mRNA expression for IL6 and TNF α , but without differences between quercetin and placebo groups (772). No anti-inflammatory effect was observed after the intake of quercetin. On the other hand, the placebo group showed a decrease in MCP1 at baseline and Post-P_{MAX} at post-intervention, possibly related to the decrease in TNF- α , as a positive correlation between MCP1 and TNF- α concentrations after short-term exercise training has been previously demonstrated (775), indicating a relationship between these 2 cytokines.

Although there is no clear pattern of improvement in inflammatory markers in 2S-hesperidin, there is an enhancement in MCP1(baseline and Post-REC) and compared to placebo in the second rectangular test at all points and at key times during recovery. However, further studies are needed to bring clarity to this question.

These 2S-hesperidin properties as antioxidant and anti-inflammatory may be related: a decrease in oxidative stress during the exercise maximum intensity could modulate the inflammatory state in the acute phase of recovery. As has been shown in

the studies presented in this publication, there is a close relationship between antioxidant and inflammatory status and their signaling pathways. Redox balance can be altered during periods of high intensity physical exercise and low rest periods, leading to a chronic oxidative stress state (534). Moreover, high oxidative stress levels can inhibit exercise physiological adaptations, reducing performance and leading to overtraining (534). Therefore, an optimal redox homeostasis is essential for a proper muscle physiological function (i.e., antioxidant status, biochemistry, signalling, bioenergetics and muscle contraction).

The effects of antioxidant supplementation on performance are a controversial topic, which still needs additional research. On one hand, it has been pointed that the use of antioxidant substances may help to maintain optimal ROS levels in the muscle, avoiding possible decreases in performance (534). On the other hand, it has been hypothesized that chronic antioxidant intake can hinder training adaptations, negatively affecting performance (745). Different studies show that antioxidant intake does not prevent exercise-induced activation of redox-sensitive signalling pathways (776). A recent publication, summarized the performance measurement that were carried out in this same intervention trial, along with the antioxidant and inflammatory markers results reported in this paper (738). In this trial, amateur cyclists supplementation (8 weeks) with 2S-hesperidin (500 mg/day) led to an increase in power production at estimated functional threshold power (2.3% = 6.40 W; $p=0.049$) and maximum power (1.9% = 7.40 W; $p=0.049$) during an incremental test after the intervention (738). Thus, 2S-hesperidin does not appear to interfere with training-induced adaptations, improving performance while avoiding oxidative stress and inflammation.

Study described in this paper has some limitations. One limitation was the short recovery time after the rectangular test (20 min after exercise) in which changes in antioxidant and inflammatory markers were evaluated. Measurements 24 and 48 h after exercise would have provided valuable additional information; however, funding constraints made it impossible. Additionally, a larger sample would have given more statistical power to reported results, due to the high individual variability in some markers. Given the few studies done in this field, future research could shed light on the effectiveness of 2S-hesperidin as an ergogenic aid with antioxidant and inflammatory effects.

Differences with current results may be related to the different stage of the season in which studies were done, in the sample used and the different aerobic and anaerobic demand profiles of the used tests. In the same way, one of the factors influencing the variability of 2S-hesperidin effects in different studies may be its pharmacokinetics, and the resulting exposure of the body to hesperidin metabolites. It has been described that the concentration of 2S-hesperidin metabolites in plasma reach

their maximum peak 5-7 hours after intake, being almost completely eliminated after 24 hours. In the urine, the maximum peak of metabolites is usually found at 24 hours of 2S-hesperidin intake and its total excretion occurs after 48 h (467).

10.5. CONCLUSION

Supplementation with 2S-hesperidin (500mg/d) for 8 weeks improves the post-rectangular test antioxidant (\uparrow SOD and \downarrow AUC-GSSG) and inflammatory status during the acute phase of post-exercise recovery (\downarrow MCP1). This modulation in antioxidant and inflammatory markers can help cyclists to improve their recovery, after intense efforts or long exercise sessions that, due to their characteristics, led to an increase of inflammation and oxidative stress. Unlike other polyphenols, 2S-hesperidin supplementation does not appear to interrupt adaptations produced by training in amateur cyclists, enhancing their performance (738).

CAPÍTULO XI.

ESTUDIO N° 6

CAPÍTULO XI. ESTUDIO N° 6: LA SUPLEMENTACIÓN DE 8 SEMANAS CON 2S-HESPERIDINA MEJORA LA MASA MUSCULAR Y REDUCEN LA GRASA EN CICLISTAS AFICIONADOS COMPETITIVOS: ENSAYO CONTROLADO ALEATORIO

11.1. INTRODUCTION

Cycling is a sport that presents a high aerobic component, but in which the anaerobic component is decisive for success in competitions (68). The key physiological factors that could predict a cyclist's performance are: oxygen consumption (VO_{2MAX}) (15), maximum power output (MPO) (49, 741), ventilatory threshold 2 (VT2) at $\sim 90\%VO_{2MAX}$ (14), power output (PO) and cycling efficiency (78). In addition, body composition control is essential for high-level athletes in order to monitor changes in different body components (fat, muscle, bone, residual, etc.) at different time points during the year, as it affects sports performance (777).

It is known that having low BF is ideal for achieving high levels of sports performance, since high BF levels require higher energy expenditure to maintain the same running speed, and may lead to a decrease in performance (778). Besides, it has been shown that a reduction in body weight can improve the climbing performance of cyclists (779). Indeed, dietary adjustments, in combination with physical exercise, are the best strategies to reduce BF, which in turn provides benefits (performance enhancement) to the athlete (780). Interestingly, in the last years, the use of phytochemical dietary supplements (including flavonoids) alone or in combination with exercise and/or diet has also shown beneficial modifications in body composition and sports performance in animals and humans (549, 738, 781).

One of the most studied flavonoids due to its antioxidant and anti-inflammatory properties is hesperidin, a flavonoid mainly found in citrus fruits (460). Hesperidin is a molecule that can be found in two isomeric forms, such as 2S and 2R, being the 2S-hesperidin the predominant form in nature (782). The biological behaviour of the two isomers is different, for example a 5.2-fold improvement in glucuronidation efficiency was observed for 2S-hesperetin compared to 2R-hesperetin in vitro, without any significant change in sulfonation kinetics (461). It should be noted that, despite a fresh squeeze orange juice mostly contains 2S-hesperidin, most commercialised hesperidin supplements contain a mixture of 2R and 2S forms.

In vitro studies have shown that hesperidin increases adenine monophosphate activated protein kinase (AMPK), that controls mitochondrial biogenesis, glycemia, and fat oxidation; and reduces triglycerides (TG) accumulation in adipocytes (509, 783).

Hesperetin, the main hesperidin metabolite, has shown to increase intracellular ATP production and mitochondrial spare capacity in human myotubes (35). In animals with high-fat diets, it has been observed that the intake of citrus flavonoids (hesperidin and naringin) increases fatty acid β -oxidation, reverses AMPK activity and decreases weight and abdominal fat (783, 784). Besides, the administration of hesperetin to aged mice completely reverted the age-related decrease of muscle fibre size and improved the running performance, due to a boost in mitochondrial energy supply and antioxidant effect (35).

In humans, intake of glucosyl hesperidin (100-500 mg/d for 6 weeks) has shown to reduce TG and low-density lipoprotein (LDL) levels (785). On the other hand, it has been observed that supplementation for 12 weeks with red-orange juice extract (400 mg/d), which is a source of 2S-hesperidin, decreased body weight, BMI, waist and hip circumference in healthy overweight human volunteers (786).

The effect of single 2S-hesperidin supplementation on body composition (fat and muscle mass) has not yet been studied. Nevertheless, 2S-hesperidin has shown performance improvements in amateur cyclists, both after acute intake (500 mg per single dose; improving anaerobic capacity (549)) and chronic intake (500 mg/d for 8 weeks; improving aerobic capacity (738) and antioxidant and inflammatory status (787)). Overall, we hypothesised that (1) 2S-hesperidin treatment would help to decrease BF, and (2) 2S-hesperidin would improve the oxidation of fatty acids during resting metabolic rate test. Therefore, the purpose of this study was to determine if the intake of 2S-hesperidin during eight weeks improved body composition by densitometry and anthropometry in amateur cyclists. A secondary objective was to evaluate the effects of 2S-hesperidin supplementation on different variables of resting metabolic rate using indirect calorimetry.

11.2. METHODOLOGY

11.2.1. Participants

Forty healthy, male amateur competitive cyclists (AMA) completed the study (Table 30). Inclusion criteria were the following: 18-55 years, 19.0-25.5 kg·m⁻² BMI, at least 3-years cycling of experience, and 6-12 h·wk⁻¹ training. Participants were excluded if they: a) were smokers (in the last 6 months) or regular alcohol drinkers (three to four days per week), b) had metabolic, cardiorespiratory or digestive pathologies or anomalies, c) had an injury in the last 6 months, d) were consuming any type of supplementation or medication in the last 2 weeks and e) had abnormal values in some parameter (haemogram, general biochemistry, transaminases, inflammation and virus) in the preliminary blood analysis. All participants were informed about the procedures

and provided signed informed consent. The study was conducted according to the guidelines of the Helsinki Declaration for Human Research (548) and the protocol was approved by the Ethics Committee/Institutional Review Catholic University of Murcia (Code: CE091802), registered with ClinicalTrials.gov (Identifier: NCT04597983).

Table 30. Baseline characteristics of male amateur competitive cyclists (N=40).

	Cardiose®	Placebo	p-value
Main Characteristics			
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Weight (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI (kg·m ⁻²)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
Training Variables			
Km total (km)	1121.12 (534.99)	1082.43 (810.46)	0.868
HRavg (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
Wavg (W)	174.86 (15.79)	163.47 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are presented as mean (SD). BMI = body mass index; BF = body fat; Km total = total distance of all the training sessions carried out during the study period; HRavg = average heart rate of all the training sessions carried out during the study period; Wavg = average power output of all training sessions during the study period.

11.2.2. Study design

A double-blinded, parallel and randomised experimental design was performed. Randomization was performed using computer software (Randomizer) to assign codes to the groups established in this study (706). Participants were divided into two groups: 2S-hesperidin (n=20) and Placebo (n=20). Depending on the group, participants took 500 mg of 2S-hesperidin (Cardiose® HTBA (HealthTech BioActives), Murcia, Spain) or 500 mg of placebo (microcellulose) per day for 8 weeks in the breakfast. Cardiose® is a natural orange extract that due to its unique manufacturing process, maintains most of the natural hesperidin isomeric form (NLT 85% 2S-hesperidin). The cyclists were instructed to continue their regular training schedule, and no significant differences between groups in the main variables of training load were observed (Table 30). Subjects in both groups were instructed not to consume foods high in citrus

flavonoids (grapefruit, lemons or oranges) for 5 days prior to and during the study, which was verified by diet recalls records and urine analysis.

11.2.3. Procedures

Participants visited the laboratory three times. The first visit (V1) consisted of a medical examination and a blood analysis to evaluate health status, while second (V2) and third visits (V3) included resting metabolic rate (RMR) test, densitometry, anthropometry, urine sample and a 3-day recall diet questionnaire (Table 31). The supplementation of both treatments started at V2 after the completion of all tests and under the supervision of an investigator.

Table 31. Between-group comparisons in dietary intake of male amateur cyclists (N=40).

	Pre-intervention			Post-intervention		
	2S-Hesperidin	Placebo	p-value	2S-Hesperidin	Placebo	p-value
Kilocalories	2163.60 (519.02)	2100.18 (515.77)	0.708	1974.09 (377.97)	2133.51 (437.98)	0.237
Carbohydrates (gr)	245.72 (73.46)	221.93 (69.68)	0.312	216.58 (63.47)	248.26 (58.15)	0.117
Protein (gr)	113.50 (25.21)	115.20 (25.37)	0.837	108.97 (23.05)	101.52 (23.67)	0.332
Lipids (gr)	80.75 (27.24)	83.52 (23.65)	0.739	71.48 (17.61)	71.59 (18.89)	0.985

Values are presented as mean (SD). The mean values correspond to the average of the data collected in the 3-day recall diet questionnaire on pre-intervention (visit 2) and post-intervention (visit 3) days. * Significant differences for $p \leq 0.05$.

11.2.4. Testing

11.2.4.1. Medical exam

The medical exam included a medical history, resting electrocardiogram, and medical examination (auscultation, blood pressure, etc.) to confirm that the participants were healthy before they were enrolled in the study.

11.2.4.2. Dual-energy x-ray absorptiometry (DXA)

Before (V2) and after (V3) intervention period, body weight and body composition (percentage of body fat (BF%), body fat (BF), lower limb fat mass (LLFM) and lean mass (LM)) were assessed in the morning with cyclists in a fasted state and

wearing light clothes. Body composition was evaluated using a whole body DXA-scan (XR-46; Norland Corp., Fort Atkinson, WI). Discrimination of BF and lean body mass (LBM) was done with a computerised analysis of DXA-scan (Software Illuminatus DXA 4.4.0, Visual MED, Inc., Charlotte, NC and Norland CooperSurgical Company, Minneapolis, MN).

11.2.4.3. Anthropometry

The same researcher (ISAK Level-1 certified) performed the anthropometric measurements for both pre- and post-test assessments. Height and body weight were measured using a digital scale with a stadiometer for clinical use (SECA 780; Vogel & Halke GmbH & Co. Hamburg, Germany). Skinfold thickness was measured using Holtain Skinfold Calipers (Holtain, Ltd. Crymych Pembrokeshire, UK), in accordance with the International Society for the Advancement of Kinanthropometry guidelines. Percentage of body fat was determined using the Faulkner Equation (671), while percentage of muscle mass was calculated using the modified Matiegka equation 27. The sum of the eight skinfolds (triceps, subscapular, bicep, crestailiac, supraspinal, abdominal, thigh and calf) was also calculated.

11.2.4.4. Resting metabolic rate (RMR)

Participants visited the laboratory in a fasted state for V2 and V3. Subjects were requested to abstain from caffeine or alcohol consumption for 24 h prior to the measurement. Diet was controlled 24 h before the visits with, using the 24-hour diet recall conducted prior to the resting metabolic rate measurement at in pre-intervention. Participants were asked to consume the same diet at post-intervention.

Resting metabolic rate (RMR) was measured by indirect calorimetry with metabolic cart (Metalyzer 3B; Cortex-medical, Leipzig, Germany). Tests were performed between 9 am and 11 am, and always at the same time in both pre- and post-intervention sessions. The room was dimly lit and quiet, and the ambient temperature was at ~25°C. Participants laid in a supine position wearing light clothing for 15 min. Data were then collected for 20 min, and only the middle 10 min were used to calculate the substrate-based utilisation of energy (carbohydrates and lipids) 28. The system was calibrated before each measurement following manufacturer recommendations.

11.2.4.5. Blood samples

Blood was collected into one 3.0-mL ethylenediaminetetraacetic acid (EDTA) tube for haemogram and another 3.5-mL polyethene terephthalate (PET) tube for overall health analysis. Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and

external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, haematocrit and haematimetric indexes were estimated. The blood extraction was performed by a nurse expert in the antecubital vein.

11.2.4.6. *Hesperidin metabolites in urine*

Urine samples, corresponding to the collection of urine 24 h before (V2) and after (V3) the supplementation in both groups for each participant, were frozen in liquid nitrogen after collection and thawed for its analysis. Fifty μL of collected urine was mixed with 100 μL of water with 1% formic acid containing the internal standard (rac-Hesperetin-d3). Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series Agilent Technologies, Sta. Clara, CA, USA). The method was validated using a pool of samples by determining the limit of detection (MDL) and quantification (MQL), repeatability (expressed as relative standard deviation RSD), and accuracy (%). Metabolites were quantified by external standard calibration using rac-Hesperetin-d3 as the internal standard.

11.2.5. Statistical analyses

Statistical analysis was carried out using IBM Social Sciences software (SPSS, v.21.0, Chicago, IL, USA). Data are presented as mean and SD. Levene and Shapiro-Wilks tests were performed in order to check the homogeneity and normality of the data, respectively. Depending on the normality and homogeneity outcomes obtained, a Paired sample T-test or Wilcoxon signed-rank test were applied for analysing within-group pre-post differences. Likewise, a between-group comparison was calculated using an ANCOVA test with pre-test values as covariates (to eliminate the possible bias caused by the initial level of each group in the different dependent variables) or Mann-Whitney U test for all body composition and RMR variables. The significance level was determined at $p \leq 0.05$. Cohen's d effect sizes (ES) (95% confidence interval) were calculated for all comparisons. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large 29. Relationships between levels of excreted hesperidin metabolites in urine and other evaluated parameters were analysed using Spearman correlation analysis ®. Significant differences were considered for $p \leq 0.05$.

11.3. RESULTS

11.3.1. DXA

The results of body composition assessed by DXA are presented in Fig 49. Within-group analysis showed a significant pre-post decrease in BF (-17.9%; $p = <0.001$; ES= 0.50), BF% (-15.3%; $p = <0.001$; ES= 0.49) and LLFM (-15.5; $p = <0.001$; ES= 0.56) in the

2S-hesperidin group, while there were no differences found in placebo ($p= 0.340$; $ES= 0.14$, $p= 0.441$; $ES= 0.11$ and $p= 0.469$; $ES= 0.10$, respectively). Furthermore, between-group comparisons revealed a significant decrease in %BF (-12.3%; $p= 0.035$; $ES= 0.67$) and LLFM (-12.6%; $p= 0.029$; $ES= 0.66$) in favour of 2S-hesperidin. Also, there was a trend with a moderate effect for a decrease in BF (-13.3%; $p= 0.055$; $ES= 0.61$) in 2S-hesperidin compared to placebo.

Regarding LM, there were no pre-post significance in both placebo group (-1.1%; $p= 0.082$; $ES= 0.13$) and in 2S-hesperidin group (0.4%; $p= 0.461$; $ES= 0.04$). In addition, no significant differences with a moderate effect were observed between groups (1.5%; $p= 0.115$; $ES= 0.60$). However, in the LM% a significant decrease (-1.8%; $p= 0.009$; $ES= 0.34$) was found in placebo, without significant change (-0.3%; $p= 0.649$; $ES= 0.06$) in 2S-hesperidin. No significant change with small effect was found when comparing groups (-1.5%; $p= 0.116$; $ES= 0.49$).

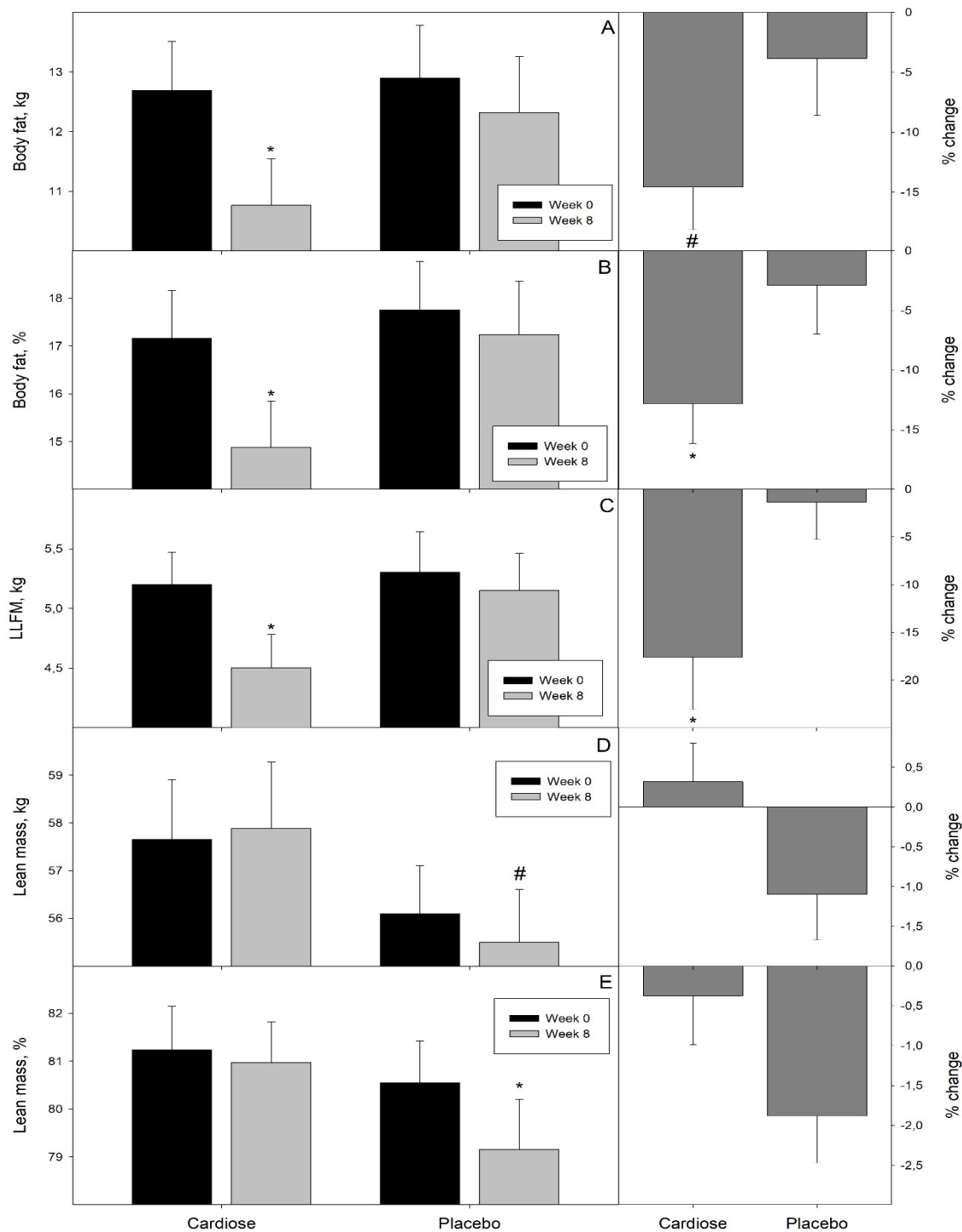


Figure 49. Changes in body composition (A – body fat mass, B – body fat mass %, C – lower limbs fat mass, D – lean mass and E – lean mass%) after the intervention, evaluated by DXA, of amateur competitive cyclists (Cardioise®, N = 20; Placebo, N = 20). * means significant difference ($p < 0.05$). # trend ($p = 0.05–0.10$).

11.3.2. Anthropometry

In anthropometry (Fig. 50), similar results to DXA were found. A significant pre-post decrease in the total body fat (TBF) (-3.1%; $p=0.047$; ES= 0.12), %BF (-3.9%; $p=0.006$; ES= 0.19) and Σ of 8 skinfolds (-6.5%; $p=0.008$; ES= 0.18) was observed in 2S-hesperidin, but no significant change in placebo ($p=0.995$; ES= 0.01, $p=0.775$; ES= 0.03 and $p=0.721$; ES= 0.03, respectively). There were no significant differences with a small effect in TBF, %BF and Σ of 8 skinfolds between groups ($p=0.221$, ES= 0.40, $p=0.129$, ES= 0.48 and $p=0.169$, ES= 0.43, respectively) in favour of 2S-hesperidin.

Regarding muscle mass (Fig. 2), a significant pre-post increase in total muscle mass (TMM) (1.8%; $p=0.011$; ES= 0.19) and percentage of muscle mass (%MM) (0.9%; $p<0.001$; ES= 0.26) was reported after ingestion of 2S-hesperidin, without any significant change in placebo ($p=0.296$; ES= 0.07 and $p=0.470$; ES= 0.07, respectively). Although there were no significant differences between groups, a trend with a moderate effect ($p=0.070$ and ES= 0.60) favouring an increase in %MM was observed in the 2S-hesperidin group.

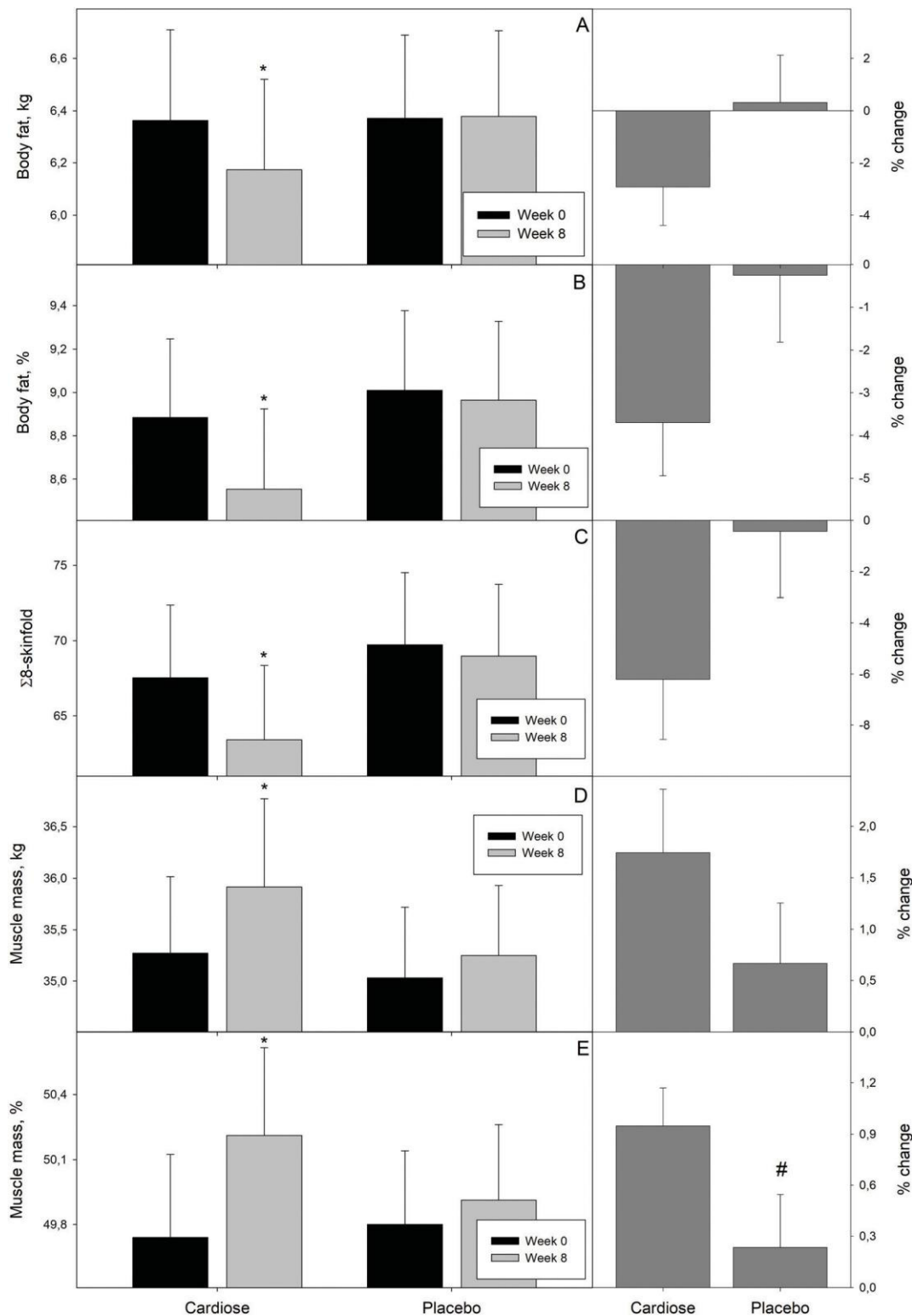


Figure 50. Changes in body composition (A – body fat mass, B – body fat mass %, C – 8 skinfold sum, D – muscle mass and E – muscle mass%) after the intervention, evaluated by anthropometry, of amateur competitive cyclists (Cardiose®, N = 20; Placebo, N = 20). * means significant difference ($p < 0.05$). # trend ($p = 0.05-0.10$).

11.3.3. Resting metabolic rate

Significant pre-post increase in CHO (Table 32) (32.4%; $p= 0.001$; $ES= 0.77$ vs 26.0%; $p= 0.004$; $ES= 0.71$, respectively) and decrease in FAT (-29.5%; $p= 0.005$; $ES= 0.68$ vs -28.0%; $p= 0.018$; $ES= 0.73$, respectively) were observed in 2S-hesperidin and placebo. Furthermore, a significant increase in RER (4.7%; $p= 0.012$; $ES= 0.72$) after supplementation was found only in placebo group. There were no significant differences between groups.

Table 32. Changes metabolic and substrate variables in resting metabolic rate (RMR) of male competitive cyclists (N=40).

	2S-Hesperidin			Placebo		
	Pre-intervention	Post-intervention	p-value	Pre-intervention	Post-intervention	p-value
VO₂ (mL·min ⁻¹)	0.27 (0.02)	0.27 (0.03)	0.912	0.27 (0.03)	0.27 (0.04)	0.813
CO₂ (L·min ⁻¹)	0.23 (0.02)	0.24 (0.03)	0.471	0.23 (0.03)	0.24 (0.04)	0.169
VO₂R (mL·kg ⁻¹ ·min ⁻¹)	3.79 (0.35)	3.78 (0.66)	0.904	3.88 (0.46)	3.82 (0.52)	0.659
RER	0.85 (0.07)	0.88 (0.05)	0.110	0.85 (0.05)	0.89 (0.04)	0.012
MET	1.08 (0.11)	1.08 (0.19)	0.966	1.11 (0.13)	1.09 (0.15)	0.600
CHO (gr·min ⁻¹)	8.48 (3.42)	11.23 (3.43)	0.001	9.12 (3.18)	11.49 (3.10)	0.004
FAT (gr·min ⁻¹)	3.71 (1.54)	2.62 (1.15)	0.005	3.64 (1.33)	2.62 (1.14)	0.018
Kcal/day	1655.20 (181.78)	1654.37 (208.25)	0.988	1697.31 (155.27)	1678.98 (227.60)	0.757

Values are presented as mean (SD). VO₂ = volume of oxygen uptake; VCO₂ = volume of dioxide of carbon uptake; VO₂R = body mass oxygen consumption; RER = resting metabolic rate; MET = metabolic equivalent; CHO = carbohydrate oxidation; and FAT = fat oxidation.

11.3.4. Hesperidin metabolites in urine

Different 2S-hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analysed in the urine of the participants after 2S-hesperidin intake. The main metabolite detected was hesperetin-3-glucuronide, representing $78.9 \pm 5.0\%$ ($n=20$) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate made up $6.9 \pm 2.9\%$ ($n=20$) and $14.7 \pm 4.1\%$ ($n=20$) of the excreted metabolites. Despite the similarities in the excreted metabolites profile, a large interindividual variability was observed in the amount of excreted hesperidin metabolites, ranging from 2.3 to 37.5 μmol . These differences between subjects indicate differences in the absorption and excretion of hesperidin, which have been previously reported 30.

11.4. DISCUSSION

This randomised, placebo-controlled trial investigated the effect of eight weeks of 2S-hesperidin supplementation on body composition (DXA and anthropometry) in forty AMA. Main results are that the chronic intake of 2S-hesperidin caused a significant decrease in BF and a significant increase in muscle mass compared to placebo. However, similar significant changes were observed in fats and carbohydrates oxidation during RMR in both groups.

The effects of 2S-hesperidin may be heavily influenced by its bioavailability. In plasma, a maximum concentration is reached 5 to 7 hours after ingestion, with a total elimination at 24 hours (467). In contrast, metabolites in urine reach their maximum peak at 24 hours after ingestion, being almost completely eliminated after 48 hours (467). Additionally, high doses of hesperidin (1 L of orange juice; 9.28 $\mu\text{mol h/L}$) led to higher area under the curve than low doses (0.5 L of orange juice; 4.19 $\mu\text{mol h/L}$), and therefore, greater exposure of the organism to its metabolites (467).

11.4.1. Body composition

Regarding DXA outcomes, this study showed improvements in body composition after 2S-hesperidin intake. Specifically, a significant decrease in the percentage of BF (-15.3%), total BF (-17.9%) and LLFM (-15.5%) were reported for the 2S-hesperidin group, while there were no changes in placebo. The power of the data obtained with the DXA has a high reliability, as it is considered a precise and accurate method for measuring body composition (788).

To the best of our knowledge, there are no previous studies that have reported the effect of hesperidin intake on fat mass. In line with described results, Dallas *et al.* (789) showed improvements in body fat mass percentage (experimental -9.7% vs placebo -3.2%; significant group differences $p < 0.001$) after a 12-week intake of

polyphenolic rich fruit extract (at least 20% of total flavanones) in combination with 30 min/week of physical activity in a healthy overweight population.

Similar results to those obtained in the DXA were found in anthropometric measurements, where a significant decrease in %BF (-3.9%), TFB (-3.1%) and Σ of 8 skinfolds (-6.5%) after ingestion of 2S-hesperidin was observed. In addition, in this group, inverse positive correlations were found between the total excretion of 2S-hesperidin metabolites in urine and fat mass percentage ($r = -0.592$; $p = 0.006$) and Σ of 8 skinfolds ($r = -0.550$; $p = 0.012$).

Both DXA and anthropometry fat component findings were similar to each other. The decrease in fat mass after the intake of 2S-hesperidin could be explained by an increase in the activation of SIRT1 and PGC-1 that would lead to over-expression of genes related to mitochondrial respiration and fatty acid oxidation at the muscular level (790). The decrease in fat mass in the experimental group may also be due to higher fat oxidation in FatMax and VT1 after intake of 2S-hesperidin in AMA, whereas the placebo group significantly decreased its fatty acid oxidation (738). These findings suggest 2S-hesperidin as a useful tool for reducing fat mass in cyclists. Taking into account described results as well as that no dietary control was included in this study, the potential of 2S-hesperidin combined with a proper dietary control may be even greater.

Legaz *et al.* (791) investigated the relationship between changes in body composition and sports performance, observing that changes in the Σ of 6 skinfolds ($r = -0.660$, $p < 0.001$) and the ratio of extremity to trunk skinfolds (triceps, front thigh, medial cal / subscapular, iliac crest, abdominal) ($r = -0.600$, $p = 0.020$), were related to changes in running performance in top-class runners after three years follow-up. This suggests that a decrease in fat mass is related to an enhancement in performance.

A recent publication by Martínez-Noguera *et al.* (738) corresponding to the performance evaluation of this intervention study, reported a positive effect on performance after the ingestion of 2S-hesperidin (8 weeks) in amateur cyclists. This group increased the power output (PO) (2.3% = 6.40 W; $p = 0.049$) at estimated functional threshold power (eFTP) and maximum power output (MPO) (1.9% = 7.40 W; $p = 0.049$) in an incremental test. Participants also showed a decrease in FAT oxidation in FatMax (-65.6%) and VT1 (-65.7%) group during the rectangular test in the placebo group but no significant changes in the 2S-hesperidin (738). These results indicate a decrease in the capacity of fatty acid oxidation in placebo, which leads to lower utilization of fats at low to moderate intensities. Since it is known that a high percentage of the training volume of cyclists is performed in low and moderate-intensity, a decrease in the capacity of fat oxidation could lead to changes in body fat mass long term (weeks or months). Therefore, the changes found by Martínez-Noguera

et al. (738) would justify the reduction of fat mass after 2S-hesperidin supplementation described in this study.

Concerning the muscle component, in DXA, the placebo group experienced a significant decrease in LM% (-1.4%; -0.597 kg), while no changes were found in 2S-hesperidin (0.3%; 0.224 kg). No-significant changes were found when comparing between groups, but a moderate effect in LM (- 1.5%; -0.822 kg) in favor of placebo was observed. In anthropometry, a significant increase in %MM (0.9%) and TMM (1.8%) was reported in the 2S-hesperidin group after the intervention period. A positive relationship was also found between urinary excretion of 2S-hesperidin metabolites and %MM ($r=0.487$; $p=0.029$), supporting the positive effect of 2S-hesperidin on muscle mass. Bieseman *et al.* found that hesperetin (main 2S-hesperidin metabolite) supplementation (50mg/kg/d), in addition to reducing oxidative stress in myotubes, completely reversed the age-related decline in muscle mass in mice and, thus, may have played a role in improving running performance (35). Regarding the relationship between hesperidin intake and muscle mass, Jeon *et al.* (792) observed pro-myogenic function of hesperidin with different regulatory mechanisms, which include promoting nuclear localisation of MyoD (transcription factor) and its interaction with target gene promoters, as well as enhancement of MyoD-mediated myogenic gene transcription and myogenic differentiation. These effects were similar *in vivo* and *in vitro*. MyoD plays a key role in modulation of myogenic precursors and induces myoblast differentiation. MyoD is a nuclear protein expressed exclusively in skeletal muscle cells and it acts as a transcriptional activator of muscle-specific gene expression during muscle differentiation (531). The interaction of MyoD and Myf5 plays an essential role in the success of myogenesis (793), this mechanism could be responsible for the anthropometric changes at the level of muscle mass described in this study. Also, it has been suggested that continuous expression of MyoD and myogenin proteins in rat muscles is an essential molecular event that induces hypertrophy of skeletal muscles (794).

Changes in body composition may be related to an important improvement in performance in amateur cyclists enrolled in this study, which has also been observed by Yoshiga *et al.* (795) in endurance athletes. This study found that fat-free mass (assessed using air displacement plethysmography) was positively correlated with absolute VO_{2MAX} in both male and female rowers and runners, suggesting that athletes with higher values of FFM presented a higher absolute aerobic capacity. Similar improvements were observed in muscle mass after an 8-week intake of 2S-hesperidin in the present study and were associated with an improvement in PO in the eFTP and MPO in an incremental test (738). It should be noted that the data provided by this study (body composition) and those by Martinez-Noguera et al. (738) were part of the same project, which was carried out at the same time, with the same sample.

Significant correlations were found between changes in MPO ($r=0.471$; $p=0.036$) and eFTP ($r=0.466$; $p=0.035$) and changes in MM% evaluated through anthropometry.

After observing an increase in MM after the ingestion of 2S-hesperidin in this study, it seems that this flavanone not only helps to conserve muscle tissue, but also increases it in AMA. Cyclists are particularly exposed to loss of muscle mass (muscle catabolism) due to large training volumes and intensities combined with caloric restriction. Described increases in %MM in the current study, which are correlated with improvement in performance, namely eFTP and MPO (738), suggest that the intake of 2S-hesperidin promotes a greater PO at high intensities of exercise due to an increase in the muscle mass. Therefore, 2S-hesperidin may be an ideal supplement for improving body composition and performance of cyclists.

Moreover, hesperidin has demonstrated a probiotic effect by promoting the growth of beneficial bacteria (*Bifidobacterium spp.*, *Lactobacillus spp.* o *Akkermansia muciniphila*) in the colon, due to an increase in the production of short-chain fatty acids (SCFA) (butyrate, propionate and acetate.) (470). Butyrate increases fat oxidation and brown adipose tissue activation (BAT), which is evidenced by increased utilization of fatty acids derived from plasma triglycerides, explained by an increased sympathetic outflow to BAT (796). In addition, butyrate has shown the ability to increase the expression of mRNA and proteins (brown fat, skeletal muscle, and liver) of PGC-1 α , supported by an increase in the activity of AMPK and p38 which leads to an increase in PGC-1 α phosphorylation (797). PGC-1 α controls energy metabolism by interaction with several transcription factors, e.g., estrogen-related receptor- α , nuclear respiratory factor-1 and -2, PPAR- α and - δ , and thyroid hormone receptor, that direct gene transcription for mitochondrial biogenesis and respiration (798). In the muscle, PGC-1 α increases oxidative (type I) fibre differentiation and enhances fatty acid metabolism, supported by an increase in proteins of type 1 myosin heavy chain and myoglobin (type I fibre marker) (797). The changes in the signaling pathways of the AMPK-PPAR α -PGC-1 α axis, together with those of the type I fibers, would improve the capacity of fat oxidation at a muscular level, which would facilitate a decrease in the fatty tissue.

11.4.2. Resting metabolic rate

The RMR outcomes reported in this study showed a significant increase in CHO oxidation (2S-hesperidin: 32.4% vs placebo: 26.0%) and a significant decrease in FAT oxidation (2S-hesperidin: -29.5% vs placebo: -28.0%) in both groups after the intervention. As with the body composition outcomes, no previous studies have evaluated the effect of hesperidin intake on substrates oxidation at rest. Regular orange juice intake (high in 2S-hesperidin) for 60 days has been shown to significantly reduce

blood serum of glucose and insulin in healthy women (484). Recent evidence has shown that the intake of 300 mL/d of orange juice during 60 days in healthy women decreased glucose (-6.5%) and insulin (-33.0%) in serum, also promoting an increase of intestinal bacteria of the species *Lactobacillus* spp., *Akkermansia* spp. and *Ruminococcus* spp., exerting a prebiotic effect (799). Also, hesperetin, a metabolite of hesperidin, has a stimulating effect on the hepatic intracellular signalling pathway on the axis sirtuin 1 (SIRT1)/AMPK, where SIRT1 mediates PPAR γ co-activator 1 α (PGC-1 α) activation, which enhances fatty acid oxidation, thereby improving glucose homeostasis and promoting insulin sensitisation (729).

No significant change in caloric output (kcal/day) was observed when intra- and inter-group comparisons were performed. Only a significant increase in RER in the post-intervention was found in the placebo group. However, it has been observed that the a 17-day intake of p-synephrine alone (50 mg; +65kcal) or in combination with hesperidin (100mg) and/or naringenin (600 mg; +122kcal) has a positive effect on calorie expenditure at rest, compared to placebo (183kcal; $p=0.039$) (800). However, intake of liquorice flavonoid oil (a single 600 mg dose) showed no change in RER at rest compared to placebo in healthy women (801).

The regulation of metabolic pathways described previously could explain the increased use of carbohydrates as a metabolic substrate, which may decrease serum glucose levels, as indicated by other human studies. These previously cited metabolic changes would explain the loss of body fat found by different measurement techniques (DXA and anthropometry) due to improved adaptations that occur at the level of fatty acid oxidation during exercise (low and moderate intensity) in cyclists with 2S-hesperidin supplementation of 8 weeks (738). Few studies have evaluated the effect of flavonoids, in our case of 2S-hesperidin, on the RMR of athletes or healthy people. Therefore, further research is needed to establish whether 2S-hesperidin may affect RER or RMR.

11.4.3. Limitations

The current study was conducted between September and December (in off-season). In this phase of the season, cyclists work at a lower training intensity and volume relative to other phases of the season. This is an important fact to be considered when comparing described results with other studies carried out during training seasons with different volumes and intensities. The selection of study dates was made based on the fact that it is usually more complicated to modify the habits of the cyclists and to control their evolution during the season, due to their own and individual periodization.

Another limitation is the sample size enrolled in this study. Despite the relatively low number of participants (n=40), they are representative of the AMA population.

Future lines of research should describe precisely the molecular mechanisms and changes in gene expression (nutrigenomic effect) involved in 2S-hesperidin action for the promotion of muscle tissue and energy metabolism. It would be also interesting to investigate how modulation of intestinal microbiome induced by 2S-hesperidin may be related to the described significant effects on body composition.

11.5. CONCLUSIONS

The chronic intake of 500 mg of 2S-hesperidin improves body composition, decreases fat mass and increases muscle mass in AMA without modifying training and diet. These changes in body composition may provide an advantage to endurance athletes in maintaining or improving their physical condition during pre-season and start the season with less fat and more muscle. Besides, 2S-hesperidin appears to prevent an increase in the RER in rest metabolic rate, post intervention. Therefore, 2S-hesperidin is postulated to be a new ergogenic aid oriented to control fat and muscle mass in endurance athletes.

CAPÍTULO XII.

ESTUDIO N° 7

CAPÍTULO XII. ESTUDIO Nº 7: LA SUPLEMENTACIÓN DE 8 SEMANAS DE 2S-HESPERIDINA MEJORA EL ESTADO ÁCIDO-BASE Y DISMINUYE EL LACTATO A INTENSIDADES BAJAS Y SUBMÁXIMAS, MEJORANDO LA RECUPERACIÓN DESPUÉS DE UN TEST INCREMENTAL EN CICLISTAS AMATEURS (BAJO REVISIÓN)

12.1. INTRODUCTION

There are several factors, mainly training, nutrition and ergogenic aids, that affect endurance performance (422). Endurance training produces a number of adaptations at the cellular and systemic level with the aim of reducing the breakdown of the whole-body homeostasis caused by exercise (802, 803). The type of training, training status, as well as the diet composition affect the exercise response and adaptations in a positive or negative way (804, 805). In addition, the intake of ergogenic aids has shown to modulate the adaptations generated via resistance training, like reactive oxygen species (ROS) signaling, acid-base balance, the redox state, training load, etc (804). These physiological mechanisms allow the body to adapt to training and improve the athlete's performance.

The major goal for endurance athletes is to increase the ability to maintain the highest speed or average power output for a given distance or time (speed/power output). This depends on the speed and efficiency with which chemical energy can be converted into mechanical energy within the skeletal muscle (806). Specifically, high-level endurance athletes want to obtain the highest rate of aerobic metabolism that is sustainable over the duration of an event, the upper limit of which is set by an athlete's maximum O₂ uptake (VO_{2MAX}) (807). However, despite having the ability to maintain high values of VO_{2MAX} for 10-15 minutes during all-out efforts, the circulatory system is unable to sustain a linear increase in the delivery of O₂ to the locomotor muscles, where the production of aerobic energy becomes restricted and, consequently, accelerates anaerobic metabolism (808).

VO_{2MAX} is limited by the ability of the cardiorespiratory system to transport O₂ to the muscles. The physiological factors that could limit VO_{2MAX} are: 1) the pulmonary diffusing capacity, 2) maximal cardiac output, 3) oxygen carrying capacity of the blood, and 4) skeletal muscle characteristics. The first three factors are classified as central factors and the fourth is categorized as a peripheral factor (809). The limitation at the lung level in highly trained athletes can be overcome with O₂-enriched air, as Powers et al. (378) observed when they compared incremental tests (VO_{2MAX}) in normal air and O₂-enriched air (26%) room conditions in highly-trained and normal subjects. They

observed that there was an increase in $\text{VO}_{2\text{MAX}}$ from 70.1 to 74.7 $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ and an increase in arterial O_2 saturation (SaO_2) from 90.6% to 95.9% during the maximum work in the O_2 -enriched air condition in highly-trained athletes, without significant changes in the normal subjects.

Another limiting factor regarding performance and $\text{VO}_{2\text{MAX}}$ of endurance athletes is the maximal cardiac output, since longitudinal studies have observed that training induces an increase in $\text{VO}_{2\text{MAX}}$ because of an increase in maximal cardiac output (810, 811). Magnetic resonance cross-sectional images showed an enhanced left ventricle (LV) mass (200 vs. 148 g) and volume (167 vs. 125 mL) in the endurance-trained group compared to matched non-athletic controls (812).

The oxygen-carrying capacity is another factor that can limit the endurance athletes' performance and $\text{VO}_{2\text{MAX}}$ (809). It's been observed that high concentration and above normal total mass of hemoglobin (Hb), an oxygen transport molecule that supports aerobic cellular metabolism, improves performance capacity in humans (813). Several authors have observed how reinfusion of 900-1.350 mL of blood increases oxygen transport capacity in the blood, demonstrating that this procedure can increase $\text{VO}_{2\text{MAX}}$ by 4-9% (814, 815). In line with previous research, an augmenting red blood cell volume by means of blood transfusions or erythropoietin injections improves exercise performance in healthy humans (816-818). In addition, several studies have found a strong relationship between the number of capillaries by fiber in the vastus lateralis and $\text{VO}_{2\text{MAX}}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) measured during cycle ergometry (819). The main significance of the training-induced increase in capillary density in skeletal muscle is to maintain or lengthen the average transit time (820). This improves the oxygen supply by maintaining the oxygen extraction (a-v O_2 difference), even at high muscle blood flow rates (809).

Furthermore, the position of the O_2 dissociation curve (ODC), conveniently described by P_{50} (PO_2 at which 50% Hb is saturated), has an important role in O_2 transport. Of course, variations in P_{50} have ramifications not only in the periphery but also during O_2 loading in the lungs. A high P_{50} opposes the association of O_2 in the lungs but favors its release to the tissues and vice versa (821).

Physiologists have done extensive work to examine whether enzyme levels in the mitochondria are a limiting factor for $\text{VO}_{2\text{MAX}}$ within the muscle fibers, where the mitochondria are the sites where O_2 is consumed in the last step of the electron transport chain (809). In theory, doubling the number of mitochondria should double the number of sites for O_2 uptake in the muscle. However, human studies show there's only a modest increase $\text{VO}_{2\text{MAX}}$, despite a 2.2 times increase in mitochondria enzymes (819). Increasing mitochondrial enzymes improves endurance performance rather than increase $\text{VO}_{2\text{MAX}}$, and low-intensity training may elicit small changes in mitochondrial enzymes without any change in $\text{VO}_{2\text{MAX}}$, and *vice versa* (822-824). In addition, the

increase in muscle mitochondria may allow a slightly greater extraction of O₂ from the blood by the working muscles, thus providing a small contribution in increasing VO_{2MAX} (825).

Additionally, skeletal muscle oxidative capacity, once thought to be indicated by percentage of type 1 (oxidative) muscle fibers (826), correlates strongly with exercise efficiency (78), which also accounts for a significant variation in exercise performance among highly trained athletes (827, 828). Two metabolic effects of an increase in mitochondrial enzymes are: 1) muscles adapted to endurance exercise will oxidize fat at a higher rate (thus sparing muscle glycogen and blood glucose) and 2) there is decreased lactate production during exercise (809). All these changes in the body are stimulus-dependent (exercise time and intensity, nutritional status, temperature, etc.), but the intake of ergogenic aids can also modify training adaptations (829).

In this sense, endurance athletes are increasing the use of ergogenic aids in the search of improving performance, which include: dietary nitrates (830), β-alanine (831), antioxidants (832), sodium bicarbonate (833), creatine (834) and polyphenols (27). Recently, there is a worldwide research interest in the pleiotropic effect of polyphenols on the immune system, chronic diseases and aging (27, 632, 835-837). The large family of polyphenols is divided into 4 groups: flavonoids (e.g., hesperidin, hesperetin, etc.), phenolic acids, stilbenes and lignans (632).

Specifically, hesperidin is a flavonoid of the flavanone family and is largely located in high concentrations in citrus fruits (655), such as sweet orange (*Citrus sinensis*) (460). S and R isomers are both found in hesperidin. 2S-hesperidin is predominantly the natural form in citrus (656), whereas processed foods with high hesperidin content undergo a transformation from S to R isomer (657). A 6-week consumption of the metabolite form of hesperidin, hesperetin (50 mg·kg⁻¹·d⁻¹) has shown to improve antioxidant status (GSH, GSSG and GSH/GSSG) and running performance (exercise time) in aged mice (35). Similar results were found with the 5-week intake of 2S-hesperidin (200 mg/kg), where improvements in the test performance (running) until exhaustion (58%) and in the antioxidant system (superoxide dismutase (SOD), glutathione peroxidase (GPx)) in the liver and lymphoid tissue were observed in rats (33). In addition, Martínez-Noguera et al. (2019) showed improvements in average power (2.27%), maximum speed (3.23%) and total energy (Σ 4 sprint test) (2.64%) during a repeated sprint test (4 sprints of 30 sec) following an acute intake of 2S-hesperidin (500 mg) in amateur cyclists (549). Recently, the same authors demonstrated significant performance improvements in estimated functional threshold power (eFTP) (2.33% = 6.40 W) and maximum power (1.93% = 7.40 W) during an incremental test after 8-weeks of 2S-hesperidin (500 mg/d) consumption in amateur cyclists (738). They also observed an increase in power at maximum speed (1.08% = 8.05 W) and a decrease in time at peak power (-11.17% = -376.5 ms) in the

Wingate test (one sprint of 30 sec) (738).

No previous articles that have studied the effect of 2S-hesperidin intake on oxygen metabolism and acid-base state, and taking into account the results obtained on the performance after intake with 500 mg of 2S-hesperidin during 8 weeks in amateur cyclists (738), carried out in our laboratory and of which the data presented in this study are part. We hypothesized that the enhancement of nitric oxide (NO) production following prolonged ingestion of 2S-hesperidin may improve peripheral blood flow to muscles, as has been demonstrated in several studies investigating the use of hesperidin and other flavonoids (531, 838-841). This would provide a better supply of oxygen and nutrients at high intensities, which can be decisive in maintaining certain levels of work.

The main objective of this intervention study was to perform an exhaustive report of the effects with 500 mg/d of 2S-hesperidin in amateur cyclists on markers related to O₂ metabolism and acid-base balance, from capillary blood sample during a rectangular test. Secondly, was to be able to give an explanation to the findings found in our recently published article (738), where was found an improvement in performance, in submaximal and maximal level exercises after 2S-hesperidin intake in amateur cyclists for 8 weeks.

12.2 METHODOLOGY

12.2.1. Participants

Forty healthy male amateur cyclists completed the study (Table 33). The inclusion criteria were: 18-55 years, 19-25.5 kg·m⁻² BMI, at least 3-years cycling experience and 6-12 h·wk⁻¹ of training. Exclusion criteria were: a) smokers or regular alcohol drinkers, b) metabolic, cardiorespiratory or digestive pathologies or anomalies, c) injury in the last 6 months, d) intake any type of supplementation or drug in the last 2 weeks and) no normal values in some parameter of the previous blood analysis. Prior to starting the study, participants were informed about the procedures and signed the informed consent. The study was conducted according to the guidelines of the Helsinki Declaration for Human Research (842) and was approved by the Ethics Committee of the Catholic University of Murcia (CE091802), registered in ClinicalTrials.gov (Identifier: NCT04597983). All participants completed the study.

Table 33. Baseline general characteristics and training variables of the cyclists.

	2S-Hesperidin	Placebo	p-value
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Body mass (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI (kg·m⁻²)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
VO_{2MAX} (L·min⁻¹)	3.99 (0.36)	3.98 (0.63)	0.971
VO_{2MAX} (mL·kg⁻¹·min⁻¹)	57.5 (6.97)	57.9 (9.53)	0.880
HR_{MAX} (bpm)	184.9 (11.11)	183.2 (8.68)	0.593
VT1 (%)	50.9 (5.63)	50.0 (4.78)	0.610
VT2 (%)	84.9 (5.85)	84.1 (5.70)	0.644
<i>Training variables</i>			
Total distance (km)	1121.12 (534.99)	1082.43 (810.46)	0.868
HR_{AVG} (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
W_{AVG} (W)	174.86 (15.79)	163.47 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are expressed as mean (SD). BMI = body mass index; BF = body fat; VO_{2MAX} = maximum oxygen volume; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); Total distance = of all the training sessions carried out during the study period; HR_{avg} = average heart rate of all the training sessions carried out during the study period; W_{avg} = average power output of all training sessions during the study period and RPE = rating of perceived exertion of all training sessions during the study

12.2.2. Study design

This research was part of a larger, previously published study investigating the effect of chronic hesperidin intake on the performance (738) and in body composition (843) in amateur cyclists. To carry out this study, a double-blind (blinding carried out by the company supplying the capsules), parallel and randomized experimental design was conducted. Randomization was performed using computer software (Randomizer)

to assign codes to the groups established in this study (706). Participants were divided in two groups: experimental (2S-hesperidin; n=20) and control (Placebo; n=20) groups. In accordance to their group assignment, participants either took two capsules of 2S-hesperidin (500 mg of 2S-hesperidin; HealthTech BioActives, Murcia, Spain) or placebo (500 mg of microcellulose) for 8 weeks. The cyclists continued their normal training schedule during the length of the study. Both groups had similar general and training characteristics at the start of the study (Table 33).

12.2.3. Procedures

The experimental protocol required 7 visits to the laboratory. Day 1 consisted of a medical examination and health status blood analysis. Days 2 and 4 entailed a reminder 24-h diet recall questionnaire and incremental test until exhaustion on a cycle ergometer. Days 3 and 5 involved of a 24-h diet recall questionnaire and rectangular test on a cycle ergometer (Figure 51 and Table 34). Participants ingested a standardized breakfast, composed of 95.16 gr carbohydrates (68%), 18.86 gr protein (14%) and 11.30 gr lipids (18%), which was prescribed by a sports nutritionist, and was consumed 2.5 hr before each testing session (visits 2, 3, 4 and 5).

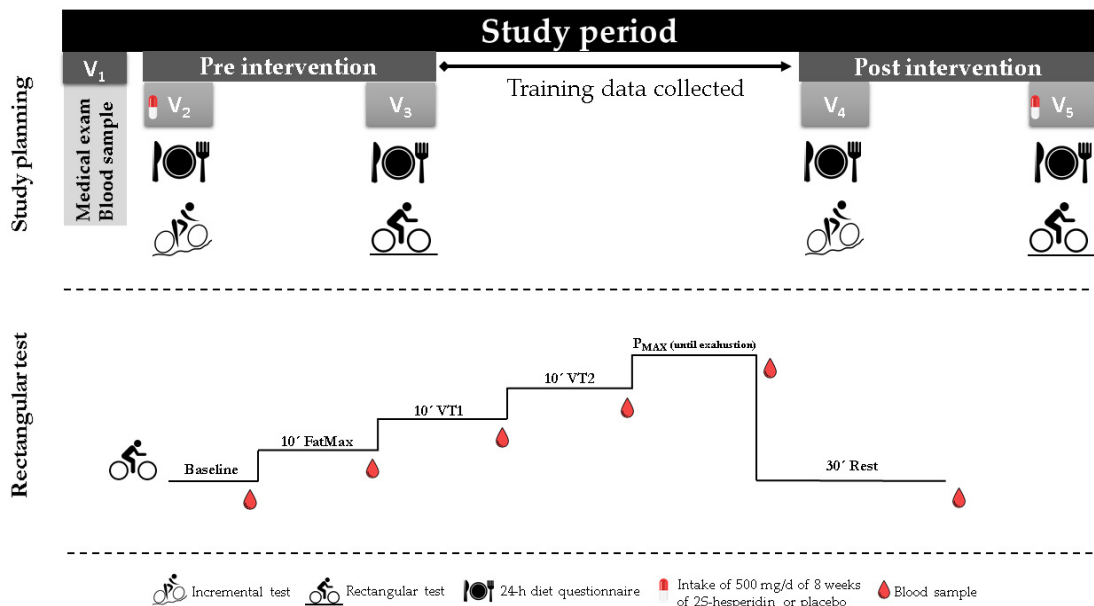


Figure 51. Study planning.

Table 34. Between-group comparisons in dietary intake of cyclists.

	Pre-intervention			Post-intervention		
	2S-Hesperidin	Placebo	p-value	2S-Hesperidin	Placebo	p-value
Kcal	2163.6 (519.02)	2100.2 (515.77)	0.708	1974.1 (377.97)	2133.5 (437.98)	0.237
Kcal/B M	31.1 (9.34)	30.2 (8.71)	0.768	27.9 (6.53)	30.3 (6.46)	0.249
CHO (g)	245.7 (73.46)	222.0 (69.68)	0.312	216.6 (63.47)	248.3 (58.15)	0.117
CHO/B M	3.5 (1.31)	3.2 (1.14)	0.416	3.1 (1.08)	3.5 (0.94)	0.173
PRO (g)	113.5 (25.21)	115.2 (25.37)	0.837	109.0 (23.05)	101.5 (23.67)	0.332
PRO/B M	1.6 (0.41)	1.7 (0.48)	0.778	1.5 (0.35)	1.5 (0.42)	0.596
LP (g)	80.8 (27.24)	83.5 (23.65)	0.739	71.5 (17.61)	71.6 (18.89)	0.985
LP/BM	1.2 (0.45)	1.2 (0.37)	0.758	1.0 (0.27)	1.0 (0.29)	0.823

Values are expressed as mean (SD). Kcal = kilocalories; CHO = carbohydrates; PRO = protein; LP = lipids; BM = body mass. The mean values correspond to the average of all 24-hour diet recall data collected at pre-intervention (visits 2, 3 and 4) and post-intervention (visits 5, 6 and 7). * indicates significant differences ($p \leq 0.05$).

12.2.4. Testing

12.2.4.1. Medical exam

The medical exam included a medical history, resting electrocardiogram and medical examination (auscultation, blood pressure, etc.), to confirm that the cyclist was healthy to participate in the study.

12.2.4.2. Maximal test

An incremental step with final ramp test with a metabolic cart (Metalyzer 3B, Leipzig, Germany) was performed to determine maximal zone of fat-burning (FatMax), ventilatory thresholds₁ (VT₁) and 2(VT₂), maximum power output (P_{MAX}) and VO_{2MAX} . The test started at 35W and was increased by 35W every 2 min, followed by a final ramp (+35W·min⁻¹), which started when the RER was higher than 1.05, and continued until exhaustion. To guarantee VO_{2MAX} value, the following criteria were checked: plateau in the final VO_2 values (increase ≤ 2.0 mL·kg⁻¹·min⁻¹ in the 2 last loads), maximal

theoretical HR $(220 - \text{age}) \cdot 0.95$) for a cycling test (550), $\text{RER} \geq 1.15$ and lactate $\geq 8.0 \text{ mmol} \cdot \text{l}^{-1}$ (551, 552). Ventilatory thresholds were obtained using the ventilatory equivalents method described by Wasserman (553).

12.2.4.3. Rectangular test

Prior to each exercise test, a resting oxygen measure was obtained by having participants in the supine position for 20 min while breathing room air into a metabolic analysis system, and only the middle 10 minutes (steady state) were used for the calculation of oxygen consumption at rest (844). The rectangular test was performed on a cycle ergometer using the power output values obtained from the maximal test (FatMax, VT1, VT2 and P_{MAX}). The test consisted of 10-min at FatMax1 (intensity at which maximum fat oxidation is given), 10-min at VT1 and 10-min at VT2, until exhaustion at P_{MAX} and 15-min at FatMax2. As soon as the exercise was over, the subjects immediately laid down on a bed so that excess post-exercise oxygen consumption (EPOC) could be measured for 30 min. Cardiorespiratory variables (VO_2 , VO_2R , carbohydrate oxidation (CHO), fat oxidation (FAT) and cycling economy) were determined for the different metabolic zones. These metabolic data have been published separately (738).

12.2.4.4. Blood samples

Blood extraction was conducted by a certified nurse, where one 3-mL tube ethylenediaminetetraacetic acid (EDTA) for hemogram and another 3.5-mL tube with polyethylene terephthalate (PET) for health analysis were obtained. Red blood cell count was carried out in an automated Cell-Dyn 3700 analyzer (Abbott Diagnostics, Chicago, IL, USA), using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, hematocrit and hematimetric indexes were estimated.

12.2.4.5. ABL-90 (blood gas analyzer)

The blood parameters of haematocrit, haemoglobin, oxyhaemoglobin, pH, lactate (amperometry electrode using the enzyme, lactate oxidase) were determined arterialized capillary blood from the fingertip at rest (pre), in 30 last seconds of FatMax1, in 30 last seconds of VT1, in 30 last seconds of VT2, Post P_{MAX} , in 30 last seconds of FatMax2 and at the end of EPOC (at rest), were measured by ABL 90 FLEX blood gas analyzer (Radiometer Medical ApS, Copenhagen, Denmark). The blood-gas analyzer was calibrated at hourly intervals throughout the day, with internal reference standards. A previous study indicated that ABL90 FLEX had good accuracy (845). The plastic capillary tubes intended for blood samples were collected were preheparinized with electrolytically balanced solid heparin. This significantly reduces the risk of clots

and helps to ensure reliable results without electrolyte bias. There are arterial and capillary (finger) blood gas analyzers that can measure biochemical, electrolyte, liver and kidney function, metabolic and hematological markers, which can detect physiological or pathological changes with a small blood sample, in case of finger sample (846).

12.2.5. Statistical analyses

Data analysis was conducted using IBM Social Sciences software (SPSS, version 21.0, Chicago, IL, USA). Descriptive statistics are presented as mean and standard deviation (SD). Levene's and Shapiro-Wilk tests were applied to check the homogeneity and normality of the data, respectively. A group \times time \times moment ANOVA was conducted to analyze within-group and between-group differences in all dependent variables and for every time-point of measurement (baseline (pre), FatMax1, VT1, VT2, P_{MAX}, FatMax2 y EPOC) and in both moments (pre-test and post-test). In addition, the area under the curve (AUC), resulting from the integration of the three time-points of measurement taken during the rectangular test, was calculated for each variable. The AUC was used to analyze pre-post differences both within groups and between groups. The within-group differences in the AUC were analyzed by repeated-measures t-test, and between-group comparisons in the AUC were conducted by applying an independent samples T-test. Cohen's d effect size (ES) (95% confidence interval) was calculated for all comparisons. Threshold values for ES statistics were as follows: > 0.2 small, > 0.5 moderate, > 0.8 large (710). Significant differences were considered when $p \leq 0.05$.

12.3. RESULTS

All subjects who participated in this study performed all the tests proposed in the study and successfully completed the research. Recruitment was carried out 2 months before the start of the study (end of September 2018).

12.3.1. Rectangular test

12.3.1.1. Oxygen and carbon dioxide metabolism (capillary blood gases)

Table 35 (Figure 52 and 53) shows the intragroup changes in biomarkers of oxygen metabolism at baseline (pre), FatMax1, VT1, VT2, P_{MAX}, FatMax2 and EPOC from the rectangular test. In the 2S-hesperidin group, there was a significant decrease at pre in O₂Hb (-1.67%; $p=0.028$; ES=2.04), sO₂ (-1.43%; $p=0.048$; ES=1.83) and an increase in RHb (26.38%; $p=0.048$; ES=1.81), after the supplementation period. On the other hand, placebo group showed a significant increase at pre in pCO₂ (4.52%;

$p=0.029$; $ES=1.81$) after the intervention. In addition, significant differences in pO_2 at Δ Baseline ($p=0.03$; ES ; 0.94) were also observed between groups (Table 37).

When assessing intragroup changes at FatMax1 during the rectangular test (Table 35) (Figure 52 and 53), there were a significant increase in pCO_2 (3.07%; $p=0.035$; $ES=1.75$) and tCO_2 (9.92%; $p=0.037$; $ES=4.83$), after the 2S-hesperidin intervention. However, the placebo group showed a significant decline in Hct (-3.99%; $p=0.043$; $ES=1.72$) and Hb (-4.01%; $p=0.042$; $ES=1.97$) at FatMax1. However, significant differences in pO_2 ($p=0.01$; ES ; 0.33) and pCO_2 ($p=0.05$; ES ; 0.50) in Δ FatMax1 were also observed between groups. (Table 37).

In a subsequent stage of the rectangular test, specifically at VT1 (Table 35) (Figure 52 and 53), the 2S-hesperidin group experienced a significant increment in pCO_2 (3.62%; $p=0.020$; $ES=1.80$) and tCO_2 (5.56%; $p<0.001$; $ES=3.21$) and a significant decrease in AaDpO₂ (-18.81%; $p=0.004$; $ES=2.87$) and a downward trend with a large effect size in Shunt (-26.58%; $p=0.057$; $ES=2.20$), after the supplementation period. When comparing intragroup pre-post-test at VT1 in placebo, we found no significant change. In addition, there was a significant difference in Hb ($p=0.04$; ES ; 1.24) at Δ VT1 and a trend with large effect size in AaDpO₂ ($p=0.09$; ES ; 1.42) were observed between groups (Table 37).

At VT2 (Table 35) (Figure 52 and 53), the placebo group showed a significant increase in COHb (32.50%; $p=0.022$; $ES=1.94$) and a significant decline in pO_2 (-7.94%; $p=0.042$; $ES=1.76$), after 8 weeks of intervention. In the 2S-hesperidin group, there was only a significant increase in COHb (21.56%; $p=0.020$; $ES=2.01$) at VT2 (Table 3). In the Δ VT2 analysis, only a trend with moderate effect size in O₂Hb ($p=0.07$; ES ; 0.63) was found when comparing the groups (Table 37).

At the maximum exercise phase of the rectangular test (P_{MAX}) the placebo group (Table 35) (Figure 53) demonstrated a significant decrease in $p50$ (-11.14%; $p=0.032$; $ES=2.25$) after the experimental period. In 2S-hesperidin group, no significant change in P_{MAX} was observed post-intervention (Table 35).

After the maximum phase of the test, the cyclists performed another exercise phase in FatMax (FatMax2) (Table 35) (Figure 52 and 53). The 2S-hesperidin group showed a significant increase in COHb (30.10%; $p=0.012$; $ES=2.63$), RHb (24.00%; $p=0.015$; $ES=3.16$), pCO_2 (6.78%; $p=0.005$; $ES=2.34$) and tCO_2 (10.58%; $p=0.003$; 2.20) and a significant decrease in O₂Hb (-1.03%; $p=0.006$; $ES=3.47$), sO_2 (-0.93%; $p=0.015$; 3.18) and a downward trend in pO_2 (-9.71%; $p=0.076$; $ES=2.24$) post-intervention. In placebo, no significant pre-post changes were observed at FaxMax2 (Table 35). There was a significant difference in Shunt at Δ FatMax2 ($p=0.05$; ES ; 1.69) between groups. (Table 37).

At resting EPOC (Table 35) (Figure 53), 2S-hesperidin had a significant increase in pCO₂ (4.03%; p=0.033; ES=2.51) and tCO₂ (6.94%; p=0.001; ES=2.61) following intervention. The placebo showed a significant increase in COHb (3.32%; p=0.049; ES=1.35) at EPOC post-intervention (Table 35). Moreover, significant differences in AaDpO₂ at ΔFatMax2 (p=0.05; ES; 1.06) were also observed between groups. (Table 37).

Finally, when comparing the intra-group areas under the curve (AUCs) (Table 35), in 2S-hesperidin, there was a significant increase in RHb (9.91%; p=0.023; ES=0.61), pCO₂ (3.73%; p=0.012; ES=0.54) and tCO₂ (6.17%; p=0.012; ES=0.73) with an upward trend in COHb (17.16%; p=0.077; ES=0.49) post-intervention. In the placebo group, there was a significant decrease in p50 AUC (-3.63%; p=0.040; ES=0.61). Moreover, we found an upward trend in COHb AUC (11.23%; p=0.088; ES=1.63) and pCO₂ AUC (3.78%; p=0.086; ES=0.50) post-intervention. When comparing the AUC between groups, there was no significant difference between groups.

ES	1.87	0.94	1.17	2.01	1.75	2.63	1.13	0.49	1.22	0.91	0.98	1.94	1.08	0.31	0.63	1.63
Pre	5.08 (0.69)	5.22 (0.43)	5.97 (0.59)	7.39 (0.77)	5.52 (0.54)	3.62 (0.26)	6.33 (0.59)	17.96 (2.71)	5.20 (0.78)	4.90 (0.49)	6.33 (0.67)	5.60 (0.88)	6.09 (0.61)	3.97 (0.29)	6.40 (0.67)	17.09 (4.24)
Post	6.42 (0.53)	5.08 (0.44)	5.74 (0.41)	7.15 (0.51)	5.85 (0.54)	4.49 (0.30)	7.04 (0.74)	19.74 (3.30)	5.71 (0.61)	4.60 (0.50)	5.29 (0.47)	6.53 (0.58)	5.41 (0.61)	3.77 (0.35)	7.17 (0.85)	18.15 (3.70)
P- value (RHb)	0.048	0.821	0.729	0.759	0.559	0.015	0.313	0.023	0.489	0.667	0.180	0.316	0.307	0.598	0.335	0.264
ES	1.81	0.30	0.37	0.30	0.59	3.16	1.13	0.61	0.59	0.56	1.41	0.97	1.01	0.62	1.05	0.23
Pre	0.677 (0.03)	0.723 (0.03)	0.608 (0.05)	0.600 (0.17)	0.700 (0.03)	0.700 (0.04)	0.700 (0.03)	4.05 (0.81)	0.750 (0.04)	0.760 (0.04)	0.750 (0.06)	0.540 (0.20)	0.810 (0.03)	0.780 (0.04)	0.760 (0.03)	4.66 (0.46)
Post	0.685 (0.03)	0.631 (0.05)	0.685 (0.03)	0.715 (0.04)	0.708 (0.03)	0.700 (0.03)	0.692 (0.03)	4.13 (0.39)	0.750 (0.03)	0.740 (0.05)	0.730 (0.03)	0.770 (0.04)	0.770 (0.04)	0.700 (0.03)	0.770 (0.03)	4.47 (0.60)
P- value (MetHb)	0.832	0.120	0.149	0.549	0.786	1.000	0.812	0.714	1.000	0.761	0.736	0.299	0.223	0.101	0.786	0.235
ES	0.23	2.58	1.41	0.63	0.24	0.00	0.28	0.09	0.00	0.48	0.31	1.08	1.06	1.68	0.31	0.38
Pre	94.84 (0.69)	94.69 (0.44)	93.94 (0.60)	92.51 (0.78)	94.38 (0.54)	96.36 (0.27)	93.57 (0.59)	566.10 (6.59)	94.73 (0.79)	95.03 (0.50)	93.59 (0.68)	94.32 (0.89)	93.83 (0.62)	95.99 (0.30)	93.50 (0.68)	566.91 (5.67)
Post	93.48 (0.54)	94.84 (0.44)	94.16 (0.42)	92.75 (0.51)	94.05 (0.55)	95.46 (0.31)	92.87 (0.75)	564.48 (6.36)	94.20 (0.61)	95.33 (0.51)	94.64 (0.47)	93.38 (0.58)	94.51 (0.62)	96.20 (0.36)	92.72 (0.86)	567.55 (6.18)
P- value (sO ₂)	0.048	0.813	0.741	0.762	0.573	0.015	0.321	0.473	0.480	0.671	0.181	0.315	0.314	0.594	0.332	0.549
ES	1.83	0.31	0.35	0.30	0.57	3.18	1.10	0.23	0.61	0.55	1.41	0.97	1.00	0.63	1.05	0.10
Pre	74.33 (4.27)	74.62 (2.21)	78.54 (4.97)	70.95 (3.29)	90.40 (4.00)	91.62 (3.67)	70.82 (2.49)	477.01 (43.84)	74.56 (4.27)	76.09 (2.21)	69.99 (4.97)	79.11 (3.29)	87.80 (4.00)	89.28 (3.67)	71.32 (2.49)	475.62 (32.00)
Post	72.05 (1.73)	74.13 (1.46)	76.63 (2.77)	69.64 (1.37)	82.84 (2.05)	82.72 (2.40)	69.55 (2.14)	454.45 (23.35)	68.57 (1.73)	76.46 (1.46)	73.48 (2.77)	72.83 (1.37)	83.82 (2.05)	87.67 (2.40)	69.32 (2.14)	465.93 (21.43)
P- value (pO ₂)	0.572	0.852	0.754	0.653	0.109	0.076	0.578	0.136	0.145	0.888	0.568	0.042	0.387	0.739	0.385	0.130

ES	0.49	0.21	0.35	0.37	1.74	2.24	0.47	0.48	1.30	0.16	0.65	1.76	0.92	0.40	0.74	0.28
Pre	42.14 (0.79)	42.01 (0.69)	41.18 (0.77)	38.59 (1.60)	35.42 (1.17)	35.38 (0.96)	39.72 (0.59)	233.55 (15.20)	39.35 (0.90)	40.42 (0.79)	40.34 (0.88)	35.19 (1.83)	34.22 (1.34)	34.12 (1.10)	38.04 (0.68)	221.69 (15.13)
Post	42.99 (0.76)	43.30 (0.73)	42.67 (0.51)	39.95 (0.73)	36.38 (1.06)	37.78 (0.81)	41.32 (0.64)	242.25 (11.97)	41.13 (0.86)	41.41 (0.83)	40.62 (0.58)	38.13 (0.83)	34.99 (1.21)	34.76 (0.92)	39.45 (0.73)	230.07 (13.63)
P value	0.213	0.035	0.020	0.421	0.433	0.005	0.033	0.012	0.029	0.145	0.681	0.133	0.581	0.468	0.091	0.086
ES	1.02	1.75	1.80	0.79	0.77	2.34	2.51	0.54	1.81	1.15	0.29	1.47	0.53	0.53	1.91	0.50
Pre	9.14 (0.17)	8.98 (0.15)	9.14 (0.16)	9.07 (0.18)	9.37 (0.16)	9.47 (0.15)	8.64 (0.16)	54.83 (2.63)	9.22 (0.18)	9.24 (0.16)	8.95 (0.18)	9.04 (0.20)	9.50 (0.18)	9.31 (0.16)	8.70 (0.18)	55.00 (2.47)
Post	9.11 (0.13)	8.98 (0.15)	8.92 (0.14)	9.14 (0.11)	9.49 (0.17)	9.28 (0.15)	8.64 (0.19)	54.81 (1.92)	9.01 (0.14)	8.89 (0.17)	8.99 (0.15)	9.13 (0.12)	9.45 (0.19)	9.34 (0.17)	8.53 (0.21)	54.57 (3.30)
P value	0.847	1.000	0.128	0.627	0.506	0.156	1.000	0.973	0.275	0.026	0.799	0.594	0.807	0.835	0.423	0.385
ES	0.18	0.00	1.27	0.38	0.72	1.20	0.00	0.01	1.05	1.95	0.20	0.41	0.26	0.17	0.88	0.16
Pre	28.39 (0.73)	26.91 (0.52)	26.28 (0.42)	21.51 (1.11)	15.06 (0.65)	20.51 (0.92)	25.52 (0.52)	137.88 (10.80)	26.98 (0.80)	26.17 (0.56)	26.74 (0.46)	19.52 (1.21)	14.07 (0.71)	19.68 (1.00)	24.68 (0.56)	131.85 (12.65)
Post	28.16 (0.74)	29.58 (1.19)	27.74 (0.38)	22.87 (0.56)	15.82 (0.66)	22.68 (0.78)	26.95 (0.43)	146.39 (9.25)	27.19 (0.36)	26.42 (1.29)	26.55 (0.41)	21.22 (0.61)	15.04 (0.71)	20.44 (0.85)	25.50 (0.47)	135.91 (10.61)
P value	0.754	0.037	<0.001	0.206	0.203	0.003	0.001	0.012	0.794	0.853	0.630	0.149	0.141	0.303	0.049	0.082
ES	0.29	4.83	3.21	1.15	1.10	2.20	2.61	0.73	0.24	0.40	0.36	1.30	1.26	0.69	1.35	0.29
Pre	26.54 (1.30)	26.29 (0.56)	29.46 (1.97)	29.37 (1.28)	34.07 (1.41)	29.60 (1.91)	26.72 (0.76)	171.66 (18.34)	25.82 (1.42)	25.90 (0.61)	25.87 (2.16)	30.99 (1.40)	34.28 (1.55)	28.99 (2.09)	26.84 (0.84)	172.38 (9.32)
Post	27.04 (0.54)	26.50 (0.61)	28.09 (0.93)	28.25 (0.54)	31.57 (0.68)	27.95 (0.76)	26.95 (0.84)	167.72 (7.17)	25.08 (0.59)	26.13 (0.67)	26.42 (1.02)	28.87 (0.59)	30.46 (0.75)	28.06 (0.83)	27.25 (0.92)	166.13 (9.11)
P value	0.753	0.791	0.473	0.395	0.114	0.429	0.843	0.483	0.666	0.788	0.790	0.150	0.032	0.683	0.744	0.040

(p50)	ES	0.35	0.35	0.65	0.82	1.64	0.80	0.27	0.20	0.48	0.35	0.23	1.39	2.25	0.40	0.44	0.61
Relative physiological Shunt	Pre	11.72 (2.64)	11.68 (1.58)	15.54 (1.72)	17.64 (2.67)	9.42 (2.21)	6.84 (0.97)	14.23 (1.66)	73.17 (19.54)	12.58 (2.64)	11.78 (1.58)	15.70 (1.72)	11.20 (2.67)	11.96 (2.21)	7.75 (0.97)	15.97 (1.66)	72.72 (19.06)
	Post	15.94 (1.38)	11.26 (1.52)	11.41 (1.35)	18.27 (1.63)	11.77 (0.95)	8.62 (0.92)	16.01 (2.02)	75.27 (12.14)	14.62 (1.38)	9.97 (1.52)	12.62 (1.35)	15.92 (1.63)	11.86 (0.95)	7.37 (0.92)	17.01 (2.02)	73.56 (14.36)
	P- value	0.135	0.856	0.057	0.818	0.349	0.180	0.284	0.747	0.459	0.437	0.146	0.097	0.968	0.769	0.527	0.785
	(Shunt)	ES	1.46	0.24	2.20	0.22	0.97	1.67	0.98	0.10	0.71	1.05	1.64	1.61	0.04	0.36	0.57
Alveolar-arterial gradient	Pre	32.84 (2.70)	29.07 (1.75)	32.81 (1.87)	33.99 (4.67)	22.27 (2.49)	23.10 (1.74)	34.63 (1.99)	168.93 (19.52)	31.48 (2.38)	27.38 (1.54)	33.50 (1.65)	33.17 (4.12)	25.31 (2.20)	20.62 (1.53)	34.66 (1.76)	170.28 (26.25)
	Post	28.87 (1.87)	26.63 (1.80)	26.64 (1.88)	32.76 (1.93)	23.39 (2.07)	21.76 (2.45)	33.96 (2.50)	161.62 (22.46)	33.86 (1.65)	25.36 (1.59)	29.74 (1.66)	33.46 (1.70)	25.61 (1.83)	23.96 (2.16)	36.30 (2.20)	167.42 (10.78)
	P- value	0.256	0.343	0.004	0.794	0.756	0.619	0.778	0.316	0.435	0.373	0.035	0.945	0.924	0.175	0.438	0.671
	ES	1.28	1.22	2.87	0.23	0.39	0.67	0.29	0.35	0.90	1.19	2.06	0.06	0.12	1.96	0.85	0.10

AaDpO₂ = difference between the alveolar concentration (A) of oxygen and the arterial (a) concentration of oxygen. In bold are p-values = ≤0.05 and trends between 0.05-0.08.

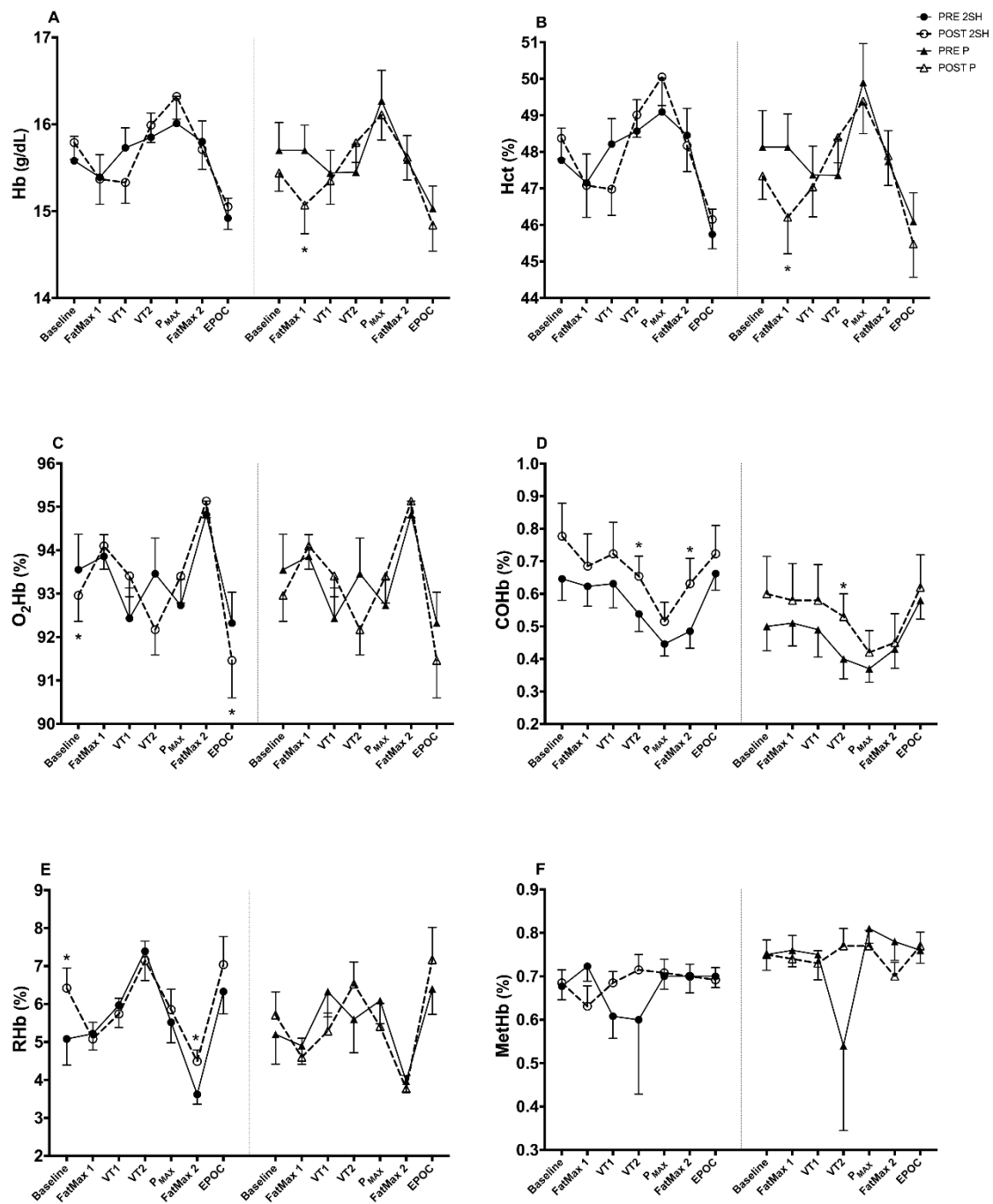


Figure 52. Differences between pre- and post-intervention within-group in finger capillary blood gas parameters at different points of the rectangular test (A-F). * $p < 0.05$.

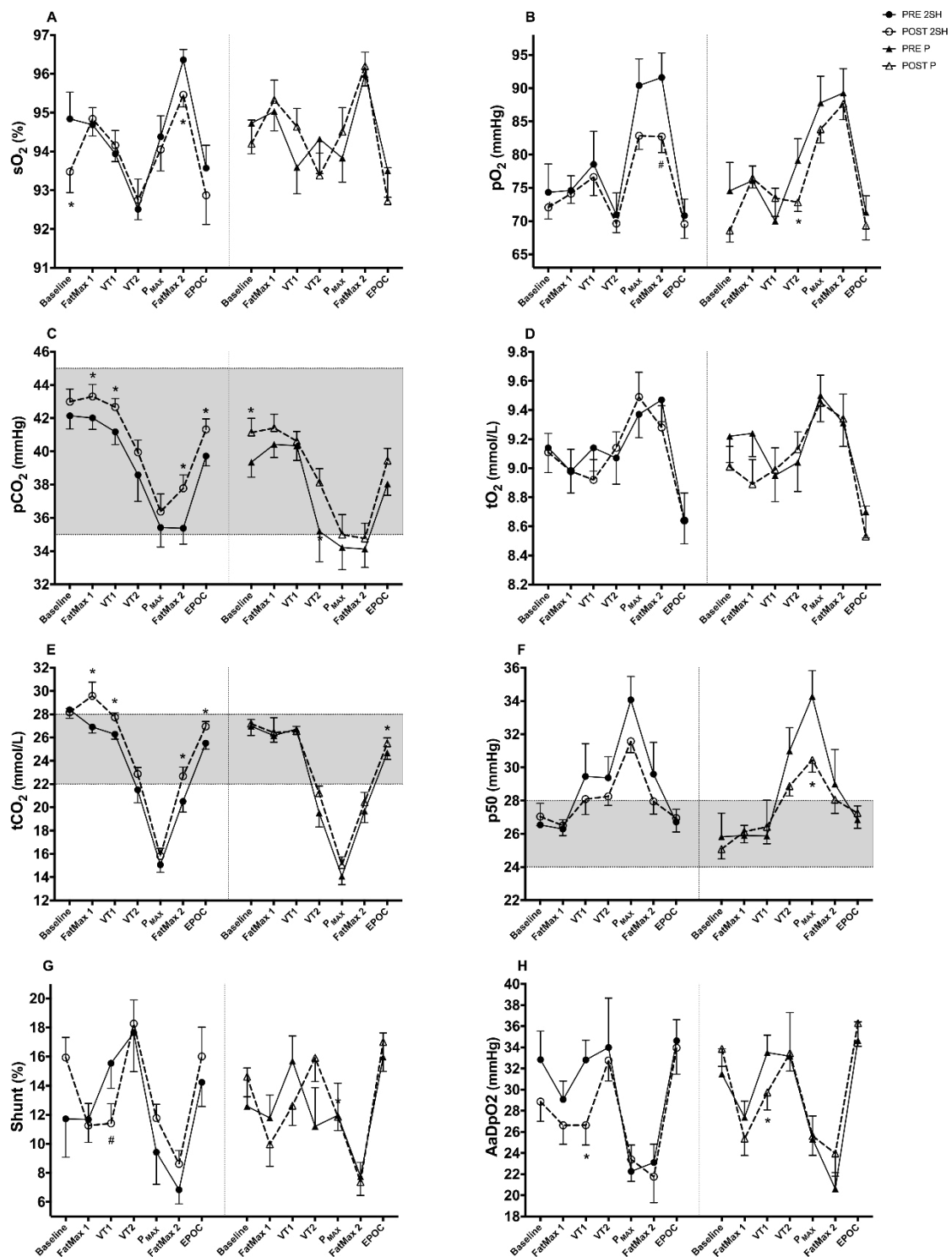


Figure 53. Differences between pre- and post-intervention within-group in finger capillary blood gas parameters at different points of the rectangular test (A-H). * $p < 0.05$. # $p = 0.05-0.08$.

12.3.1.2. Acid-base status (capillary blood gases)

Table 36 (Figure 54) shows the intragroup changes in biomarkers of acid-base status at baseline (pre), FatMax1, VT1, VT2, P_{MAX}, FatMax2 and EPOC achieved during the rectangular test. In 2S-hesperidin we no found significant changes post-intervention. Similarly, no significant changes were found at pre (Table 36) in placebo.

When we measured acid-base status at FatMax1 (Table 36) (Figure 54), the 2S-hesperidin group showed a significant increase in HCO₃⁻ (10.30%; p=0.040; ES=4.85), Lac (-29.39%; p=0.010; ES=2.26) and SBE (424%; p=0.046; ES=4.87) and an upward trend with a large effect size SBC (10.36%; p=0.076; ES=5.26) and ABE (534%; p=0.059; ES=4.82) post-intervention. In the placebo group, we found a significant decreased in Lac (-21.91%; p=0.041; ES= 1.71) post-intervention. However, in Δ FatMax1, a trend with large effect size in SBC (p=0.07; ES; 1.81) were observed between groups. (Table 37).

When we analyzed the changes in VT1 (Table 36) (Figure 54), 2S-hesperidin had a significant increase in HCO₃⁻ (5.55%; p<0.001; ES=3.20), SBC (4.65%; p=0.001; ES=3.59), ABE (6500%; p=0.001; ES=3.48) and SBE (1913%; p=0.001, ES=3.42) and a significant decrease in Lac (-30.83%; p=0.003; ES=2.98), after the supplementation period. On the other hand, no significant changes were found in the placebo group post-intervention. A significant difference in SBE (p=0.01; ES; 4.59) in Δ VT1, a trend with large effect size in ABE (p=0.08; ES; 5.05) were also observed between groups. In the Δ VT1 analysis, although there were no significant differences in HCO₃⁻ (p=0.31; ES; 5.48), SBC (p=0.81; ES; 5.07) and Lac (p=0.18; ES; 3.39) between groups, these parameters had a large effect size (Table 37).

In the submaximal exercise stage measurements (VT2) (Table 36) (Figure 54), the 2S-hesperidin group did not show any significant changes in acid-base state post-intervention. Similarly, there were no significant changes in VT2 in placebo after the 8-week intervention.

At P_{MAX} (Table 36) (Figure 54), the 2S-hesperidin group showed no significant pre-post change in acid-base status. However, in the placebo group (Table 36), an upward trend was observed at P_{MAX} in SBC (5.88%; p=0.077; ES=1.34) post-intervention.

During the rectangular test, after P_{MAX} decreased in intensity to FatMax2 (Table 36) (Figure 54), 2S-hesperidin showed a significant increase in pH (0.26%; p=0.028; ES=1.48), HCO₃⁻ (10.81%; p=0.003; ES=2.19), SBC (7.68%; p=0.006; ES=2.01), and ABE (34.23%; p=0.034; ES=1.62). Conversely, we found a significant decrease in Lac (-18.56; p=0.018; ES=1.88) post-intervention. In placebo, no significant change was found at FatMax2 after intervention.

Finally, at EPOC (Table 36) (Figure 54), the 2S-hesperidin group showed a significant increase in HCO₃⁻ (5.76%; p=0.001; ES=2.63), SBC (4.48%; p=0.001; ES=2.39), ABE (246.15%; p=0.001; ES=2.42) and SBE (248.39%; p<0.001; ES=2.53) and a significant

decrease in Lac (-18.56%; $p=0.039$; ES=1.51), post-intervention. In placebo (Table 36) (Figure 54), we found a significant increase in HCO_3^- (3.40%; $p=0.045$; ES=1.36) at resting EPOC after intervention.

When comparing the intra-group AUCs of the acid-base state (Figure 36), in 2S-hesperidin there was a significant increase in pH (0.16%; $p=0.016$; ES=0.54), HCO_3^- (6.34%; $p=0.012$; ES=0.74) SBC (5.07%; $p=0.017$; ES=0.79), with a downward trend in Lac (12.58%; $p=0.057$; ES=0.51), post-intervention. After comparing AUCs in placebo, there was no significant change after the intervention (Table 36). When comparing AUC between groups, a significant change in pH (0.16%; $p=0.022$; ES=0.99) was found in favour of 2S-hesperidin, post-intervention. In the analysis of ΔAUC , significant differences were observed in pH ($p=0.02$; ES; 1.03) (Table 36).

T _v	(mmol/L)		1.68	1.85	1.75	7.05	14.21	5.49	2.94	32.10	1.84	2.28	2.30	8.35	14.82	7.22	3.65	37.47
	Post	(Lac)	(0.13)	(0.24)	(0.23)	(0.52)	(0.79)	(0.67)	(0.35)	(6.58)	(0.15)	(0.26)	(0.25)	(0.56)	(0.86)	(0.73)	(0.38)	(8.54)
	P-value		0.871	0.010	0.003	0.134	0.730	0.018	0.039	0.057	0.680	0.041	0.833	0.741	0.503	0.702	0.098	0.391
	ES		0.15	2.26	2.98	1.49	0.28	1.88	1.51	0.51	0.38	1.71	0.19	0.32	0.53	0.28	1.17	0.25
	Pre		100.00	86.25(2.97)	93.58	96.83	129.08	114.25	101.00	616.46	101.82	87.00(3.10)	87.82	94.27	123.73	112.73	93.00	611.50
			(2.91)		(3.28)	(3.37)	(6.17)	(6.08)	(6.45)	(68.76)	(3.04)		(3.43)	(3.52)	(6.44)	(6.35)	(6.74)	(73.23)
	Post	Glucose (mg/dL)	100.00	87.75(3.24)	95.42	95.08	118.67	109.83	99.58	598.91	98.55	91.09(3.38)	89.00	93.82	116.91	106.73	92.73	591.40
		(Glu)	(2.57)		(2.79)	(3.58)	(5.92)	(5.38)	(3.62)	(69.45)	(2.69)		(2.91)	(3.74)	(6.19)	(5.62)	(3.78)	(64.12)
	P-value		1.000	0.677	0.589	0.616	0.090	0.487	0.782	0.248	0.231	0.282	0.738	0.900	0.277	0.368	0.959	0.255
	ES		0.00	0.47	0.52	0.48	1.57	0.68	0.20	0.24	0.99	1.22	0.32	0.12	0.98	0.87	0.04	0.25
	Pre		2.14	0.50	-0.02	-5.21	-13.13	-5.20	-0.52	26.57	1.23	0.18	0.75	-7.02	-14.25	-6.09	-0.91	30.22
			(0.79)	(0.52)	(0.35)	(1.14)	(0.78)	(1.03)	(0.50)	(8.72)	(0.90)	(0.59)	(0.40)	(1.30)	(0.89)	(1.17)	(0.57)	(10.04)
	Post	Actual base excess (mmol/L)	1.72	3.17	1.28	-3.84	-12.28	-3.42	0.76	24.13	0.92	-0.12(1.49)	0.30	-5.71	-13.00	-5.12	-0.35	26.76
		(ABE)	(0.28)	(1.31)	(0.36)	(0.57)	(0.74)	(0.79)	(0.38)	(8.72)	(0.32)		(0.41)	(0.65)	(0.85)	(0.90)	(0.44)	(8.15)
	P-value		0.604	0.059	0.001	0.200	0.169	0.034	0.001	0.472	0.739	0.846	0.238	0.279	0.082	0.290	0.154	0.103
	ES		0.50	4.82	3.48	1.13	1.02	1.62	2.42	0.26	0.31	0.46	1.04	0.92	1.28	0.76	0.90	0.32
	Pre		2.59	0.72	0.08	-5.67	-14.04	-5.93	-0.62	29.22	1.31	0.06	0.79	-7.67	-15.25	-5.59	-1.31	33.13
			(0.85)	(0.59)	(0.42)	(1.25)	(0.81)	(1.43)	(0.57)	(9.82)	(0.92)	(0.64)	(0.45)	(1.36)	(0.88)	(1.56)	(0.62)	(10.89)
	Post	Standard base excess (mmol/L)	2.14	3.77	1.61	-4.10	-10.71	-3.88	0.92	26.76	1.21	0.15	0.51	-5.94	-14.08	-6.24	-0.62	29.67
		(SBE)	(0.33)	(1.41)	(0.41)	(0.65)	(1.79)	(0.90)	(0.46)	(9.37)	(0.36)	(1.54)	(0.45)	(0.71)	(1.95)	(0.97)	(0.50)	(8.62)
	P-value		0.606	0.046	0.001	0.187	0.106	0.128	<0.001	0.509	0.916	0.954	0.500	0.180	0.591	0.651	0.104	0.174
	ES		0.50	4.87	3.42	1.17	3.87	1.34	2.53	0.23	0.10	0.13	0.57	1.18	1.23	0.38	1.03	0.29
	Pre	Lactate	1.66	2.62	2.53	8.45	14.46	6.98	3.61	36.72	1.77	2.92	2.25	8.68	15.35	7.46	4.23	39.32
			(0.14)	(0.32)	(0.24)	(0.88)	(0.86)	(0.74)	(0.42)	(8.50)	(0.15)	(0.34)	(0.27)	(0.96)	(0.93)	(0.81)	(0.45)	(6.76)

In bold are p-values ≤ 0.05 and trends between 0.05-0.08.

Table 37. Comparison of pre-post-intervention differences between each of the rectangular test points (Baseline, FatMax1, VT1, VT2, P_{MAX}, FatMax2, EPOC) and AUC between groups.

Between-Group Comparison									
	Δ Baseline	Δ FatMax1	Δ VT1	Δ VT2	Δ P _{MAX}	Δ FatMax2	Δ EPOC	Δ AUC	
Hct (%)	Differences	-0.252 (0.64)	0.334 (0.94)	0.990 (1.04)	0.040 (0.74)	1.485 (1.82)	0.189 (0.90)	0.343 (0.59)	3.402 (4.35)
	P-value	0.70	0.72	0.35	0.96	0.39	0.83	0.56	0.44
	Effect size	1.26	2.23	1.17	0.81	1.56	0.66	1.19	0.27
Hb (g/dL)	Differences	-0.252 (0.33)	0.393 (0.27)	-0.568 (0.26)	0.234 (0.32)	0.334 (0.42)	-0.124 (0.25)	0.127 (0.26)	0.829 (1.40)
	P-value	0.70	0.15	0.04	0.47	0.44	0.62	0.62	0.56
	Effect size	1.28	1.77	1.24	0.86	1.51	0.58	1.15	-0.25
O₂Hb (%)	Differences	-6.230 (5.48)	-5.609 (3.98)	3.321 (4.45)	-7.165 (3.89)	1.546 (5.55)	-0.931 (3.68)	-2.655 (2.97)	-3.282 (2.26)
	P-value	0.26	0.17	0.46	0.07	0.78	0.80	0.38	0.16
	Effect size	1.39	0.08	1.27	0.63	1.76	2.19	0.15	0.50
COHb (%)	Differences	5.315 (5.19)	4.444 (4.13)	-3.763 (5.30)	5.395 (3.09)	-0.031 (5.93)	-3.753 (4.07)	-1.099 (2.70)	0.264 (0.37)
	P-value	0.31	0.29	0.48	0.09	1.00	0.36	0.69	0.49
	Effect size	0.43	0.13	0.04	0.31	0.46	2.25	0.45	0.23
RHb (%)	Differences	-0.825 (1.88)	-0.732 (2.06)	-0.379 (2.53)	0.145 (1.67)	-1.981 (4.24)	2.405 (2.47)	2.220 (1.94)	0.718 (1.10)
	P-value	0.66	0.73	0.88	0.93	0.65	0.34	0.26	0.52
	Effect size	1.22	0.25	1.16	1.40	1.68	3.07	0.09	0.25
MethHb (%)	Differences	-0.060 (0.72)	0.138 (0.61)	-0.963 (0.72)	0.785 (0.97)	-1.048 (0.85)	-0.381 (0.45)	-0.584 (0.78)	0.274 (0.28)
	P-value	0.93	0.82	0.19	0.43	0.23	0.40	0.46	0.34

	<i>Effect size</i>	1.19	1.78	0.57	1.61	1.83	0.53	0.49
	<i>Differences</i>	-0.968 (0.84)	0.500 (0.84)	-1.574 (1.60)	-0.165 (1.81)	1.371 (0.83)	0.352 (0.86)	-2.263 (2.67)
sO₂ (%)	<i>P-value</i>	0.26	0.56	0.33	0.93	0.11	0.69	0.41
	<i>Effect size</i>	1.20	1.17	1.39	1.65	3.08	0.11	0.26
	<i>Differences</i>	1.898 (0.86)	0.801 (0.87)	2.380 (1.55)	1.634 (2.22)	0.765 (1.17)	1.028 (0.75)	-12.868 (15.19)
pO₂ (mmHg)	<i>P-value</i>	0.03	0.36	0.13	0.47	0.52	0.18	0.41
	<i>Effect size</i>	0.94	0.90	1.72	0.80	1.53	0.32	0.28
	<i>Differences</i>	0.001 (0.01)	0.012 (0.01)	-0.020 (0.02)	-0.001 (0.02)	0.008 (0.01)	0.005 (0.01)	0.330 (5.01)
pCO₂ (mmHg)	<i>P-value</i>	0.96	0.11	0.23	0.96	0.43	0.46	0.95
	<i>Effect size</i>	1.31	1.92	0.91	0.15	2.18	0.25	0.14
	<i>Differences</i>	-0.005 (0.04)	0.026 (0.05)	-0.023 (0.18)	0.010 (0.06)	0.024 (0.04)	-0.030 (0.04)	0.409 (0.80)
tO₂ (mmol/L)	<i>P-value</i>	0.91	0.63	0.90	0.88	0.60	0.43	0.61
	<i>Effect size</i>	0.99	1.79	0.09	0.91	1.64	0.86	0.00
	<i>Differences</i>	0.142 (0.50)	-0.010 (0.42)	-0.125 (0.52)	2.992 (6.71)	0.561 (0.52)	0.146 (0.38)	4.457 (3.64)
tCO₂ (mmol/L)	<i>P-value</i>	0.78	0.98	0.81	0.66	0.29	0.70	0.24
	<i>Effect size</i>	0.58	4.60	0.31	0.33	2.07	1.65	0.54
	<i>Differences</i>	0.085 (0.18)	0.126 (0.28)	0.059 (0.41)	-0.646 (0.50)	-0.097 (0.28)	-0.068 (0.15)	2.308 (6.43)
p50 (mmHg)	<i>P-value</i>	0.64	0.65	0.89	0.21	0.73	0.66	0.72
	<i>Effect size</i>	0.77	0.98	0.74	0.84	0.34	0.16	0.23
	<i>Differences</i>	0.011 (0.03)	-0.033 (0.04)	-0.009 (0.01)	-0.013 (0.02)	-0.041 (0.02)	0.012 (0.02)	1.268 (7.49)
Shunt (%)	<i>P-value</i>	0.74	0.37	0.53	0.47	0.05	0.53	0.87
	<i>Effect size</i>	0.81	0.52	1.52	1.00	1.69	0.46	0.01
AaDpO₂ (mmHg)	<i>Differences</i>	-0.250 (0.53)	-3.316	-0.433	-2.060	-1.300 (0.93)	-1.176	-4.448

<i>Effect size</i>	0.39	1.98	4.59	0.14	1.05	2.00	2.17	0.07
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Hct = hematocrit, *Hb* = hemoglobin, *O₂Hb* = oxyhemoglobin, *COHb* = carboxyhemoglobin, *RHb* = deoxyhemoglobin, *MetHb* = methemoglobin, *sO₂* = oxygen saturation, *pO₂* = oxygen partial pressure, *pCO₂* = carbon dioxide partial pressure, *tO₂* = total blood oxygen concentration, *tCO₂* = total blood carbon dioxide concentration, *p50* = oxygen partial pressure at 50% oxygen saturation, *Shunt* = relative physiological shunt, *AaDpO₂* = alveolar-arterial gradient, *HCO₃⁻* = bicarbonate anion, *SBC* = standard bicarbonate, *Lac* = lactate, *Glu* = glucose, *ABE* = actual base excess and *SBE* = standard base excess. In bold are *p*-values ≤ 0.05 and trends between 0.05-0.08.

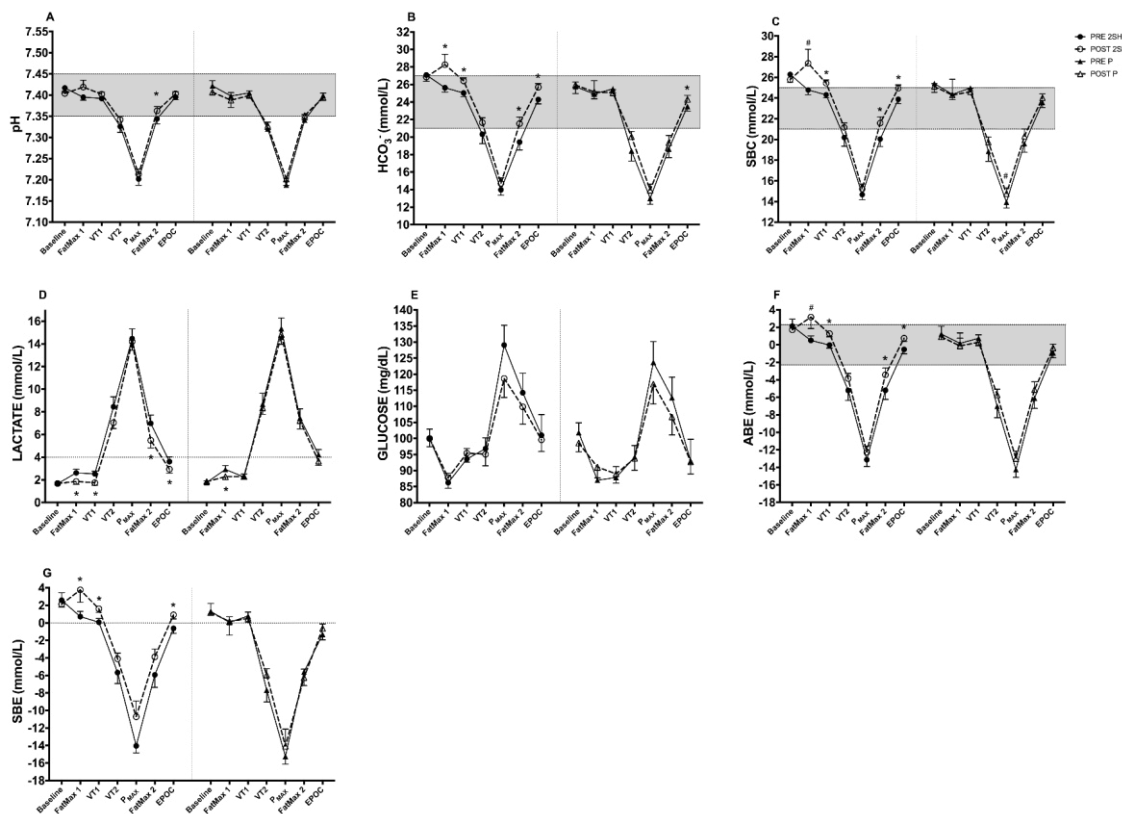


Figure 54. Differences between pre- and post-intervention intra-group differences in acid-base status parameters, lactate and glucose at different points of the rectangular test (A-G). * $p < 0.05$. # $p = 0.05-0.08$.

12.4. DISCUSSION

This study primarily aimed to determine if 8-weeks of 2S-hesperidin supplementation (500 mg/d) has the ability to modify biomarkers of oxygen and carbon dioxide metabolism and acid-base state and whether these possible changes can affect performance in trained amateur cyclists. The second objective was to determine if any of the changes in the previously mentioned parameters could explain improvement in performance, as observed in our recently published article (738), of which this research is a part. The most important findings of this study showed that the chronic intake of 2S-hesperidin: i) increased RHB and decreased in oxygen parameters at baseline (0) ii) increased dioxide carbon metabolism and state acid-base markers at FatMax1, iii) increased dioxide carbon metabolism and state acid-base markers and decreased Shunt, AaDpO₂ and Lac at VT1, iv) increased dioxide carbon metabolism and state acid-base markers and decreased oxygen metabolism markers and Lac in FatMax2, v) increased dioxide carbon metabolism and state acid-base markers and decreased Lac in EPOC post intervention, and vi) increased dioxide

carbon metabolism and state acid-base markers and decreased Lac when comparing pre-post intervention AUCs. However, there were no pre-post changes in the aforementioned parameters in placebo.

12.4.1. Blood gas changes and acid-base at baseline (pre)

At baseline, the 2S-hesperidin group showed a decrease in O₂Hb and sO₂ following the intervention. However, there were pre-intervention significant differences in pO₂ (2S-hesperidin -2.28 vs placebo -8.90, mmHg) at baseline between groups (Δ baseline; Table 5), indicating a greater decrease in resting pO₂. This difference may be due to the effect of detraining because the study was conducted off-season to pre-season, where the cyclists had decreased their training load. Detraining is defined as the partial or complete loss of physiological, anatomical, and performance adaptations due to the reduction or cessation of training (847). In young top-level road cyclists, after 5 weeks of training cessation, a decrease in red blood cell count ($-6.6 \pm 4.8\%$) and Hb ($-5.4 \pm 4.3\%$) at rest were observed (848). However, in our study, there were no significant changes in Hct and Hb in both groups. The rapid decrease in blood volume after the first days of cessation of training possibly has an important role in the processes that decrease maximal cardiac output and, consequently, VO_{2MAX} (848).

Moreover, cessation of training results in a rapid decline in plasma volume primarily mediated by a loss in intravascular protein content. Furthermore, cessation of training results in a rapid decrease in plasma volume, mediated mainly by a loss of intravascular protein content, which directly affects blood volume (849). However, to our knowledge, there are studies that have observed the decrease in pO₂ after cessation or reduction of training volume. Therefore, based on the findings of this study, chronic intake of 2S-hesperidin could prevent the decline in resting pO₂ during a period when cyclists reduce their training volume. Although, further studies are still needed in this regard.

12.4.2. Blood gas changes and acid-base at FatMax1 and VT1

Exercise performed in FatMax1 intensity that produces maximum fat oxidation rate, translated as the % VO_{2MAX}, uses the oxidation of free fatty acids and intramuscular triglycerides in skeletal muscle as its main source of energy (850). Both peak oxidative maximum and FatMax are related to better-prolonged endurance performance, being an important determinant of performance in elite endurance athletes (851). On the other hand, being a zone close to the physiological-metabolic level of the FatMax, VT1 represents the first increase in minute ventilation (VE) that is proportional to the increase in CO₂ output (VCO₂) generated by the HCO₃⁻ buffering of lactic acid. As a result, the ventilatory equivalent for oxygen (VE·VO₂) increases with

no change in the ventilatory equivalent for carbon dioxide ($V \cdot VCO_2$) (7). In these, two exercise zones (FatMax and VT1), energy production is predominantly aerobic.

In our study, at FatMax1 and VT1, there were increases in pCO_2 and tCO_2 in 2S-hesperidin. Moreover, this group also showed a decrease in $AaDpO_2$ and a downward trend in Shunt after the in capillary blood intervention. No previous studies have evaluated polyphenol intake on blood gas biomarkers. At intensities close to 50% of the lactate threshold (LT) or VO_{2MAX} (FatMax and VT1), the values of arterial CO_2 pressure ($PaCO_2$) and partial pressure of exhaled carbon dioxide ($PETCO_2$) remain stable (852). It is well known that CO_2 is the obligatory product of aerobic catabolism in tissues and, as it is produced at a higher rate, higher concentrations of H^+ will also be generated, thereby increasing the production of HCO_3^- from carbon dioxide (853). But unlike H^+ , the effect on total bicarbonate concentration is minimal (853). Although in the 2S-hesperidin group the ΔpH values increased (alkalinisation), the placebo group showed a decrease in ΔpH (acidification), without significant changes between groups, but there was a large effect size in FatMax1 ($ES=2.13$) and VT1 ($ES=2.50$). Therefore, the increased pCO_2 and tCO_2 in the 2S-hesperidin group is not responsible for acidification ($\downarrow pH$) of the blood, although at low-moderate intensities (FatMax1 and VT1) this situation does not have much influence on performance. However, elevated pCO_2 post-intervention (2S-hesperidin) could be a consequence of alterations in pulmonary ventilation, which was observed in our recently published study (that is part of the same project as the present research), where there was no worsening or improvement in VT1 performance (W) in amateur cyclists following 8 weeks of 2S-hesperidin (500 mg/d) intake (738).

We detected an increase in HCO_3^- in FatMax1 and VT1 in the 2S-hesperidin group. In addition, we observed an increase in SBC, ABE and SBE, with a decrease in Lac, indicating an improvement in acid/base status and a lower contribution of glucose to energy production at low intensities. These results could explain how the 8-week intake of 2S-hesperidin was able to maintain fatty acid and carbohydrate oxidation compared to placebo (2S-hesperidin: -12.9% and -0.45% vs placebo: -34.3% and 17.7%; respectively) at VT1 intensities in amateur cyclists in a period where there was lower intensity and volume of training) (738). This decrease in fatty acid oxidation and increase in carbohydrate oxidation in placebo in VT1 found by Martínez-Noguera *et al.* (738) is linked to the negative changes in aerobic metabolism ($\downarrow Hct$, $\downarrow Hb$, $\downarrow tO_2$ and $\downarrow AaDpO_2$) from this study, although there were no significant changes in $sO_2\%$ and pO_2 . In fact, changes in Hb or by plasma volume expansion can slightly alter O_2 supply and thus O_2 kinetics in humans (852).

The consequences of these changes would be a higher carbohydrate utilization at low-to-moderate intensities in the placebo-supplemented cyclists. Therefore, chronic ingestion of 2S-hesperidin could improve acid/base and lactate status, which may

influence the efficiency of the energy substrate used at low-to-moderate intensities (VT1), leading to conservation of muscle glycogen for higher intensity phases in the final phase of a competition in amateur cyclists.

12.4.3. Blood gas changes and acid-base at VT2

In some athletes, high-intensity endurance exercise induces a time-dependent decrease in sO_2 of more than 5% compared to resting levels ($\sim 98\%$), where extreme declines in the 80% range have been reported (854). The desaturation of O_2Hb during exercise is based on respiratory and non-respiratory factors. Non-respiratory factors, like metabolic acidosis and hyperthermia, produces a rightward shift in the O_2Hb dissociation curve (855). In high performance athletes (856), arterial desaturation of O_2Hb is due to a fall in PaO_2 (857) secondary to an abnormally widening of $AaDpO_2$ (854). In healthy untrained individuals, $AaDpO_2$ is reported to decline to 20 to 30 mmHg during maximal exercise; however, in some elite athletes, this difference can be as high as 35-50 mmHg (858).

In contrast, our study found no post-intervention changes in $AaDpO_2$ in both groups (2S-hesperidin: -1.23 mmHg vs placebo: 0.29 mmHg) at VT2. However, only the placebo group showed a decrease in pO_2 (-7.94%) with a non-significant decrease in O_2Hb , sO_2 and increase in $COHb$ but with a large effect size. In addition, we found a trend with a moderate effect size between groups in O_2Hb for $\Delta VT2$. These results are in line with our previously reported study that showed a decrease in VO_2 (L/min) (-8.3%; $p \leq 0.01$) and oxygen consumption relative to weight (VO_2R) (mL/kg/min) (-8.9%; $p \leq 0.01$) at VT2 (10 min) when performing a rectangular test (cycloergometer) amateur cyclists who ingested placebo (8-weeks) but not in the 2S-hesperidin group (738). In this same study, we also performed an incremental test where we found a non-significant decrease in power output at VT2 (WVT2) (-3.1%; -8.9W; $p=0.264$) in placebo and a non-significant increase (1.0% = 2.9 W; $p=0.642$) in 2S-hesperidin post-intervention. It is important to make clear that this study (738) and the data presented in this paper are part of the same project and were carried out at the same time. There are currently no other studies that have used other polyphenols as markers of oxygen metabolism and with which we can compare our results. There is evidence that a decrease in SpO_2 (77.9%; O_2Hb saturation estimated using a pulse oximeter) manipulated by a low FIO_2 (0.15) led to a decrease in mean power output (MPO) (-23.3%) and increase in time (32 s) in trained male cyclists performing a 5-K time trial (TT) compared to normoxic conditions (FIO_2 , 0.21) (859). In addition, they observed that, when generating a hyperoxia state by means of a FIO_2 of 1.0 reaching a SpO_2 of 100%, cyclists increased MPO (18.1%) and decreased time (-19.5 s) in a 5K TT compared to normoxic conditions. This demonstrates that changes in arterial oxygen can affect

performance. It has been shown that for every 1% reduction in SaO₂ below 95%, there is a 1-2 decrease in VO_{2MAX} (860).

Arterial desaturation during exercise may be due to an insufficient hyperventilatory response secondary to a low chemo-responsiveness as a consequence of a low response to circulating chemical stimuli, such as catecholamines, adenosine, protons or potassium (861) and O₂ and CO₂ (862) and/or mechanical constraints on the airway (854). Inadequate ventilatory responses during exercise reduces the alveolar partial pressure of O₂ (PAO₂), which negatively affects arterial blood gas status and SaO₂ (863). Previously, it has been reported that EIAH (SaO₂≤91%), involves an increase in AaDpO₂, which combined with a minimal alveolar hyperventilatory response, results in a reduction in pO₂ (864). Several authors suggest that one consequence of EIAH is that even small amounts of EIAH have a significant negative effect on limiting the transport and utilization of O₂ during maximal exercise (860, 865). Dempsey and Wagner et al. (854) observed SaO₂ values <93% (moderate and severe EIAH) in highly trained athletes, which was related to decreases in ventilatory equivalents for CO₂, showing that a worsening in the hyperventilatory response may be associated with the development of EIAH.

Several authors have reported that hesperidin may prevent the decline in pO₂ during maximal exercise through different mechanisms. Liu et al. (531) showed that hesperetin (hesperidin metabolite) increases NO release from endothelial cells in a dose-dependent manner and up-regulates endothelial nitric oxide synthase (eNOS) expression. In addition, hesperetin plus naringenin ameliorates airway structural remodeling (improved basement membrane thickness and smooth muscle hypertrophy) with a significant reduction of inflammatory cells and lowering of mucus plug formation in murine chronic asthma mode (840).

Furthermore, flavonoids (a subgroup of polyphenols to which hesperidin belongs) can facilitate an increase in mitochondrial Ca²⁺ levels by acting on the mitochondrial Ca²⁺ uniporte (839). This mechanism can up-regulate respiratory rate and ATP production and stimulate eNOS, thus increasing NO synthesis (838, 841, 866). NO-induced vasodilation may increase oxygen supply to active muscles, which could improve performance (841, 867).

Another factor that can affect blood oxygenation is the Shunt, which is defined as blood that enters the arterial system without coming in contact with ventilated areas of the lung (868, 869). In our study, no significant intra-group pre-post differences were found, but placebo did show a non-significant increase with a large effect size (ES= 1.61) in VT₂. Another important limiting factor for arterial pO₂ is the diffusion problem caused by the presence of pulmonary edema during high-intensity exercise, as fluid in the lungs increases the effective thickness of the alveolar wall and decreases the area of gas exchange (870, 871). The edema prevents air from reaching pulmonary

capillaries, resulting in perfusion without ventilation and a physiologic right-to-left shunt, thereby creating greater hypoxemia (872). One possible mechanism of action of hesperidin in oxygen metabolism is the inhibition of histamine and expression of mRNA and proteins histamine receptor H1 in the hypothalamus and brainstem regions in rats that consumed hesperidin (873). In addition, the anti-inflammatory effect of hesperidin has been demonstrated in a rat model with lung damage, where there was a decreased presence of TGF- β 1, IL-1 β , IL-4, IL-10 and TNF- α in the lung tissues (874). The activation of the immune system may influence the lung's response. For example, basophilic, granulocytes and mast cells are degranulated, as indicated by the enhanced ratio of plasma histamine to total histamine in the blood during intense exercise, and may contribute to the increased permeability of the lung microvasculature (875). In addition, Martinez-Noguera *et al.* (787) observed a decrease in monocyte chemoattractant protein 1 at basal levels after a period of recovery from exertion to exhaustion and decline in the area under the curve of the rectangular test performed by amateur cyclists.

Wei *et al.* (876) showed that the administration of hesperidin significantly decreased the number of infiltrating inflammatory cells and Th2 cytokines in bronchoalveolar lavage, goblet cell hyperplasia and mucus hypersecretion compared with the ovalbumin-induced group of mice. On the other hand, hesperidin methylchalcone acts as an inhibitor of increased bradykinin-induced microvascular permeability and prevents leukotriene B₄-histamine, which induces vascular leakage, thus reducing the formation of edema in an animal model of venous insufficiency (877). In particular, an elevated concentration of IL-1 β and IL-8 in plasma during exercise may partly explain the increase in %H associated with EIAH in highly trained athletes, showing how a reaction of the inflammatory system can influence histamine concentrations and consequently EIAH (878). Therefore, chronic intake of 2S-hesperidin could prevent a decrease in pO₂, thus avoiding a decrease in oxygen consumption and performance in cyclists in a period of decreased training intensity and volume (off-season to preseason).

We also observed in this clinical trial, a non-significant intra-group increase in both treatments but with a large effect size in pH for the 2S-hesperidin group (2S-hesperidin: 0.22% vs placebo: 0.11%) and no significant changes but a moderate effect size (ES= 0.52) (between-group comparison) in Δ VT2. Together with these changes, we also found a non-significant intra-group decrease in both groups but with a large effect size in 2S-hesperidin (2S-hesperidin: -16.6% vs placebo: -3.8%) and no significant changes but a large effect size in Δ VT2.

Changes in pO₂ in the placebo group in VT2 could be largely contributed by anaerobic metabolism, but this is not evident in our study, as there were no significant changes in Lac post-intervention. However, the lower lactate production in the 2S-

hesperidin group seems to indicate a lower anaerobic contribution of glycolysis or a better lactate clearance, thereby leading to better pH levels. Due to the influence of acidosis on the development of fatigue, an increase in pO_2 , a lower intracellular lactate accumulation and an increase in intracellular pH can increase exercise capacity independently of VO_{2MAX} (879).

Administration of lemon peel flavonoids (LPF) for 4 weeks in mice decreased post-intervention lactic acid levels following a swim to exhaustion test, improving time-to-exhaustion and endogenous antioxidant status (880). As mentioned above, Martínez-Noguera et al (738) found an improvement in VT2 ($\uparrow W$) performance after 8 weeks of 2S-hesperidin intake in amateur cyclists using the same rectangular test as the current study, which could be linked to lower levels of Lac in VT2 post-intervention.

Therefore, chronic intake of 2S-hesperidin could have the capacity to prevent a decrease in pO_2 in VT2, in which anaerobic metabolism is mainly involved and can be described as "hard" or "high intensity" exercise (881). This parameter plays an important role in the performance of extreme endurance competitions, such as 3-week races, like Tour of France(134).

12.4.4. Blood gas changes and acid-base at P_{MAX}

The P_{MAX} during an incremental test until exhaustion is a mechanical expression of aerobic capacity (54) In addition, it is known that cycling at an output power equivalent to VO_{2MAX} or P_{MAX} is not sustainable in endurance events, and that sustainable output powers measured on the ergometer are around the lactate threshold, the onset of blood lactate accumulation (OBLA) or the ventilation threshold (typically 75 to 90% of P_{MAX}) (11). Maintaining maximum cycling power production is an important quality for performance in competitive cyclists, due of the strong relationship between power production and endurance cycling performance (11, 14, 882).

When assessing changes in capillary blood samples at P_{MAX} , we found a decrease in $p50$ in the placebo group (-11.14%) coupled with a non-significant increase with a large effect size in sO_2 (0.72%) and decrease in pO_2 (-4.53%). However, there was no significant change in any parameter in 2S-hesperidin, but a non-significant decrease with large effect size in pO_2 (-8.33%) and $p50$ (-7.34%). To our knowledge, this study is the first to examine blood gas parameters following polyphenol supplementation. It is known that the $p50$, indicating the affinity of Hb- O_2 , is expressed as the pO_2 value at 50% saturation of Hb with O_2 (883). The standard $p50$ in humans is 26.9 mmHg at pH 7.4 and 37°C (884), which was the methodology used in our study. There are factors that can modify the $p50$ (Hb- O_2 affinity) due to an increase in hydrogen ions (acidosis), temperature, 2,3-bisphosphoglycerate (2,3-BPG) and pCO_2 that decrease Hb- O_2 affinity

with higher $p50$ values and a rightward shift of the ODC (885). During physical exercise and depending on the intensity of the exercise, an increase in local temperature occurs at the muscle level and produces more H^+ and CO_2 , which leads to a better release of oxygen (885). This is mainly due to the conversion of CO_2 to bicarbonate and H^+ by carbonic anhydrase, consequently decreasing pH and Hb- O_2 affinity (885). In addition, CO_2 has a specific effect on Hb- O_2 affinity at constant pH (399). However, an increase in $p50$ in venous blood (decrease in Hb- O_2 affinity) would benefit oxygen-deficient tissues (883). In our study, although the $p50$ finger capillary blood (ΔP_{MAX} ; $ES=0.84$, between groups) decreased, there were no changes in markers of acid-base status in placebo, which could indicate a higher rate of fatigue that would anticipate physical exhaustion in P_{MAX} . Nevertheless, in the study we recently published, the placebo group decreased performance and the 2S-hesperidin group improved performance during the rectangular test after eight weeks of supplementation (738).

During maximal exercise, the SaO_2 appears to be affected by the reduction in pH, and this is of particular importance when PaO_2 is low (886). Maximal exercise produces an extreme lactate spill-over to blood which decreases the pH to below 7.1 and, according to the ODC, this is critical for SaO_2 (887). Indeed, to test the effect of pH on SaO_2 , it has been found that the infusion of sodium bicarbonate maintains a stable blood buffer capacity, thereby attenuating acidosis and increasing SaO_2 from 89% to 95%, and enabling exercise capacity to increase (887).

Although both groups had a non-significant decrease in pO_2 , a significant decrease in $p50$ was only observed in placebo, indicating a leftward shift of ODC, leading to an increase in Hb- O_2 affinity, in the capillary blood sample. This effect is in line with the non-significant increase but with a large effect size of sO_2 . Therefore, it is plausible that 2S-hesperidin intake could prevent a decrease in $p50$ in finger capillary blood and would improve O_2 delivery at the tissue level at maximal exertion ($P_{MAX} \rightarrow$ rectangular test), which could be associated with an increase in maximal power in 2S-hesperidin vs placebo, as we observed in a recently published study but an incremental test (study which is part of the same project as the current project) (738). But this is only a hypothesis, as we did not measure $p50$ in venous blood, which would be the most appropriate to verify ODC changes at the tissue level. More studies are needed in this regard.

12.4.5. Blood gas changes and acid-base at FatMax2

At FatMax2 were increase in variables of the CO_2 metabolism (pCO_2 and tCO_2), hemoglobin (COHb and RHb) and Shunt (19%), but with inverse direction, there was a decreased in oxygen metabolism markers (O_2Hb , sO_2 , pO_2 and tO_2) in 2S-hesperidin.

When evaluating the changes in the placebo group at FatMax2, no significant changes were found. In view of the results, we cannot clearly say that the decrease in markers of oxygen metabolism is due to an increase in AaDpO₂ in 2S-hesperidin, but there were significant differences in Shunt at Δ FatMax2 in the comparison between groups.

Previous studies have demonstrated that lung volume and function are temporarily impaired after exercise, suggesting small-airway closure and possible subclinical edema, all contributing to a decreased diffusion capacity (888, 889). In addition, hydrogen ion accumulation and CO₂ decrease pH, which induces metabolic acidosis during and post-exercise, which subsequently stimulates hyperventilation due to respiratory compensation and decreases the arterial pCO₂ concentration (890). Based on the classic concept of Hill and Lupton (891), elevated post-exercise oxygen uptake is indicative of oxygen debt, which is the result of phosphocreatine resynthesis and lactate metabolism processes. These mechanisms could be responsible for the increase in CO₂ markers and decrease in O₂ markers in 2S-hesperidin in finger capillary blood, coupled with changes in ventilation (hypoventilation) and an increased oxygen deficit generated from post P_{MAX} to FatMax2, as no changes in p50 were observed post-intervention.

However, only the 2S-hesperidin group enhanced acid-base status markers (\uparrow pH, \uparrow HCO₃⁻, \uparrow SBC) and decreased Lac in FatMax2 post-intervention, suggesting that there was a lower anaerobic contribution, as Lac is a sensitive biomarker of non-oxidative glycolysis (104), as well as indicating a better washout of Lac in the transition from post P_{MAX} (very high intensity exercise) to FatMax2 (low-moderate intensity exercise). In addition, a marker of Lac and H⁺ clearance is VCO₂ production (nonmetabolic) relative to VO₂ (402, 892), and this is in line with our results, as CO₂ increased and O₂ decreased markers in FatMax2 in the 2S-hesperidin group. The decrease in Lac could be explained by improvements in the intracellular to extracellular transfer of lactate for oxidation or conversion to glucose and glycogen (893, 894).

Some phytomolecules with high flavonoid content have shown lower lactate concentrations compared to placebo after exercise to exhaustion, for example after 6 weeks of drone pupae extract intake in mice (-27%) (895) and after 30 days of pericarpium citri reticulatae extract supplementation in rats (896). In addition, consumption of 500 mL/d of orange juice and 1 hour of aerobic training (3 times a week for 3 months) in women decreased blood Lac concentration by 27% in the experimental group compared to 17% in the control group after exercise to exhaustion (665). These findings suggest that the experimental group showed less muscle fatigue and better response to training. On the other hand, when purified Chestnut flower flavonoids were administered to mice, a decrease in Lac and an increase in lactate dehydrogenase (LDH) were observed after a swim to exhaustion test (897). LDH is expressed in several tissue cells and is involved in the glycolytic pathway by facilitating the redox reaction

between pyruvic acid and lactic acid (reversible reaction) with concomitant actions of NADH and NAD⁺, playing an important role in the quenching of Lac during high endurance exercise (898). However, more research is needed to explain precisely the mechanisms by which flavonoids can decrease Lac after high-intensity exertion.

Another mechanism that may have elicited post-intervention decrease in Lac in FatMax2 is the increased peripheral blood flow in leg muscles via increased production of NO, as mentioned above, enhancing recovery, which is characterized by greater changes in Lac allowing transport of lactate to other tissues for oxidation and allowing greater phosphocreatine resynthesis and less accumulation of metabolites (899). Therefore, based on our findings, chronic intake of 2S-hesperidin can decrease Lac levels and improve markers of acid-base status, which offers cyclists greater recovery after maximal exertion.

12.4.6. Blood gas changes and acid-base at EPOC

In the post-exercise recovery period (EPOC), there was an increase in oxygen consumption, which consists of a rapid and a prolonged component. A review in 2003 concluded that EPOC can last for several hours after exercise, but it can also be transient and minimal (900). A relative shift from carbohydrate to fat as a substrate source is a consistent finding after prolonged strenuous exercise (900). Since the energy equivalent of oxygen is lower with fat as a substrate compared to carbohydrate (free fatty acids: ~4.7 mol ATP/mol oxygen; glucose: ~5.1 mol ATP/mol oxygen), some of the EPOC may be explained by this substrate shift. It has been estimated that the substrate changes after exhaustive submaximal exercise accounts for 10-15% of the observed EPOC (901).

In the last phase of the protocol that was performed at rest (EPOC), 2S-hesperidin reported an increase in markers of CO₂ metabolism (pCO₂, tCO₂) and acid-base status (pH, HCO₃⁻, SBC, ABE and SBE) and a decrease in Lac. However, in the placebo group, only an increase in HCO₃⁻ and an increase in tCO₂ post-intervention were detected. Furthermore, when evaluating changes between groups, significant differences in AaDpO₂ at ΔEPOC were found.

In general, the changes observed in EPOC are in line with those found in FatMax2, where the 2S-hesperidin group improved acid-base status and decreased post-intervention Lac, giving 2S-hesperidin a recuperative effect after high-intensity exertion followed by low-intensity exertion. In addition, reduced Lac levels in 2S-hesperidin indicate a lower energy contribution from carbohydrates via the anaerobic pathway.

12.4.7. Blood gas changes and acid-base in AUCs

When assessing the AUCs, which reflect the overall changes, the 2S-hesperidin group showed an increase in capillary blood markers in RHb, pCO₂, tCO₂, pH, SBC, and HCO₃⁻, but a decrease in Lac. In contrast, the placebo only showed a decrease in p50 post-intervention. In addition, significant changes in pH were found in Δ AUC (p=0.02; ES=1.03) when comparing the groups.

These findings partially reaffirm the changes found in some phases of the rectangular protocol, as the changes shown in pCO₂, tCO₂, HCO₃⁻ and SBC in FatMax1, VT1, FatMax2 and EPOC are related to significant differences in the AUC in 2S-hesperidin. However, changes in Lac shown in FatMax1, VT1, FatMax2 and EPOC were related to a downward trend with a moderate effect size in the AUC in 2S-hesperidin.

Overall, our data confirm improvements in acid-base status (low and moderate intensity exercise) and decrease in Lac (low-moderate and high intensity exercise) after 8 weeks 2S-hesperidin (500 mg/d) supplementation in amateur cyclists.

12.4.8. Limitations

Ideally, blood gases should be obtained in arterial blood. However, indwelling arterial catheters for sampling arterial blood are not always feasible and desirable. Thus, indirect methods were used to assess blood gases in the present study. Therefore, the degree to which the measurements provided an accurate proxy for arterial measures should be considered. Arterial blood gases (pO₂ and pCO₂) during exercise could be estimated by using capillary blood samples (pO₂ and pCO₂). Previous studies found that capillary blood samples are in good agreement with arterial blood samples for partial pressure of carbon dioxide, but not for partial pressure of oxygen. The main cause of underestimation of pO₂ in capillary blood samples could be insufficient arterialization of blood due to venous admixture. The capillary blood sample method requires adequate blood flow in the arm to enable a sufficient volume of blood to be sampled without additional external pressure during sampling.

The period of the season in which the study was conducted was in late September and mid-December, also known as their pre-season. During the pre-season, cyclists decrease their training volume and intensity with respect to other times of the season. Thus, our results should be taken with caution, considering the pre-season. If the study had been carried out in another period of the season where cyclists had more training volume and intensity, the results could have been different, in a positive way. Our sample size could have been larger, but due to budget constraints and planning to run the tests in 2 months, it made it unfeasible to recruit more subjects.

12.5. CONCLUSIONS

Chronic ingestion of 2S-hesperidin improved acid-base status (\uparrow pH, HCO_3^- and SBC) at low-moderate exercise intensities (FatMax1, VT1, FatMax2 and EPOC) and decreased Lac at low-moderate and submaximal intensities (FatMax1, VT1, VT2, FatMax2 and EPOC) in amateur cyclists. In addition, 2S-hesperidin intake prevented the decrease in pO_2 at VT2, which is associated to the prevention of a decrease in VO_2 and performance in periods of less training volume and load (738). These findings position 2S-hesperidin as a new ergogenic aid, which may help cyclists to improve performance at high intensities (VT2) and recovery after very high intensity exercise (P_{MAX}). Furthermore, the improvements in acid-base status and decrease in Lac in FatMax1 and VT1 generated after 2S-hesperidin ingestion are linked to the maintenance of fatty acid oxidation in FatMax1 and VT1 (738), indicating a greater contribution of the aerobic pathway at low-moderate intensities relative to placebo.

CAPÍTULO XIII.
DISCUSIÓN GENERAL

CAPÍTULO XIII. DISCUSIÓN GENERAL

La búsqueda de nuevas ayudas ergogénicas que permitan a los deportistas, independientemente de su nivel, mejorar tanto el rendimiento como los procesos de recuperación, es un hecho que está documentado desde hace tiempo, con una amplia búsqueda de moléculas de diferente procedencia (902). Sin embargo, en los últimos años, la comunidad científica se está centrando en averiguar si ciertas moléculas denominadas polifenoles, procedentes de las plantas (principalmente, frutas y verduras) tienen la capacidad de modular alguno de los mecanismos que regulan el rendimiento y la recuperación del deportista (27, 903, 904). En este sentido, los hallazgos encontrados muestran diferentes tipos de evidencia dependiendo de la sustancia utilizada. Ya que, los polifenoles son un grupo muy amplio de moléculas y están clasificados en varios subgrupos según su estructura química: ácidos fenólicos, estilbenos, curcuminoides, lignanos, elagitaninos, ácido elágico, cumarinas y flavonoides, además, el último subgrupo se divide en otros subgrupos: flavonoles, flavan-3-oles, flavonas, isoflavonas, antocianinas, dihidrochalconas, proantocianidinas y flavanonas, encontrándose en este último subgrupo de flavonoides la 2S-hesperidina (905), que es la molécula protagonista de esta Tesis Doctoral.

Debido al creciente interés sobre la relación entre la ingesta de flavonoides (polifenoles) y el rendimiento deportivo y la recuperación a la vista de los efectos positivos en modelos patológicos (30, 658, 906, 907).y debido a la escasa información que existe en la utilización de la hesperidina para tal fin, se decidió evaluar la ingesta de 2S-hesperidina tanto a corto plazo (500 mg/d en una sola dosis) o a largo plazo (500 mg/d durante 8 semanas) en ciclistas amateur, atendiendo a los siguientes objetivos: 1) determinar si existían diferencias en el sistema antioxidante endógeno entre ciclistas profesionales y amateur, y averiguar si éstas tienen relación con el rendimiento deportivo; 2) determinar si existían diferencias en la composición corporal (masa ósea y muscular) entre ciclistas profesionales y amateur, y averiguar si éstas tienen relación con el rendimiento deportivo; 3) evaluar el efecto de la ingesta aguda (una dosis de 500 mg/d) de 2S-hesperidina en el rendimiento anaeróbico, cambios metabólicos y energéticos en VT1 y en el sistema antioxidante endógeno en ciclistas amateur; 4) evaluar el efecto de la ingesta crónica (500 mg/d para 8 semanas) de 2S-hesperidina en marcadores de rendimiento mediante un test incremental, y el rendimiento anaeróbico, además de evaluar marcadores metabólicos y de eficiencia energética en ciclistas amateur; 5) evaluar el efecto de la ingesta crónica (500 mg/d para 8 semanas) de 2S-hesperidina en marcadores del estado antioxidante/oxidante endógeno, además de marcadores inflamatorios en ciclistas amateur; 6) evaluar el efecto de la ingesta crónica

(500 mg/d para 8 semanas) de 2S-hesperidina en marcadores de la composición corporal, además, de los marcadores medidos en la tasa metabólica en reposo pre y post intervención en ciclistas amateur; y 7) evaluar el efecto de la ingesta crónica (500 mg/d para 8 semanas) de 2S-hesperidina en marcadores de gasometría y del estado ácido base en ciclistas amateur. Estos objetivos se establecieron, con el fin de averiguar que mecanismos podrían ser los responsables de una posible mejora del rendimiento o recuperación tras los test realizados.

Tras realizar el estudio 1 (741), encontramos diferencias significativas en la producción de potencia (W) y VO_2 en VT_1 , VT_2 y en VO_{2MAX} , tanto en valores absolutos como relativos en ciclistas profesionales (PRO) comparado a ciclistas amateur (AMA). Lo que indica mejores valores de rendimiento en PRO. Sin embargo, solo encontramos diferencias significativas en el sistema antioxidante endógeno, específicamente, niveles superiores en CAT, GSSG y ratio %GSSG/GSH, pero inferiores SOD en PRO comparado a AMA. Mostrando que no hay un perfil definido de antioxidantes endógenos entre ambos grupos de ciclistas estudiados, que previamente no se había investigado. Además, y cumpliendo uno de los objetivos del estudio, encontramos una relación entre el ratio %GSSG/GSH y W_{VT_1} y W_{VT_2} en PRO, por lo tanto, apreció un vínculo entre un menor estado oxidante (\downarrow %GSSG/GSH) y un mayor rendimiento (W) en VT_1 y VT_2 . La exploración de las características de la muestra, a nivel de rendimiento y niveles de enzimas antioxidantes endógenas, creíamos que era esencial, ya que, diferencias en los niveles de ambos marcadores podrían generar un sesgo tras la evaluación y análisis de los resultados. Desde el primer momento, la idea era evaluar de una misma manera la 2S-hesperidina tanto en una muestra de ciclista PRO y AMA. Pero debido al difícil acceso a equipos profesionales de ciclismo, sobre todo por las dificultades a la hora de ejecutar las intervenciones debido a su ajustado planing de entrenamientos y viajes, fue imposible. Por este motivo, los ensayos clínicos sólo se realizaron en ciclistas amateurs.

En el estudio 2 (908), buscábamos establecer posibles diferencias en la composición corporal que pudieran influir en los resultados de los posteriores estudios planificados (549, 738, 787, 843). En este estudio observamos que todos los PRO tenían niveles inferiores del valor de 1.033 g/cm^2 que es el valor de normalidad establecido por la NHAMES III (594), por el contrario, sólo 7 de 15 AMA tenían un valor inferior. Además, los niveles de BMD, BMC y BA fueron inferiores en PRO comparado a AMA. Previamente, otros autores han descrito esta situación cuando han investigado la BMD, encontrando niveles bajos en ciclistas élite y master (593, 595). Lo que indica que ciclismo puede afectar de forma negativa en diferentes variables de salud ósea.

Además, también encontramos niveles inferiores de FFM en PRO comparado a AMA, y correlaciones positivas entre FFM y variables del estado óseo (BMD y CMO), además, hubo una mejora del modelo de regresión lineal al comparar las variables de

rendimiento y de composición corporal ajustadas a la FFM en ambos grupos, excepto para la relación W_{MAX} y BMD. Esto nos hace pensar que existe una relación entre el eje hueso-masa muscular-potencia, que anteriormente no había sido descrita en la bibliografía científica. Lo cual nos hace pensar que habría que realizar algún tipo de vigilancia del estado óseo de los ciclistas en general, poniendo especial atención en los PRO. Tras finalizar este estudio, el análisis descriptivo se terminó y comenzamos con los estudios de intervención, primero utilizando una ingesta aguda (una sola dosis de 500 mg/d de 2S-hesperidina) y después utilizando una ingesta crónica (500 mg/d durante 8 semanas) en ciclistas amateur.

En el estudio 3 (549), el objetivo fue averiguar si la ingesta de una sola toma de 500 mg/d de 2S-hesperidina en el desayuno podría mejorar el rendimiento anaeróbico mediante un test de Wingate y cambios en marcadores metabólicos y energéticos mediante un test rectangular en VT1. En el test de Wingate el grupo que ingirió 2S-hesperidina tuvo mejores datos de sprint que placebo en potencia media (567.84 ± 55.44 vs 555.25 ± 51.81), revoluciones a velocidad máxima (132.86 ± 9.59 vs 128.70 ± 9.24) y la energía total producida (J) (Σ de los 4 sprints). Estos resultados van en línea con la mejora del rendimiento físico observada en ratas entrenadas tras la suplementación con 2S-hesperidina (33) o en el rendimiento en pruebas de contrarreloj de ciclismo en atletas masculinos entrenados tras la suplementación con 2S-hesperidina (500 mg/día) durante 4 semanas (32). Estos estudios informaron de mejoras del 58% en el tiempo de la prueba de agotamiento y del 5% en la potencia absoluta (W) de una contrarreloj de 10 minutos, respectivamente. Por lo tanto, estos datos demuestran que la suplementación con 2S-hesperidina mejora el rendimiento físico en una prueba anaeróbica como el test de sprint repetido (test de Wingate).

En este estudio también se encontró pequeños cambios en diferentes enzimas antioxidantes (CAT y SOD), péptidos con actividad antioxidante (GSSG/GSH) y marcadores de oxidación (MDA-TBARS) entre el grupo de 2S-hesperidina y placebo. Precisamente, se observó un pequeño aumento no significativo de la actividad de la CAT, frente al placebo, tras la suplementación aguda con 2S-hesperidina (Cardiose®). Asimismo, se observó una correlación inversa entre los niveles excretados de metabolitos de hesperidina en orina y las variaciones porcentuales de la actividad de CAT $\% \Delta$ 01-03 ($p = 0.013$). En ratas sometidas a un ejercicio intenso, la suplementación con 2S-hesperidina contribuyó a mantener la actividad de la CAT y a evitar los cambios inducidos por la actividad física (33). En base a los resultados encontrados, podemos establecer que la ingesta aguda de 2S-hesperidina (500 mg/d) podría promover la actividad de esta enzima antioxidante (CAT). Un aumento de la actividad de CAT durante el ejercicio puede ofrecer una ventaja en los esfuerzos de alta intensidad (por ejemplo, el sprint), donde hay una alta tasa de producción de ROS (651), disminuyendo el daño a la célula muscular.

Además, se observó una pequeña disminución no significativa de la actividad de la SOD tras la realización del test de esprint repetido en el grupo 2S-hesperidina (-5,9%) pero no en el placebo (-0,9%). Esta disminución se mantuvo 24 h después del final de la sesión de ejercicio. En estudio anterior, la suplementación con 2S-hesperidina disminuyó la actividad de la SOD en ratas entrenadas después de un test hasta el agotamiento y tras un periodo de entrenamiento de 56 días (33). La 2S-hesperidina parece reducir la sobreexpresión de SOD inducida por el ejercicio físico. Esto puede ser debido a su actividad eliminadora de radicales libres por parte de la SOD (714), la hesperidina neutraliza las especies reactivas de oxígeno -como el anión superóxido- generadas durante condiciones de estrés oxidativo, como el ejercicio físico intenso. La disminución de la actividad de la SOD puede estar relacionada con la menor necesidad de esta enzima endógena cuando se proporciona un antioxidante exógeno, como la hesperidina u otros flavonoides (687).

La suplementación con Cardiose® no produjo un aumento significativo de los niveles de GSH, pero se observó una correlación significativa (GSH % Δ 01-02, $r = 0,551$; $p = 0,033$) entre los niveles de metabolitos de hesperidina en la orina y las variaciones porcentuales de los niveles de GSH. Además, se observó una tendencia diferente en GSH y GSSG en el grupo suplementado. El placebo mostró un aumento de la relación GSSG/GSH tras el protocolo de ejercicio, mientras que la suplementación con 2S-hesperidina disminuyó la relación GSSG/GSH. La razón de estas modificaciones en GSH y GSSG podría ser causada por las bajas concentraciones de lipoperóxidos (\downarrow TBARS: -5.7% tras el test de esprints repetidos y -2.3% tras el protocolo rectangular en 2S-hesperidina) y peróxido de hidrógeno en el grupo que ingirió 2S-hesperidina, que son metabolizados por la GPX, generando un aumento de GSSG (692). Este aumento es neutralizado por el aumento de la actividad de la glutatión reductasa (GR) (693). Estos resultados sugieren que la ingesta aguda de 2S-hesperidina promueve la función antioxidante del glutatión tras un ejercicio de alta intensidad, lo que indica un mejor estado antioxidante en el grupo experimental en ciclistas amateur.

En el estudio 4 (738), el objetivo de esta investigación fue evaluar la ingesta crónica de 2S-hesperidina (500 mg/d para 8 semanas) en marcadores de rendimiento aeróbico y anaeróbico mediante un test incremental y el rendimiento anaeróbico mediante un test de Wingate, además de evaluar cambios en marcadores metabólicos durante un test rectangular en ciclistas amateur. En el test incremental la ingesta de 2S-hesperidina mejoró el rendimiento en el FTP estimado (2.3% = 6.4W; $p = 0.049$) y potencia máxima (1.9% = 7.4 W; $p = 0.049$), sin embargo, en el grupo placebo no se observaron cambios en el FTP estimado (-0.9 % = -2.51W; $p = 0.387$) y potencia máxima (-0.8% = -2.9W; $p = 0.388$). Además, en la comparación entre grupos se encontramos un incremento en el FTP estimado (3.2% = 8.9W; $p = 0.042$; $\eta^2 = 0.107$; ES = 0.68) y en la potencia máxima (2.7% = 10.3W; $p = 0.042$; $\eta^2 = 0.107$; ES = 0.68) en 2S-hesperidina

comparado a placebo. Esta mejora del rendimiento por la 2S-hesperidina fue confirmada por el hallazgo de una correlación positiva entre los niveles de metabolitos de hesperidina excretados en la orina y la diferencia en la potencia máxima ($r = 0.701$; $p < 0.001$) y el FTP estimado ($r = 0.725$; $p < 0.001$) en el grupo suplementado. En lo que respecta a la suplementación con flavonoides, un estudio anterior informó de un aumento del 5% en la producción de potencia absoluta en una prueba de tiempo (TT) de 10 minutos tras 4 semanas de ingesta de 2S-hesperidina (500 mg) en ciclistas (32). Otros autores también han encontrado mejoras en el rendimiento del 58% (tiempo hasta el agotamiento) en animales (33, 34). Recientemente, se ha publicado un artículo donde la suplementación crónica con un extracto de flavonoides de cítricos (alto contenido en 2S-hesperidina) mejoró la capacidad anaeróbica y la potencia máxima durante un ejercicio de alta intensidad en individuos moderadamente entrenados (909).

Algunos autores han demostrado que la hesperidina tiene un efecto antioxidante y promueve la síntesis de óxido nítrico en diferentes modelos de estudio patológico (31, 33, 531, 711, 712). En un modelo de rata con pleuritis, la actividad antioxidante de la hesperidina redujo la producción de ROS en el hígado y aumentó las actividades hepáticas de CAT y SOD (711). Estruel-Amades et al. (33) observaron que cinco semanas de suplementación con 2S-hesperidina (200 mg/kg 3 días a la semana) prevenían el aumento de ROS y la disminución de la actividad de SOD y CAT tras una prueba hasta el agotamiento en el timo y el bazo de ratones con un plan de entrenamiento intensivo. Esta actividad "scavenging" de la hesperidina neutraliza las especies reactivas del oxígeno, como el anión superóxido, generadas durante condiciones de estrés oxidativo, como el ejercicio físico intenso (714).

Como se ha comentado anteriormente, la 2S-hesperidina tiene la capacidad de aumentar la producción del NO, aumentando la vasodilatación que es un mecanismo fisiológico utilizado no sólo para el suministro de sangre oxigenada, sino también para la entrega de glucosa, lípidos y otros nutrientes a una variedad de tejidos (715). Teóricamente, el aumento del flujo sanguíneo incrementaría el suministro de O₂ y nutrientes (por ejemplo, aminoácidos y glucosa) al músculo esquelético que se ejercita, ayudando así al rendimiento del ejercicio durante la alta intensidad (condiciones de hipoxia) (716). Este mecanismo sumado a una mejora del estado antioxidante endógeno pueden ser los responsables de la mejora del rendimiento en el FTP estimado y la potencia máxima en el test incremental en el grupo que consumió 2S-hesperidina.

Además, en el test rectangular encontramos diferencias entre la ingesta de 2S-hesperidina y placebo, indicando desajustes mediados por la reducción del volumen y la intensidad del entrenamiento durante el periodo del estudio (718, 719), identificados como una disminución de la FAT (FatMax y VT1) y una disminución del VO₂R

(mL/kg/min) (VT2) en el placebo. Estos hallazgos están en línea con los encontrados en el test incremental, donde los desajustes al entrenamiento (potencia generada en el FTP estimado y potencia máxima) también estuvieron presentes. Estos cambios pueden justificar el descenso del rendimiento a intensidades submáximas y máximas del placebo en nuestro estudio. Sin embargo, la ingesta de 2S-hesperidina no pudo evitar la pérdida de rendimiento en VT1, aunque no fue significativa, pero sí mantuvo el rendimiento en VT2 y lo mejoró en el FTP estimado y en la potencia máxima. Las posibles vías utilizadas para que la ingesta crónica de 2S-hesperidina podría disminuir los cambios fisiológicos derivados del desentrenamiento estarían relacionados con componentes moduladores génicos, como AMPK y PGC-1 (35, 720, 721), que controlan la producción de energía, la utilización de sustratos metabólicos (grasas y carbohidratos), la biogénesis mitocondrial y la capacidad oxidativa (722, 723). Esto apoya nuestra hipótesis de que la ingesta crónica de 2S-hesperidina podría ayudar a generar o mantener las adaptaciones a nivel mitocondrial y del sistema antioxidante endógeno en un periodo en el que el volumen y la intensidad del entrenamiento disminuyen, como en el estudio realizado (finales de septiembre-mediados de diciembre), manteniendo los niveles de rendimiento en el ejercicio de alta intensidad en ciclistas amateurs.

Nuestros resultados sugieren que la ingesta crónica de 2S-hesperidina puede prevenir la disminución del VO_2R (mL/kg/min) (VT2) que se asocia a una disminución de la capacidad de producir potencia en los ciclistas amateur y a un descenso de la FAT (FatMax y VT1) con una disminución de la capacidad de producción de energía en los ciclistas. Por lo que, una disminución en la oxidación de grasas en FatMax y VT1 y aumento de la utilización de carbohidratos a intensidades bajas-moderadas podría anticipar la fatiga en un posterior trabajo de alta intensidad, como en una competición ciclista.

Los resultados obtenidos (estudio 4) en el test de Wingate tras la ingesta de 2S-hesperidina mostraron una mejora tanto en la potencia inicial absoluta como en la relativa en comparación con el placebo. Por otra parte, ambos grupos mejoraron ambas variables de potencia para un esprint de 30 s, sin que hubiera diferencias al comparar los grupos. En la actualidad, no existen otros estudios que hayan evaluado la ingesta crónica de 2S-hesperidina mediante un test de Wingate, excepto nuestro estudio de efectos agudos (549), donde se observaron mejoras en la potencia media (2.3%) y en la velocidad máxima (3.2%) durante una prueba repetida de esprint de 30 s en ciclistas amateur tras una ingesta aguda de 2S-hesperidina. Sin embargo, no hay estudios anteriores que hayan evaluado el efecto de la ingesta crónica de hesperidina en la capacidad anaeróbica máxima (no oxidativa). Sin embargo, la ingesta combinada de mangiferina y luteolina (polifenoles) durante 15 días también ha mostrado mejoras en la potencia media (5.0%) durante un test de Wingate (654). En las pruebas de esfuerzo

máximo de corta duración, algunos de los cambios pueden explicarse por un efecto de aprendizaje inicial, seguido de una variación típica dentro de las pruebas (730). Teniendo en cuenta que las diferencias significativas entre las dos condiciones experimentales han sido pequeñas en las medidas evaluadas en el test de Wingate, hay que tener en cuenta que en este tipo de pruebas pueden ser susceptibles a los efectos del placebo, nocebo o Hawthorne (731, 732). Consideramos que en este tipo de test (Wingate) la familiarización puede tener un efecto importante en los resultados finales, por lo que para futuras investigaciones introduciremos una comparación entre los valores obtenidos en la familiarización y el placebo, con el fin de observar las variaciones que puedan afectar al resultado final o al comparar los grupos experimentales teniendo en cuenta la variabilidad del test (734).

En el estudio 5 (787), los objetivos fueron evaluar la ingesta crónica (500 mg/d para 8 semanas) de 2S-hesperidina en marcadores del estado antioxidante/oxidante endógeno e inflamatorios pre, al finalizar y tras 30 min después de finalizar un test rectangular en ciclistas amateur. Durante el test rectangular, observamos un mantenimiento de la SOD desde los valores basales hasta Post-PMAX y un aumento hasta Post-REC en la primera visita, con aumentos significativos en Post-PMAX y la Post-REC pre-post-intervención en el grupo que ingirió 2S-hesperidina. Sin embargo, se observó un aumento en los niveles de actividad de la SOD, evaluados como el AUC, para ambos grupos durante la intervención. En ciclistas amateur (Estudio 1), la ingesta aguda de 2S-hesperidina (dosis única; 500 mg) no provocó una disminución significativa de la SOD en la línea de base (549). En animales, la suplementación con 2S-hesperidina (200 mg/kg durante 3 días a la semana), junto con un programa de entrenamiento de 5 semanas, tampoco produjo cambios significativos en la actividad de la SOD en ratas tras una prueba de ejercicio exhaustivo (33).

El aumento de la capacidad antioxidante de la 2S-hesperidina puede explicarse por las características antioxidantes de esta molécula, relacionadas con los grupos hidroxilos de su anillo B (748). Además, Parhiz et al. (749) descubrieron que la hesperidina tenía una importante actividad de “scavenging” (neutralizadora) de radicales y evitaba el daño oxidativo inducido por el H₂O₂ en las membranas celulares de los glóbulos rojos, con actividades de “scavenging” de radicales comparables a las del ácido ascórbico y trolox (un derivado de la vitamina E). Además, la 2S-hesperidina ha mostrado un efecto neutralizador de la peroxidación lipídica no enzimática y del radical superóxido, hidroxilo, peroxinitrito y óxido nítrico (454, 748), lo que conduce a un menor agotamiento de las enzimas antioxidantes y permite el mantenimiento de altos niveles de antioxidantes, incluso después del estrés oxidativo inducido por el ejercicio. En base a los hallazgos encontrados, la mayor actividad de la SOD al final del esfuerzo máximo y tras un breve período de recuperación tras la intervención, indica

que la ingesta crónica de 2S-hesperidina mejora la capacidad antioxidante en el esfuerzo máximo y en la fase aguda de recuperación en los ciclistas aficionados.

Por otro lado, al comparar ambos grupos los valores basales pre post-intervención, el grupo de 2S-hesperidina tenía valores más bajos del GSSG que el placebo, lo que indica niveles inferiores de estrés oxidativo. Esto está en consonancia con la disminución encontrada en el AUC (GSSG) en 2S-hesperidina, lo que indica una disminución del estrés oxidativo cuando se considera la prueba rectangular completa, que puede estar relacionada con la adaptación al desentrenamiento. De hecho, los volúmenes e intensidades de entrenamiento más bajos se asocian con niveles más bajos de GSH y GSSG en ciclistas profesionales (568), lo que también se encontró Post-PMAX en el placebo. A pesar de que no existen estudios previos en humanos que evalúen los efectos de la ingesta crónica de hesperidina sobre el GSH y GSSG, en cambio, se observaron disminuciones no significativas del GSH, GSSG y la relación GSSG/GSH tras una prueba de sprint repetida en ciclistas aficionados después de una dosis única de 2S-hesperidina (500 mg) (549). Del mismo modo, los modelos animales patológicos han demostrado el efecto positivo de la suplementación con hesperidina sobre estos marcadores del glutatión (\uparrow GSH y \downarrow GSSG) (694, 754). Por lo tanto, la ingesta crónica de 2S-hesperidina podría disminuir los niveles de GSSG (evidenciados por el \downarrow del AUC), indicando un mejor estado antioxidante en la prueba rectangular, pero específicamente inmediatamente después del ejercicio. Esto facilitaría una más rápida recuperación post-entrenamiento o competición para los ciclistas. La principal ventaja de incorporar el AUC en este estudio es que permite definir con precisión la duración y la magnitud de la variable evaluada, lo que no puede hacerse en una comparación punto por punto (753).

En este estudio, los niveles de IL6 aumentaron durante la primera y la segunda prueba rectangular desde la línea de base hasta el Post-PMAX en ambos grupos, pero hubo diferentes tendencias desde el Post-PMAX hasta el Post-REC en la segunda prueba rectangular (\downarrow 2S-hesperidina y \uparrow placebo). Se observó una disminución significativa de la IL6 durante la etapa de recuperación en el placebo, después de la intervención. Hasta donde sabemos, ningún estudio ha evaluado los efectos de la ingesta de 2S-hesperidina sobre marcadores inflamatorios en humanos. Pero, otros flavonoides, como los flavanoles derivados del cacao, tampoco han logrado inhibir el aumento de la IL6 tras un ejercicio intenso (75% de la potencia máxima durante 30 minutos) en ciclistas (763). Creemos que la alta variabilidad de los datos de IL6 fue un factor que no nos permitió encontrar diferencias significativas intra e intergrupo. Además, los valores de IL6 en el placebo fueron cuantitativamente más altos que los de la 2S-hesperidina, lo que puede favorecer una disminución significativa de la IL6 tras la reducción de la carga de entrenamiento realizada por los ciclistas de la postemporada a la pretemporada, periodo en el que se realizó el estudio (desde finales

de septiembre a finales de diciembre). Sin embargo, un estudio reciente en animales entrenados demostró que la ingesta de hesperidina (200 mg/kg durante 3 días a la semana) durante 5 semanas previno el aumento de los niveles de IL6 en los macrófagos peritoneales tras un ejercicio hasta el agotamiento (34). Curiosamente, en este estudio, se observó un aumento significativo de la IL6 después de un ejercicio agotador, desde antes del entrenamiento hasta después de la intervención, en el grupo de placebo. Además, la ingesta de hesperidina también ha sido capaz de disminuir los niveles de IL6 en un modelo de rata con artritis reumatoide (703).

En el apartado de marcadores inflamatorios, nosotros observamos niveles inferiores de MPC-1 durante todo el ejercicio (AUC) tras la suplementación en ambos grupos. Esta disminución fue estadísticamente significativa en los valores basales (-20.2%) y durante la fase de recuperación (-26.1%) para el grupo suplementado con 2S-hesperidina. Además, cuando se comparó entre los grupos los diferentes momentos del test rectangular tras la intervención, el grupo de 2S-hesperidina tuvo niveles más bajos (-17.6%, -17.4% y -18.4%, respectivamente) en comparación con el placebo. En estudios anteriores en modelo de daño pulmonar agudo, tanto in vitro como in vivo, la hesperidina ha mostrado efectos inmunomoduladores, regulando a la baja la expresión de MCP1 así como de otras citoquinas proinflamatorias, como IL6 y TNF (764, 773). Precisamente, se ha observado que el tratamiento con hesperetina-7-O-glucuronido (5 mg·kg⁻¹) disminuye la expresión del ARNm de MCP1 en células endoteliales de la aorta de rata (773). Por otra parte, la administración oral de 100 o 200 mg/kg de hesperidina 3 veces por semana durante 4 semanas en ratas produjo una disminución de las citoquinas proinflamatorias citoquinas proinflamatorias interferón-gamma- γ y MCP1 en linfocitos del ganglio linfático mesentérico (33). En particular, la hesperidina puede aumentar la abundancia de *Faecalibacterium prausnitzii*, que inhibe la activación del NF-B y, en consecuencia, atenúa la respuesta inflamatoria (774). La capacidad inhibidora de la hesperidina en algunas bacterias puede modificar la composición de la microbiota intestinal actuando como inmunomodulador y antiinflamatorio (\downarrow IL-1, TNF e IL6), con una relación directa entre ambos efectos (470).

Los efectos de la administración de suplementos antioxidantes sobre el rendimiento son un tema controvertido, que todavía necesita más investigación. Por un lado, se ha señalado que el uso de sustancias antioxidantes puede ayudar a mantener niveles óptimos de ROS en el músculo, evitando posibles disminuciones del rendimiento (534). Por otro lado, se ha planteado la hipótesis de que la ingesta crónica de antioxidantes puede dificultar las adaptaciones al entrenamiento, afectando negativamente al rendimiento (745). Sin embargo, un estudio ha mostrado como la ingesta de antioxidantes no inhibe la activación de las vías de señalización sensibles al estado redox inducidas por el ejercicio (776). No obstante, en el estudio 4 (738) donde se realizaron las mediciones de marcadores de rendimiento se llevó a cabo en el mismo

proyecto que el estudio 5 (787), donde se realizaron las mediciones de los marcadores antioxidantes e inflamatorios, estos estudios fueron realizados de forma paralela. Pues bien, en el estudio 4 (738) la suplementación en ciclistas amateurs (8 semanas) con 2S-hesperidina (500 mg/día) mostró un aumento de la producción en el FTP estimado ($2.3\% = 6.40W$; $p = 0.049$) y en la potencia máxima ($1.9\% = 7.40W$; $p = 0.049$) durante una prueba incremental después de la intervención (738). Por lo tanto, la suplementación crónica con 2S-hesperidina no parece interferir con las adaptaciones inducidas por el entrenamiento, mejorando el rendimiento y evitando el estrés oxidativo y la inflamación después de un ejercicio máximo y en la fase aguda de la recuperación en ciclistas amateur.

En el estudio 6 (843), el objetivo fue evaluar el efecto de la ingesta crónica (500 mg/d para 8 semanas) de 2S-hesperidina en marcadores de la composición corporal mediante DXA y antropometría, además de los cambios metabólicos en la tasa metabólica en reposo. En cuanto a los resultados del DXA, este estudio mostró mejoras en la composición corporal tras la ingesta de 2S-hesperidina. En concreto, se observó una disminución significativa del porcentaje de BF (-15.3%), BF total (-17.9%) y LLFM (-15.5%) en el grupo de 2S-hesperidina mientras que no hubo cambios en el grupo de placebo. Hay que mencionar, que la potencia de los datos obtenidos con la DXA tiene una alta fiabilidad, ya que es un método preciso y exacto para medir la composición corporal (788). Resultados similares a los obtenidos en la DXA fueron encontradas en las mediciones antropométricas, donde se observó una disminución significativa del %BF (-3.9%), del TFB (-3.1%) y del Σ de 8 pliegues cutáneos (-6.5%) tras la ingestión de 2S-hesperidina de forma crónica en ciclistas amateur. Además, en este grupo se encontraron correlaciones positivas inversas entre la excreción total de metabolitos de 2S-hesperidina en orina y el porcentaje de masa grasa ($r = -0.592$; $p = 0.006$) y el Σ de 8 pliegues cutáneos ($r = -0.550$; $p = 0.012$).

Hasta donde sabemos, no hay estudios anteriores que hayan informado del efecto de la ingesta de hesperidina sobre la masa grasa. Pero en línea con los resultados descritos, Dallas et al. (789) encontraron mejoras en el porcentaje de masa grasa corporal (experimental -9.7% vs. placebo -3.2%; diferencias significativas entre grupos $p < 0.001$) tras 12 semanas de ingesta de extracto de fruta rico en polifenoles (al menos un 20% de flavanonas totales) en combinación con 30 min semanales de actividad física en una población sana con sobrepeso. Los resultados del componente graso tanto medidos con la DXA como con la antropometría fueron similares.

La disminución de la masa grasa tras la ingesta de 2S-hesperidina podría explicarse por un aumento de la activación de SIRT1 y PGC-1 que llevaría a la sobreexpresión de genes relacionados con la respiración mitocondrial y la oxidación de ácidos grasos a nivel muscular (790). Por lo tanto, la disminución de la masa grasa en el grupo experimental también podría deberse a una mayor oxidación de grasas en

FatMax y VT1 (estudio 4) tras la ingesta de 2S-hesperidina en ciclistas amateur, mientras que el grupo placebo disminuyó significativamente su oxidación de ácidos grasos (FatMax; -65.6% y VT1; -65.7%) (738). Además, como ya hemos comentado anteriormente, en el estudio 4 se observó un aumento de la potencia en el FTP estimado (2.3%) y en la potencia máxima (1.9%) en una prueba incremental.

Estos resultados indican una disminución de la capacidad de oxidación de los ácidos grasos en el placebo, lo que conduce a una menor utilización de las grasas a intensidades bajas y moderadas. Dado que se sabe que un alto porcentaje del volumen de entrenamiento de los ciclistas se realiza en intensidad baja y moderada, una disminución de la capacidad de oxidación de las grasas podría conducir a cambios en la masa grasa corporal a largo plazo (semanas o meses). Por tanto, los cambios encontrados en el estudio 4 (738) justificarían la reducción de la masa grasa tras la suplementación con 2S-hesperidina descrita en este estudio. La combinación de los efectos de una mejora de la composición corporal unido a la mejora del rendimiento tras la ingesta de 2S-hesperidina, son 2 de los factores claves en el éxito de los ciclistas en sus respectivas competiciones, sobre todo en etapas de montaña, donde la relación peso/potencia gana relevancia. Por todo ello, la suplementación con 2S-hesperidina sería una herramienta útil para reducir la masa grasa en los ciclistas. Pero Teniendo en cuenta los resultados descritos, así como, que no se incluyó ningún control dietético, el potencial de la 2S-hesperidina para perder masa grasa combinada con un control dietético adecuado, puede ser aún mayor.

En cuanto al componente muscular, en la DXA, el grupo placebo experimentó una disminución significativa del LM% (-1.4%; -0.597 kg), mientras que no se encontraron cambios en la 2S-hesperidina (0.3%; 0.224 kg). Tampoco se encontraron cambios significativos en la comparación entre grupos, pero se observó un tamaño del efecto moderado en la LM (-1.5%; -0.822 kg) a favor del placebo. En la antropometría, se registró un aumento significativo del %MM (0.9%) y del TMM (1.8%) en el grupo de la 2S-hesperidina tras el período de intervención. Además, se encontró una relación positiva entre la excreción urinaria de metabolitos de 2S-hesperidina y el % MM ($r = 0.487$; $p = 0.029$), apoyando el efecto positivo de la 2S-hesperidina sobre la masa muscular. Biesemann et al. (35) encontraron que la hesperetina (principal metabolito de la 2S-hesperidina) ($50 \text{ mg kg}^{-1} \cdot \text{d}^{-1}$), además de reducir el estrés oxidativo en los miotubos (células musculares) invirtió completamente la disminución de la masa muscular relacionada con la edad en ratones y, por lo tanto, puede haber desempeñado un papel en la mejora del rendimiento en carrera. En cuanto a la relación entre la ingesta de hesperidina y la masa muscular, Jeon et al. (792) observó una función promiogénica de la hesperidina con diferentes mecanismos de regulación, que incluyen la promoción de la localización nuclear de MyoD y su interacción con los promotores de

los genes diana, así como la mejora de la transcripción de genes miogénicos mediada por MyoD y la diferenciación miogénica.

Además, teniendo en cuenta que los datos aportados por este estudio (composición corporal) y los del estudio 4 (738) formaban parte del mismo proyecto, que se realizó al mismo tiempo y con la misma muestra se decidió realizar un estudio correlacional entre las variables. En este sentido, se encontraron correlaciones significativas entre los cambios en la potencia máxima ($r = 0.471$; $p = 0.036$) y el FTP estimado ($r = 0.466$; $p = 0.035$) y cambios en el MM% evaluado a través de la antropometría. Tras observar un aumento de la MM tras la ingesta de 2S-hesperidina en este estudio, parece que esta flavanona no sólo ayuda a conservar el tejido muscular, sino que también lo aumenta en los AMA. Los ciclistas están especialmente expuestos a la pérdida de masa muscular (catabolismo muscular) debido a los grandes volúmenes e intensidades de entrenamiento combinados con la restricción calórica. Los aumentos descritos en %MM en el estudio actual, que se correlacionan con la mejora en el rendimiento (FTP estimado y potencia máxima) del estudio 4 (738), sugieren que la ingesta de 2S-hesperidina promueve una mayor producción de potencia a altas intensidades de ejercicio debido al mantenimiento e incluso el aumento de la masa muscular. Por lo tanto, la 2S-hesperidina puede ser un suplemento ideal para mejorar la composición corporal y el rendimiento de los ciclistas.

En el estudio 7 (bajo revisión), el objetivo fue evaluar el efecto de la ingesta crónica (500 mg/d para 8 semanas) de 2S-hesperidina en marcadores del metabolismo del oxígeno y estado ácido-base en sangre capilar antes, durante (FatMax1, VT1, VT2, P_{MAX}, FatMax2) al finalizar y después de 30 min de finalizar (EPOC) un test rectangular en ciclistas amateur. Los hallazgos principales estuvieron relacionados con la mejora del sistema tampón (\uparrow HCO₃⁻, SBC y ABE) y \downarrow de Lac en FatMax1, VT1, FatMax2 y EPOC, más un aumento de pCO₂ y tCO₂ a estas intensidades de ejercicio post-intervención en el grupo que se suplementó con 2S-hesperidina. También se encontró un descenso no significativo del Lac en VT2, pero con un tamaño del efecto grande ($p = 0.135$; ES = 1.49) en el grupo 2S-hesperidina, pero no en el grupo placebo ($p = 0.741$; ES = 0.32) post-intervención. Estos últimos resultados, van en línea con los encontrados en el estudio 4, donde se encontró una mejora de la potencia generada en el FTP estimado (2.3% = 6.4W) en el grupo que ingirió de forma crónica 2S-hesperidina, que es una zona de ejercicio cercana al VT2.

Otro hallazgo importante, fue el descenso en VT2 de la pO₂ en el grupo placebo tras la intervención. Este último hallazgo va en línea con el encontrado en el estudio 4 (738), donde se encontró un descenso significativo en el VO₂ (L/min) (\downarrow 8.3%; $p = 0.01$) and VO₂R (mL/kg/min) (\downarrow 8.9%; $p = 0.01$) en VT2 en el grupo placebo tras la ingesta de 8 semanas de 2S-hesperidina pero utilizando un test incremental .

Ningún estudio ha evaluado la ingesta ni aguda ni crónica de 2S-hesperidina sobre marcadores del estado ácido-base ni del metabolismo del oxígeno a nivel capilar ni en deportistas ni en población general. Pero la ingesta de 4 semanas de un extracto de cítricos (flavonoides de la semilla de limón) ha mostrado disminuir el lactato tras un ejercicio (corriendo a 1.8 km/h) hasta el agotamiento, en ratones (880). En este sentido, un estudio ha mostrado que el aumento de la expresión del factor de transcripción PGC-1 α es capaz de disminuir la producción de lactato en el músculo esquelético, mediante la regulación al alza de la expresión de la LDH B y la reducción de la LDH A (910). Por lo tanto, PGC-1 α promueve el rápido suministro de energía mediante la oxidación del lactato. Además, se ha visto que la hesperidina es un potente activador de PGC-1 α “*in vitro*” (células musculares), con aumentó del ATP intracelular en un 33% y la capacidad de reserva mitocondrial en un 25% (35). Aunque sólo es una hipótesis a falta de confirmar en humanos, podríamos establecer que un aumento de la activación de PGC-1 α mediado por la ingesta de 2S-hesperidina podría ser la responsable de la disminución del Lac post-intervención (durante el test rectangular y EPOC, menos en P_{MAX}) debido a un aumento de la LDH. Por lo tanto, nuestro actual estudio sería el primero que demuestra que la ingesta crónica de 2S-hesperidina es capaz de disminuir la producción de lactato a intensidades de ejercicio moderadas-submáxima y tras la recuperación (EPOC) de un ejercicio máximo seguido por otro de intensidad baja (FatMax2).

Además, los cambios en el Lac fueron acompañados por un aumento del HCO₃⁻, SBC y ABE a intensidades moderadas tras la ingesta de 2S-hesperidina, pero no en el grupo placebo. Estos cambios fueron unidos a un aumento significativo del pH en FatMax2 y el AUC en el grupo que ingirió 2S-hesperidina en el análisis intragrupo y una diferencia significativa en AUC al comparar los grupos. Esto indica una mejora de la capacidad de taponamiento del organismo, a la misma vez, que una menor utilización de la vía glucolítica anaeróbica. Según nuestro conocimiento, y tras la búsqueda en las bases de datos científicos, no hemos encontrado un estudio donde la ingesta de cualquier tipo de flavonoide mejore marcadores del estado ácido-base. Pero lo que sí está demostrado, es que la administración de sustancias alcalinizantes como el bicarbonato o citrato de sodio (aumentan el bicarbonato y pH sanguíneo) mejoran el rendimiento en pruebas de ciclismo de 4 y 30 km contrarreloj (911, 912). Hay que mencionar, que la alta tasa de recambio de ATP del músculo esquelético que se observa durante el ejercicio de alta intensidad aumenta la producción de iones de H⁺, lo que conduce a la acidosis muscular que se asocia con la pérdida de rendimiento (12). Esto se debe a que una acidosis metabólica inducida por el ejercicio, caracterizada por una mayor producción de H⁺ en lugar de una tasa de eliminación, puede disminuir la generación de sustratos energéticos a través de las vías glucolíticas al reducir la actividad de las enzimas clave como la glucógeno fosforilasa y la PFK (913). Además,

los H^+ también compiten con los iones de Ca^+ por el sitio de unión en la troponina, dificultando directamente la capacidad de contracción del músculo (914). La fosforilación oxidativa también puede ser inhibida por la acidosis (913), mientras que la resíntesis de PC también puede verse comprometida a un pH bajo (915).

Por lo tanto, unos niveles de pH más altos y más bajos de Lac en intensidades bajas-moderadas (FatMax1, VT1 y FatMax2), como encontramos en nuestro estudio tras la ingesta de 2S-hesperidina, indican una menor utilización de la vía anaeróbica láctica para la obtención de energía, lo que conlleva una menor acumulación Lac en estas fases previas al ejercicio de alta intensidad. Esta situación conlleva una tasa de utilización más baja de hidratos de carbono por unidad de tiempo que ha pH más bajo, lo que permite disponer de mayores depósitos de glucógeno para fases del ejercicio donde la intensidad es mayor y son claves para el éxito en las competiciones. Y para finalizar, una mejora en el descenso del Lac post-ejercicio (tras P_{MAX}) tanto en FatMax2 como EPOC tras la intervención, indica una mejor recuperación tras el ejercicio de alta intensidad (P_{MAX}).

CAPÍTULO XIV.
LIMITACIONES

CAPÍTULO XIV. LIMITACIONES

Las limitaciones de los estudios que componen la presente Tesis Doctoral, se exponen a continuación:

- En el estudio **1** y **2** las limitaciones fueron en cuanto al número de la muestra, ya que fue más difícil reclutar atletas PRO que atletas de nivel inferior (AMA). Además, las diferencias en marcadores antioxidantes endógenos entre este estudio y los trabajos anteriores pueden estar influidas por la instrumentación y la metodología utilizadas, el momento de la temporada en el que se realizaron las mediciones, y el estado de entrenamiento de los ciclistas.
- En el estudio **3** la principal limitación también fue el tamaño de la muestra. Ya que, una muestra mayor podría mejorar la potencia de nuestros resultados. Además, de la falta de estudios previos sobre el tema, dificultó la comparación de nuestros resultados.
- En el estudio **4** una limitación fue la falta de disponibilidad de biopsias musculares debido a recursos financieros, para examinar los posibles mecanismos que podrían explicar las mejoras del rendimiento. Ya que, podrían haber aportado una información valiosa.
- En el estudio **5** una de las limitaciones fue el corto tiempo de recuperación después de la prueba rectangular, en el que se evaluaron los cambios en los marcadores antioxidantes e inflamatorios. Las mediciones realizadas 24 y 48 h después del ejercicio habrían proporcionado una valiosa información adicional; sin embargo, las limitaciones en el presupuesto lo hicieron imposible. Además, una muestra más grande nos habría dado más poder estadístico a los resultados reportados debido a la alta variabilidad individual en algunos marcadores. También la falta de estudios que hayan evaluado el efecto de la hesperidina en marcadores antioxidantes e inflamatorio en humanos, hizo complicada la comparativa de nuestros resultados. Las diferencias de nuestros resultados comparado con otros estudios pueden estar relacionadas con la diferente etapa de la temporada en la que se realizaron los estudios en la muestra utilizada, y los diferentes perfiles de demanda aeróbica y anaeróbica de las pruebas utilizadas. Además, otro de los factores que influyen en la variabilidad de los efectos de la 2S-hesperidina en la comparación con otros estudios puede ser su farmacocinética y la consiguiente exposición del organismo a los metabolitos de la hesperidina.

- El estudio 6, al igual que el 4 y 5, se realizó entre septiembre y diciembre (desde final hasta el inicio de la temporada). En esta fase de la temporada, los ciclistas trabajan a menor intensidad y volumen de entrenamiento en relación con otras fases de la temporada. Este es un hecho importante a tener en cuenta al comparar los resultados descritos con otros estudios realizados durante las temporadas de entrenamiento con diferentes volúmenes e intensidades. La selección de las fechas del estudio se hizo en base a el hecho de que suele ser más complicado modificar los hábitos de los ciclistas y controlar su evolución durante la temporada, debido a su periodización individual de cada ciclista.
- El estudio 7, se realizó en las mismas fechas que el estudio 4, 5 y 6, se realizó entre septiembre y diciembre (desde final hasta el inicio de la temporada) Lo ideal es que la gasometría se obtenga en sangre arterial. Sin embargo, los catéteres arteriales permanentes para la toma de muestras de sangre arterial no siempre son factibles ni deseables. Por lo tanto, en el presente estudio se utilizaron métodos indirectos para evaluar los gases sanguíneos. Por lo tanto, debe considerarse el grado en que las mediciones proporcionaron una aproximación precisa a las medidas arteriales. Los gases sanguíneos arteriales (pO_2 y pCO_2) durante el ejercicio podrían estimarse utilizando muestras de sangre capilar (pO_2 y pCO_2). Pero en estudios anteriores se comprobó que las muestras de sangre capilar concuerdan bien con las muestras de sangre arterial para la presión parcial de dióxido de carbono, pero no para la presión parcial de oxígeno. La causa principal de la subestimación de la pO_2 en las muestras de sangre capilar podría ser la insuficiente arterialización de la sangre debido a la mezcla venosa. El método de toma de muestras de sangre capilar requiere un flujo sanguíneo adecuado en el brazo para permitir que se tome un volumen suficiente de sangre sin presión externa adicional durante la toma de muestras. El periodo de la temporada en el que se realizó el estudio fue entre finales de septiembre y mediados de diciembre. Durante este periodo, los ciclistas disminuyen su volumen e intensidad de entrenamiento con respecto a otros momentos de la temporada. Por lo tanto, nuestros resultados deben tomarse con precaución. Ya que, si el estudio se hubiera realizado en otro periodo de la temporada en el que los ciclistas tuvieran más volumen e intensidad de entrenamiento, los resultados podrían haber sido diferentes, en sentido positivo. El tamaño de nuestra muestra podría haber sido mayor, pero debido a las limitaciones presupuestarias y a la planificación de realizar las pruebas en 2 meses hizo inviable el reclutamiento de más sujetos.

CAPÍTULO XV.
CONCLUSIONES

CAPÍTULO XV. CONCLUSIONES

En base a los resultados obtenidos en los diferentes estudios que componen la presente Tesis Doctoral y teniendo en cuenta que los hallazgos deben ser aplicables a muestras de similares características, presentamos las siguientes conclusiones:

15.1. CONCLUSIONES GENERALES

- Existen diferencias en parámetros de rendimiento deportivo, composición corporal y algunas enzimas antioxidantes entre ciclistas PRO y AMA.
- La ingesta aguda de 2S-hesperidina (500 mg en una sola toma) mejora el rendimiento anaeróbico unido a pequeñas modificaciones en el estado antioxidante endógeno de ciclistas amateur.
- La ingesta crónica de 2S-hesperidina (500 mg durante 8 semanas) mejora el rendimiento deportivo en altas intensidades en un test incremental maximal, el estado antioxidante e inflamatorio después de un ejercicio máximo en un test rectangular y disminuye la masa grasa, a la vez que aumenta la masa muscular tras la intervención en ciclistas amateur. Disminuye el Lac a intensidades bajas-moderadas-submáximas y tras un ejercicio hasta el agotamiento. Además, aumenta marcadores del balance ácido-base a intensidades bajas-moderadas y tras un ejercicio maximal.

15.2. CONCLUSIONES ESPECÍFICAS

ESTUDIO 1: Determinar si existen diferencias en el sistema antioxidante endógeno entre ciclistas profesionales y amateur, y averiguar si estas tienen relación con el rendimiento deportivo.

- Los ciclistas PRO presentan valores mayores de CAT, GSSG y GSSG/GSH en comparación con los AMA.
- Se encontró por primera vez una correlación inversa entre WVT1 y WVT2 con la ratio %GSSG/GSH en reposo sólo en PRO. Esto indica un mejor estado antioxidante que permite un mayor rendimiento con respecto a la producción de energía.

ESTUDIO 2: Determinar si existen diferencias en la composición corporal (masa ósea y muscular) entre ciclistas profesionales, y averiguar si estas tienen relación con el rendimiento deportivo.

- El 100% de los PRO tenían valores de DMO inferiores a los valores normales (1033 g/cm²) propuestos por la NHANES III en los hombres.
- Los PRO tenían valores inferiores de DMO, CMO y FFM, pero superiores de W y VO₂ en VT1, VT2 y VO₂MAX en comparación con los AMA.
- Correlaciones positivas en PRO entre FFM con BMC y BMD, lo que indica que la masa muscular puede promover mayores niveles de variables óseas.

ESTUDIO 3: Evaluar el efecto de la ingesta aguda (una dosis) de 500 mg de 2S-hesperidina en el rendimiento anaeróbico medido mediante un test de Wingate en ciclistas amateur.

- La suplementación aguda con Cardiose® (500 mg de 2S-hesperidina) mejora el rendimiento en el esfuerzo anaeróbico máximo, modulando el estado oxidativo en ciclistas semiprofesionales.

ESTUDIO 4: Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores de rendimiento aeróbico mediante un test incremental, y el rendimiento anaeróbico mediante un test de Wingate en ciclistas amateur.

- La suplementación con 2S-hesperidina durante 8 semanas mejora en el FTP estimado y la potencia máxima en ciclistas amateur durante un test incremental.
- La suplementación con 2S-hesperidina durante 8 semanas previene un descenso del VO₂R (VT2) y de la FAT (FatMax y VT1) en un test rectangular en períodos de entrenamiento con menos volumen y carga.
- La suplementación con 2S-hesperidina durante 8 semanas mejora la potencia producida en los primeros 5 s del test de Wingate.

ESTUDIO 5: Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores del estado antioxidante/oxidante endógeno y marcadores inflamatorios pre, después del test rectangular (FatMax, VT1, VT2 y potencia máxima) y 30 min después de finalizar el test rectangular en ciclistas amateur.

- La suplementación con 2S-hesperidina (500mg/d) durante 8 semanas mejora el estado antioxidante (↑SOD y ↓AUC-GSSG) durante un test rectangular y el estado inflamatorio durante la fase aguda de la recuperación post-ejercicio (↓MCP1).

- A diferencia de otros polifenoles, la suplementación con 2S-hesperidina no interrumpe las adaptaciones producidas por el entrenamiento en los ciclistas aficionados, mejorando su rendimiento.

ESTUDIO 6: Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores de la composición corporal mediante densitometría (DXA) y antropometría, además, de la tasa metabólica en reposo pre y post intervención en ciclistas amateur.

- La suplementación con 2S-hesperidina (500mg/d) durante 8 semanas mejora la composición corporal, disminuye la masa grasa y aumenta la masa muscular en ciclistas amateur.
- La suplementación con 2S-hesperidina (500mg/d) durante 8 semanas no afectó a ninguno de los parámetros de la tasa metabólica en reposo a ciclistas amateur.

ESTUDIO 7: Evaluar el efecto de la ingesta crónica (500 mg/d durante 8 semanas) de 2S-hesperidina en marcadores del metabolismo del oxígeno y del estado ácido-base mediante gasometría de sangre capilar pre, durante (FatMax1, VT1, VT2, potencia máxima y FatMax2), tras finalizar y 30 min después de finalizar el test rectangular en ciclistas amateur.

- La suplementación con 2S-hesperidina (500mg/d) durante 8 semanas previene una caída de la pO_2 en VT2 en ciclistas amateur, vinculado con una mejora de la potencia en el FTP estimado en el estudio 4.
- La suplementación con 2S-hesperidina (500mg/d) durante 8 semanas disminuye el Lac en intensidades bajas, moderadas y submáximas durante un test rectangular y en la fase aguda de recuperación en ciclistas amateur.
- La suplementación con 2S-hesperidina (500mg/d) durante 8 semanas aumenta el HCO_3^- , SBC y ABE en intensidades bajas y moderadas durante un test rectangular y en la fase aguda de recuperación en ciclistas amateur.

CAPÍTULO XVI.
FUTURAS LÍNEAS DE
INVESTIGACIÓN

CAPÍTULO XVI. FUTURAS LÍNEAS DE INVESTIGACIÓN

En base a los resultados de la presente Tesis Doctoral, a continuación, se describen las futuras líneas de investigación para intentar dar explicación desde varios puntos de vista, los posibles mecanismos responsables de los efectos de la 2S-hesperidina:

- Generar una línea de investigación con el objetivo de describir con precisión los posibles mecanismos moleculares y cambios en la expresión génica (efecto nutrigenómico) implicados en los efectos de la 2S-hesperidina en los cambios del tejido muscular y graso, rendimiento, estado antioxidante e inflamatorio y del metabolismo energético. Utilizando para este nivel de precisión muestras de biopsias musculares y otros tejidos.
- Investigar cómo la modulación del microbioma intestinal inducida por la 2S-hesperidina y los metabolitos producidos por estos, puede estar relacionado con los efectos descritos en el cualquiera de los marcadores estudiados en esta investigación. Ya que, se ha visto una interacción de ciertas especies bacterianas y sus metabolitos producidos con algunas funciones fisiológicas o bioquímicas en humanos.
- Investigar si la ingesta de algunas cepas bacterianas mejora la biodisponibilidad de la 2S-hesperidina y ver cómo afectaría a los marcadores analizados en este estudio.
- Investigar como la suplementación con 2S-hesperidina puede afectar a nivel neurocognitivo y si algún posible efecto en este sentido puede ser uno de los factores que mejore el rendimiento deportivo.
- Investigar si la suplementación con 2S-hesperidina es capaz de mejorar el rendimiento y producir modificaciones metabólicas, en otro momento de la temporada que no sea el final de temporada.
- Determinar si la encapsulación con otras moléculas puede mejorar la biodisponibilidad y farmacocinética de la 2S-hesperidina.

CAPÍTULO XVII.
APLICACIONES PRÁCTICAS

CAPÍTULO XVII. APLICACIONES PRÁCTICAS

En base a los resultados obtenidos en las investigaciones presentadas en la presente Tesis Doctoral y tras su discusión con la bibliografía relacionada, los profesionales de la nutrición deportiva, y de forma precisa, aquellos que trabajan con ciclistas o deportistas de resistencia, podrían tener en cuenta las siguientes recomendaciones para aplicar en su práctica diaria:

- Teniendo en cuenta los resultados encontrados en el estudio 3, la ingesta aguda de 2S-hesperidina (500 mg en una toma) sería interesante tomarla 5 horas antes de un ejercicio con un alto componente anaeróbico como un esprint de 30 s o que se aproximen a ese tiempo, ya que, es el que ha sido evaluado, con el fin de mejorar el rendimiento en ciclistas o deportistas de resistencia.
- Teniendo en cuenta los resultados obtenidos en el estudio 4, la ingesta crónica de 2S-hesperidina (500 mg/d durante 8 semanas) no solo previene la pérdida de adaptaciones debido al desentrenamiento en un periodo donde se reduce el volumen e intensidad (off-season), sino que en algunos marcadores de rendimiento los mejora (FTP estimada y potencia máxima). Además, también previene el descenso del VO_2 en VT_2 debido al desentrenamiento en deportes de resistencia.
- Teniendo en cuenta los resultados encontrados en el estudio 5, la ingesta crónica de 2S-hesperidina (500 mg/d durante 8 semanas) se podría utilizar para mejorar la recuperación, ya que, es capaz de mejorar el estado antioxidante e inflamatorio tras ejercicio de alta intensidad y en la fase aguda de la recuperación en ciclistas o deportistas de resistencia. Por lo tanto, sería muy interesante para competiciones de varios días seguidos como las vueltas por etapas.
- Teniendo en cuenta los resultados encontrados en el estudio 6, la ingesta crónica de 2S-hesperidina (500 mg/d durante 8 semanas), se podría indicar su toma para mejorar el componente graso y muscular en un periodo de entrenamiento donde se baja el volumen e intensidad. Es muy interesante el efecto en el componente muscular, ya que, en los deportistas de resistencia, en particular, en los ciclistas conservar la masa muscular es un factor muy importante, en un deporte donde se expone a muchas horas de entrenamiento y competiciones, generando grandes déficits energéticos.

CAPÍTULO XVIII.
REFERENCIAS BIBLIOGRÁFICAS

CAPÍTULO XVIII. REFERENCIAS BIBLIOGRÁFICAS

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CAPÍTULO XIX.

ANEXOS

CAPÍTULO XIX. ANEXOS



Article

Differences between Professional and Amateur Cyclists in Endogenous Antioxidant System Profile

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Citation: Martínez-Noguera, F.J.; Alcaraz, P.E.; Ortolano-Ríos, R.; Dufour, S.P.; Marín-Pagán, C. Differences between Professional and Amateur Cyclists in Endogenous Antioxidant System Profile. *Antioxidants* **2021**, *10*, 282. <https://doi.org/10.3390/antiox10020282>

Academic Editors: Gareth Davison and Conor McClean

Received: 16 January 2021
 Accepted: 10 February 2021
 Published: 12 February 2021

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Abstract: Currently, no studies have examined the differences in endogenous antioxidant enzymes in professional and amateur cyclists and how these can influence sports performance. The aim of this study was to identify differences in endogenous antioxidants enzymes and hemogram between competitive levels of cycling and to see if differences found in these parameters could explain differences in performance. A comparative trial was carried out with 11 professional (PRO) and 15 amateur (AMA) cyclists. All cyclists performed an endogenous antioxidants analysis in the fasted state (visit 1) and an incremental test until exhaustion (visit 2). Higher values in catalase (CAT), oxidized glutathione (GSSG) and GSSG/GSH ratio and lower values in superoxide dismutase (SOD) were found in PRO compared to AMA ($p < 0.05$). Furthermore, an inverse correlation was found between power produced at ventilation thresholds 1 and 2 and GSSG/GSH ($r = -0.657$ and $r = -0.635$; $p < 0.05$, respectively) in PRO. Therefore, there is no well-defined endogenous antioxidant enzyme profile between the two competitive levels of cyclists. However, there was a relationship between GSSG/GSH ratio levels and moderate and submaximal exercise performance in the PRO cohort.

Keywords: catalase; superoxide dismutase; oxidized glutathione; reduced glutathione; hemoglobin; power output

1. Introduction

Competitive cycling is highly stressful on both aerobic and anaerobic metabolisms. Road cycling races require the riders to produce high relative power output (W/kg) for short duration (i.e., less than 1 min at the start, during steep climb and at the end of the race) while also sustain efforts that last for several minutes to several hours [1]. Overall, professional cyclists (PRO) perform high training volumes (~32,500 Km) during the competitive season, which include 90–100 race days [2]. On the other hand, amateur competitive cyclists (AMA) can be defined as cyclists that train 3–7 times per week, with daily training volumes of 60–120 min and that compete about 20 times in a year [3]. During training sessions and competitions (aerobic and anaerobic exercise), there is a rise in reactive oxygen species (ROS) and subsequent oxidative stress, which can lead to a favorable adaptation in the body's antioxidant defense system [4]. This improvement in the endogenous antioxidant system is generally associated with lower levels of oxidative stress biomarkers [5].

Within the endogenous antioxidant system, superoxide dismutase (SOD) is the first line of enzymatic defense that transforms the superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) [6]. Then, H_2O_2 , which is also harmful to cells, can be metabolized in a couple of ways: (1) conversion into water by glutathione peroxidase (GPx) with the

2. Methodology

2.1. Selection of Participants

A total of 26 male cyclists (11 PRO, 15 AMA) were recruited and completed the study. The PRO were competing at the *Union Cycliste Internationale* (UCI) PRO TOUR level and have participated in UCI major stage races (*Vuelta a España*, *Giro d'Italia*, *Tour de France*). The 15 AMA were from the southeast region of Spain. The PRO riders were selected based on the following criteria: (1) 20 to 40 years of age, (2) enrolled in a professional licensed team and (3) competed in at least one of the main 3-week stage races in the last years. Subjects for the AMA group had to meet the following inclusion criteria: (1) 20 to 40 years of age, (2) had at least 3 years of cycling experience and (3) performed specific training 6–12 h/week.

All subjects signed the informed consent document before their participation. The study was performed following the guidelines of the Helsinki Declaration for Human Research [19] and was approved by the Ethics Committee of the Catholic University of Murcia (CE091802).

2.2. Study Protocol

The experimental design of the study required each rider to visit the laboratory twice between the end of October and December (i.e., post-season period). In the first visit, a medical exam and blood analysis were completed to check their state of health. In the second visit (post-48 h), the cyclists performed a maximal incremental test. The 2 h prior to this latter test, they ingested a standardized breakfast, which was based relative to body mass (557.7 kcal) and composed of 95.2 g of carbohydrates (68%), 19.0 g of protein (14%) and 11.3 g of lipids (18%), established by a sports nutritionist. All subjects were instructed to refrain from high-intensity training 48 h before each visit.

2.3. Incremental Test

An incremental step test with final ramp until exhaustion was performed on a cycle ergometer (Cyclus 2TM, RBM elektronik-automation GmbH, Germany) using a metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine VT1, VT2 and $\text{VO}_{2\text{max}}$, as well as the associated levels of power output. The testing protocol started with 35 W and increased by 35 W every 2 min until $\text{RER} > 1.05$ was reached, from which the final ramp ($+35 \text{ W}\cdot\text{min}^{-1}$) until exhaustion was initiated [20]. To ensure that $\text{VO}_{2\text{MAX}}$ was achieved, at least 2 of the following criteria had to be met: plateau in the final VO_2 values (increase $\leq 2.0 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in the two last loads), maximal theoretical HR (220-age) $\cdot 0.95$ [21], $\text{RER} \geq 1.15$ and lactate $\geq 8.0 \text{ mmol}\cdot\text{L}^{-1}$ [22,23]. Ventilatory thresholds were obtained using the ventilatory equivalents method described by Wasserman [24].

2.4. Blood Analysis

A total of 21.5 mL of blood were withdrawn from the antecubital vein for analyses: one 3.0 mL tube with ethylenediaminetetraacetic acid (EDTA) for hemogram and another 3.5 mL tube with polyethylene terephthalate (PET) for biochemical parameters. For the measurement of antioxidant parameters, five 3.0 mL EDTA tubes were obtained, where one tube was immediately centrifuged at 3500 rpm at 4 °C for 10 min. All tubes were temporarily stored at 2–4 °C and then sent to an external laboratory for analysis. Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, hemoglobin, hematocrit and hematometra indexes (mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC)) were estimated.

2.5. Oxidative Stress and Antioxidant Status Markers

2.5.1. Catalase

The activity of catalase was measured in the whole blood using a UV-VIS spectrophotometer. The catalase enzyme extracts the peroxides from the region of the gel it occupies,

following the isolation of the native protein. The removal of peroxide does not cause potassium ferricyanide (yellow substance) to be reduced to potassium ferrocyanide, which reacts with ferric chloride to form a blue Prussian precipitate. The catalase positive control activity is defined in international unit equals (1 unit) to the amount of catalase necessary to decompose 1.0 μM of H_2O_2 per minute at pH 7.0 at 25 °C while H_2O_2 concentration falls from ≈ 10.3 mM to 9.2 mM. The absorbance of H_2O_2 decreases at 240 nm proportional to its decomposition so that the concentration of H_2O_2 is critical in this determination. The decrease in absorbance per time unit is the measure of catalase activity [25]. Results were expressed in U/g of Hb.

2.5.2. SOD

Superoxide dismutase (SOD) activity was measured using an SD125 Ransod kit (Randox Ltd. Crumlin, Reino Unido) in whole blood. Xanthine and xanthine oxidase were used to produce superoxide anion ($\text{O}_2^{\bullet -}$), which responded with the 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) reactive and formed a red complex that is detectable at 420 nm. The SOD activity was measured as the inhibition degree of this reaction [26]. Results were expressed in U/g of Hb.

2.5.3. Glutathione

The analysis of reduced glutathione (GSH) was performed using the glutathione-S-transferase assay described by Akerboom and Sies [27]. Calculation of GSH was performed from lymphocytes treated with perchloric acid at a final concentration of 6%, collecting the supernatant after vortexing and subsequent centrifugation for 10 min at 10,000 rpm. Following collection of the supernatants in vials, high-performance liquid chromatography (HPLC) coupled to a Waters NH2 ODS S5 column (0.052, 25 cm) was conducted. Oxidized glutathione (GSSG) was analyzed using a similar method described by Asensi. [28].

2.6. Statistical Analyses

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 21.0, International Business Machines Chicago, IL, USA). Descriptive statistics are presented as mean \pm standard deviation (SD). Levene and Shapiro-Wilks tests were performed to check for homogeneity and normality of the data, respectively. A Student's t-test for unpaired data was used to evaluate differences between groups. Additionally, the standardized mean differences were calculated using Cohen's effect size (ES) (95% confidence interval) for all comparisons. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large [29]. The different correlations between the parameters were evaluated using Pearson's correlation (r). Significance level was set at $p \leq 0.05$.

3. Results

3.1. Subject Characteristics

The general characteristics and hemogram results are presented in Table 1. Age, body mass and height were not different between PRO and AMA groups. Interestingly, PRO had higher MCH (4.8%, $p < 0.001$) and MCHC (3.6%, $p < 0.001$) compared to AMA. There were no group differences in RBC, Hb, HCT and MCV (Table 1). No correlation was found between age and antioxidant markers in both groups. However, there were correlations found between age and Hb, HCT and MCHC ($r \leq -0.597$, $p < 0.05$).

3.2. Antioxidant Parameters

Table 2 shows the outcomes of CAT, SOD, GSSG, GSH, %GSSG/GSH and GSSG+GSH, which were measured at baseline before the incremental tests. Higher levels in CAT (30.0%, $p < 0.001$), GSSG (63.2%, $p < 0.001$) and %GSSG/GSH (70.1%, $p < 0.001$), and lower levels in SOD (-16.2% , $p = 0.009$) were found in PRO compared to AMA. However, no differences in GSH (-4.3% , $p = 0.216$) and GSSG+GSH (-3.5% , $p = 0.317$) values were observed between PRO and AMA.

Table 1. Baseline general characteristics and hemogram variables of professional and amateur cyclists.

	PRO	AMA	<i>p</i> -Value	Cohen's <i>d</i>	Effect Size
Age (years)	28.3 (4.65)	29.3 (6.54)	0.671	0.17	Trivial
Body mass (kg)	68.5 (4.43)	69.9 (5.50)	0.488	0.28	Small
Height (cm)	178.0 (6.93)	175.0 (6.71)	0.274	0.44	Small
HEMOGRAM					
RBC ($10^6 \cdot \mu\text{L}^{-1}$)	5.06 (0.281)	5.15 (0.260)	0.441	0.08	Trivial
Hb ($\text{g} \cdot \text{dL}^{-1}$)	15.6 (0.827)	15.1 (0.676)	0.107	0.49	Small
HCT (%)	44.5 (2.28)	44.6 (1.57)	0.866	0.13	Trivial
MCV (fl)	87.9 (2.19)	86.8 (2.92)	0.305	1.10	Large
MCH (pg)	30.8 (0.35)	29.4 (1.03)	<0.001	1.44	Large
MCHC (%)	35.0 (0.74)	33.8 (0.60)	<0.001	1.19	Large

Values are expressed as mean (SD). Abbreviations: RBC = red blood cell; Hb = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration and SD = standard deviation.

Table 2. Endogenous antioxidant enzymes from professional and amateur cyclists.

	PRO	AMA	<i>p</i> -Value	Cohen's <i>d</i>	Effect Size
CAT (U/g Hb)	32.5 (5.34)	25.0 (4.51)	<0.001	1.55	Large
SOD (U/g Hb)	1213 (233.0)	1447 (184.4)	0.009	1.13	Large
GSSG (nmol/mg protein)	0.524 (0.103)	0.321 (0.077)	<0.001	2.28	Large
GSH (nmol/mg protein)	24.4 (2.00)	25.5 (2.17)	0.216	0.50	Moderate
GSSG/GSH	2.16 (0.436)	1.27 (0.279)	<0.001	2.52	Large
GSSG+GSH (nmol/mg protein)	24.9 (2.02)	25.8 (2.19)	0.317	0.41	Small

Values are expressed as mean (SD). Abbreviations: CAT = catalase; SOD = superoxide dismutase; GSH = reduced glutathione; GSSG = oxidized glutathione; % GSSG/GSH = oxidized/reduced glutathione ratio and SD = standard deviation.

3.3. Physiological and Metabolic Parameters at VT1

VO_2 , W, WR, $\% \text{VO}_{2\text{MAX}}$, HR and RER values at VT1 are shown in Table 3. Significant group differences in VO_2 (76.0%, $p < 0.001$), W (90.4%, $p < 0.001$), WR (92.5%, $p < 0.001$), $\% \text{VO}_{2\text{MAX}}$ (53.3%, $p < 0.001$) and HR (12.9%, $p = 0.004$), but not for RER (0.78%, $p = 0.707$) were observed.

Table 3. Metabolic and performance variables of professional and amateur cyclists.

	PRO	AMA	<i>p</i> -Value	Cohen's <i>d</i>	Effect Size
VT1					
VO_2 ($\text{mL} \cdot \text{min}^{-1}$)	3593 (271.0)	2041 (401.0)	<0.001	4.40	Large
W	299 (32.9)	157 (36.1)	<0.001	4.07	Large
WR ($\text{W} \cdot \text{kg}^{-1}$)	4.37 (0.42)	2.27 (0.56)	<0.001	4.14	Large
$\% \text{VO}_{2\text{max}}$	76.2 (3.91)	49.7 (5.58)	<0.001	5.36	Large
HR (beats $\cdot \text{min}^{-1}$)	149 (14.7)	132 (13.2)	0.004	1.25	Large
RER	0.906 (0.05)	0.899 (0.04)	0.707	0.15	Trivial

Table 3. Cont.

	PRO	AMA	p-Value	Cohen's d	Effect Size
VT2					
VO ₂ (mL·min ⁻¹)	4259 (234.0)	3389 (505.0)	<0.001	2.10	Large
W	379 (34.0)	286 (45.1)	<0.001	2.28	Large
WR (W·kg ⁻¹)	5.54 (0.41)	4.13 (0.74)	<0.001	2.28	Large
%VO _{2max}	90.3 (2.36)	84.7 (5.67)	0.005	1.24	Large
HR (beats·min ⁻¹)	168 (11.1)	171 (9.4)	0.467	0.29	Small
RER	1.01 (0.05)	1.03 (0.03)	0.323	0.40	Small
VO_{2max}					
VO ₂ (mL·min ⁻¹)	4714 (241.0)	4066 (580.7)	0.002	1.38	Large
VO ₂ /R (mL·kg ⁻¹ ·min ⁻¹)	69.0 (3.94)	58.7 (9.58)	0.003	1.34	Large
W	474 (31.5)	383 (49.2)	<0.001	2.13	Large
WR (W·kg ⁻¹)	6.93 (0.44)	5.51 (0.81)	<0.001	2.09	Large
HR (beats·min ⁻¹)	186 (7.42)	186 (7.62)	0.966	0.02	Trivial
RER	1.22 (0.04)	1.14 (0.06)	0.001	1.49	Large

Values are expressed as mean (SD). Abbreviations: VO₂ = oxygen uptake; VO_{2max} = maximum oxygen consumption; VO₂/R = maximum oxygen consumption relative to weight; W = power output; WR = power output relative to weight; %VO_{2max} = percentage of VO_{2max}; HR = heart rate (beats·min⁻¹); RER = respiratory exchange ratio; VT1 = ventilatory threshold 1; VT2 = ventilatory threshold 2 and SD = standard deviation.

GSSG/GSH was significantly correlated with W_{VT1} and VO_{2VT1} (r = -0.657 and r = -0.651; p < 0.05, respectively) in PRO (Table 4) (Figure 1).

Table 4. Correlation between endogenous antioxidant enzymes and performance-metabolic variables from professional and amateur cyclists.

		CAT	SOD	GSSG	GSH	%GSSG/GSH	GSSG + GSH
PRO (n = 11)							
W _{VT1}	r	-0.120	0.305	-0.449	0.425	-0.657	0.397
	p-value	0.72	0.36	0.17	0.19	0.03	0.23
VO _{2VT1}	r	0.001	0.378	-0.442	0.457	-0.651	0.429
	p-value	0.998	0.252	0.173	0.157	0.030	0.188
W _{VT2}	r	-0.253	0.183	-0.575	0.116	-0.635	0.085
	p-value	0.45	0.59	0.06	0.73	0.04	0.80
VO _{2VT2}	r	-0.319	0.423	-0.518	0.277	-0.622	0.247
	p-value	0.34	0.20	0.10	0.41	0.04	0.46
W _{MAX}	r	-0.045	0.186	-0.342	0.239	-0.443	0.219
	p-value	0.90	0.58	0.30	0.48	0.17	0.52
VO _{2MAX}	r	-0.375	0.422	-0.312	0.304	-0.414	0.284
	p-value	0.26	0.20	0.35	0.36	0.21	0.40

Table 4. Cont.

		CAT	SOD	GSSG	GSH	%GSSG/GSH	GSSG + GSH
AMA (n = 15)							
W _{VT1}	r	0.181	0.172	0.206	-0.102	0.256	-0.098
	p-value	0.52	0.54	0.46	0.72	0.36	0.73
VO _{2VT1}	r	0.360	0.159	0.182	-0.108	0.230	-0.105
	p-value	0.19	0.57	0.52	0.70	0.41	0.71
W _{VT2}	r	0.414	0.113	-0.002	0.047	-0.046	0.040
	p-value	0.13	0.69	0.99	0.87	0.87	0.89
VO _{2VT2}	r	0.358	0.234	-0.104	-0.068	-0.097	-0.077
	p-value	0.19	0.69	0.71	0.81	0.73	0.78
W _{MAX}	r	0.180	0.173	-0.136	-0.379	0.009	-0.386
	p-value	0.52	0.54	0.63	0.16	0.97	0.16
VO _{2MAX}	r	0.289	0.278	-0.118	-0.334	0.001	-0.339
	p-value	0.30	0.32	0.66	0.22	0.10	0.22

Values are expressed as mean (SD). Abbreviations: CAT = catalase (U/g Hb); SOD = superoxide dismutase (U/g Hb); GSH = reduced glutathione (nmol/mg protein); GSSG = oxidized glutathione (nmol/mg protein); % GSSG/GSH = oxidized/reduced glutathione ratio; VO₂ = oxygen uptake; VO_{2MAX} = maximum oxygen consumption; VT1 = ventilatory threshold 1; VT2 = ventilatory threshold 2 and W = power output.

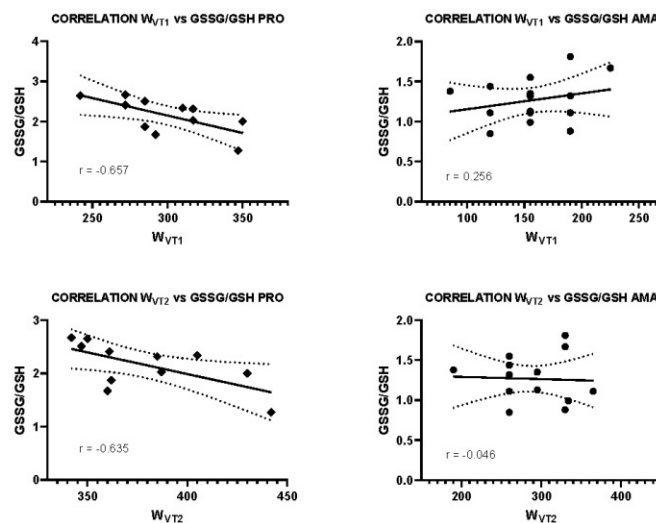


Figure 1. Correlations between the power generated at the ventilation threshold 1 and 2 between the GSSG/GSH ratio in PRO and AMA.

3.4. Physiological and Metabolic Parameters at VT2

Table 3 demonstrates the VT2 results of VO₂, W, WR, %VO_{2MAX}, HR and RER. Significant group differences in VO_{2VT2} (25.6%, $p < 0.001$), W_{VT2} (32.5%, $p < 0.001$), WR_{VT2} (34.1%, $p < 0.001$) and %VO_{2MAXVT2} (6.6%, $p = 0.005$) were observed.

GSSG/GSH was significantly correlated with W_{VT2} and VO_{2VT2} ($r = -0.635$ and $r = -0.622$; $p < 0.05$, respectively) in PRO (Figure 1). GSSG tended to correlate with W_{VT2} ($r = -0.575$; $p = 0.06$) in PRO (Table 4).

3.5. Physiological and Metabolic Parameters at VO_{2max}

Maximal values of VO_2 , VO_2/R , W , WR , HR and RER are presented in Table 3. Significant group differences in VO_{2MAX} (15.9%, $p = 0.002$), VO_2/R_{MAX} (17.5%, $p = 0.002$), W_{MAX} (23.8%, $p < 0.001$), WR_{MAX} (25.8%, $p < 0.001$), and RER_{MAX} (7.0%, $p = 0.001$), but not for HR_{MAX} ($p = 0.966$) were found.

In VO_{2MAX} , no correlation with any antioxidant marker was observed (Table 4).

4. Discussion

This study provides the first direct comparison of endogenous antioxidant, hematological, performance and metabolic biomarkers ($VT1$, $VT2$ and VO_{2MAX}) between PRO and AMA cyclists. Our results demonstrate that: (i) PRO have higher values in MCH, MCHC, CAT, GSSG and GSSG/GSH but lower values in SOD than AMA; (ii) PRO have higher levels of absolute and relative power output and oxygen consumption in all intensity zones ($VT1$, $VT2$ and VO_{2MAX}) than in AMA, with the largest differences found at $VT1$; (iii) inverse correlations were identified in W_{VT1} , VO_{2VT1} , W_{VT2} and VO_{2VT2} with GSSG/GSH in PRO.

Differences in Antioxidant Enzymes and Hemogram

When intense physical exercise is performed (especially in untrained or those not familiar with the exercise), there is an increase in the production of reactive oxygen species, which are neutralized by our complex endogenous antioxidant defense system (GSH, GSSG, CAT, SOD, GPx and GR) and by exogenous antioxidants (vitamin C, vitamin E, carotenes) [30].

Regarding EAE, we observed higher levels of CAT activity, GSSG and GSSG/GSH, but lower levels of SOD activity in PRO versus AMA. Mena et al. [31] found higher resting levels of SOD, CAT and GPx in a sample of PRO cyclists compared to sedentary people. Tauler et al. [32] also showed differences in antioxidant enzyme activity in erythrocyte between PRO and AMA at rest. In the same study, a decrease in CAT (−12%), GPx (−14%) and GR activity (−16%) but an increase in SOD activity of about 25% after a submaximal test (80% VO_{2MAX} ; 1 h 30 min) was reported [32].

Long distance runners have been shown to have a three-fold higher CAT activity compared to short distance runners [18]. Similarly, it was observed that marathon runners had twice as high catalase activity compared to sprinters [12]. In this study, we also demonstrated higher levels of CAT in PRO than in AMA, and this may be largely explained by the fact that PRO perform greater volume, intensity and competitions (higher aerobic load and prolonged periods of exercise) than AMA, which induces higher levels of exposure to ROS and, consequently, adaptations of EAE [6]. When CAT levels increase, it is possible that GPx activity is not sufficient to neutralize high levels H_2O_2 (endurance exercise) [7].

Regarding SOD, Mena et al. [31] observed lower levels of SOD activity (−32.1%) in PRO than in elite cyclist, but in the case of CAT (80.0%) and GPx (149.0%), the levels were higher, reporting an ascending behavior of SOD during a stage race (2800 km in 17 stages) in PRO. Tauler et al. [32] has also found lower levels of SOD activity in PRO (−19.8%) than in AMA at baseline, which are in line with the results of our study. Antioxidant enzyme activity can be modified either by an initial increase (adaptation) or a decrease if the oxidative stress of long duration (utilization) [33]. Therefore, the low basal levels of SOD activity in professional cyclists could be overwhelmed and the high concentration of superoxide anions could activate CAT, allowing compensated metabolism of H_2O_2 . This may be the reason why PRO has lower levels of SOD activity than AMA, as PRO have higher levels of exercise exigency that sometimes get close to exhaustion, which can lead a decrease in the working capacity of SOD.

On the other hand, there is evidence to suggest that GSH or GSH/GSSG decreases during exercise because of its utilization against ROS [33]. Ultra-endurance exercise depletes erythrocyte GSH levels by ~66% for 24 h and levels remain ~33% lower than normal 1 month later [34]. PRO frequently compete in longer distance events than AMA,

which can lead to lower levels of GSH in PRO than in AMA, although no differences were observed in GSH between PRO vs. AMA in our study. In addition, the muscle can import GSH from plasma during exercise, and as a result, there is a change in the GSH/GSSG ratio after exercise with a decrease in the GSH/GSSG ratio at the time of exhaustion [35]. Furthermore, it is important to mention that tissues are not only capable of importing GSH but also exporting GSSG under oxidative stress [35]. Moreover, GSH is a molecule that is key in cellular redox status regulation, and consequences of prolonged GSH depletion may include a compromise in immunity, where lower GSH is associated with decreased lymphocyte proliferation and increased viral reactivation [34].

GSSG levels are a biomarker of cellular oxidative stress, since GSH is an important antioxidant in many tissues and oxidizes in the catalyzed reduction of H_2O_2 to H_2O to become GSSG [36]. The increase in GSH (mainly) and GSSG in plasma after exercise could be explained by an efflux from the liver to other tissues, including skeletal muscle [37]. GSSG levels in skeletal muscle have previously been shown to increase by ~50% in rats after running on a treadmill at moderate intensity [38] and by ~20% after cycling in humans (workload corresponding to 90% of VO_{2peak} ; 10×4 min) [39]. Leonardo et al. [40] observed an increase in both GSSG and GSSG/GSH after a period of intense PRO training, which returned to their baseline levels after a period of tapering. We found similar baseline values of GSSG in our study. In addition, we found higher levels of GSSG and GSSG/GSH in PRO than in AMA.

The efforts made during cycling competitions produce oxidative stress in lymphocytes, leading to a reduction in GSH levels and an increase in GSSG levels. The decrease in GSH and increase in GSSG during exercise may be explained by an increase in H_2O_2 formation, as reported by Wang et al. who found that high-intensity exercise (80% VO_{2MAX}) decreased GSH levels while lipid peroxidation increased immediately and after 24h of exercise [41]. Furthermore, in this study, lymphocytes were incubated with H_2O_2 for 2 and 4 h, promoting an increase in DNA fragmentation immediately and 24 h after high intensity exercise. Thus, H_2O_2 would cause a failure of the endogenous antioxidant system leading to DNA damage in lymphocytes. Ferrer et al. [42] found that high intensity exercise (swimming) increased GPx activity (converts GSH to GSSG) in lymphocytes, in the same way as other authors found after a cycling stage [43,44]. This supports the decrease in GSH and increase in GSSG after high intensity exercise. Therefore, the higher levels of GSSG and GSSG/GSH in PRO vs. AMA in our study may be due to a higher production of ROS, which leads to a higher production of GSSG and, consequently, of GSSG/GSH together with a decrease in GSH.

In addition, our study is the first to show correlations between GSSG/GSH with W_{VT1} ($r = -0.657$) and W_{VT2} ($r = -0.635$) in PRO. This is also supported by a trend towards a significant correlation between GSSG and WVT2 ($r = -0.575$; $p = 0.06$) in PRO. These relationships suggest that cyclists who generate more power at VT1 and VT2 have lower GSSG/GSH levels, and therefore, less oxidative stress, as GSSG/GSH ratio is known to be a marker of antioxidant status [20].

In response to strenuous physical working conditions, the body's antioxidant capacity may be temporarily diminished, as its components are used to scavenge the harmful radicals that are produced [45]. It is well known that exercise-induced ROS are detrimental to physiological function, including decreased performance and immune function and increased fatigue [45]. Moreover, it has been shown that the response of antioxidant capacity to exercise responds in a similar way to the activity of EAE [45]. Therefore, the antioxidant defense system may be temporarily reduced in response to increased ROS production but may increase during the recovery period as a result of the initial prooxidant insult [46]. However, contradictory findings have been reported where increases in GPx, SOD, and CAT, as well as decreases in GPx, GR, SOD have been observed [45]. Evidently, this controversy may depend on the moment of sampling (i.e., period of the season), as well as on the duration and intensity of the exercise, which varies considerably between studies.

It could be that there is an undefined optimal level of ROS production and oxidative damage required for adaptations in antioxidant defenses and other physiological parameters, leading to health and performance improvements [45]. However, overproduction of ROS and oxidative damage due to chronic long-term exercise and/or overtraining may exceed the above-mentioned optimal level, resulting in irreparable oxidative damage, which can lead to the development or progression of poor health and/or disease [47]. Therefore, the measurement of the antioxidant capacity (CAT, SOD, GSH, GSSG and GSSG/GSH) of the body is used as a marker of oxidative stress and can provide us insight on how it affects performance. Given the results of our research and the evidence shown in the scientific literature, there is no endogenous antioxidant profile defined in PRO compared to AMA.

There are also other antioxidant proteins, such as peroxiredoxin (PRX) and thioredoxin (TRX) containing thiol groups, with a high capacity to neutralize reactive oxygen and nitrogen species and decrease oxidative stress [48]. One study showed how moderate and high-intensity exercise and a low volume high intensity interval training trial increased TRX (85%, 64% and 206%, respectively); however, PRX only increased during high intensity exercise (moderate: -6229% ; high: 203% and low volume high intensity interval: -23% , respectively) in peripheral blood mononuclear cells [48]. In addition, an increase in nuclear transcription factor kappa B was found during all exercises, suggesting an activation of the inflammatory system, probably due to increased oxidative stress. Future studies should examine whether there are differences in these antioxidant proteins between PRO and AMA and their relationship with performance.

Regarding hematological parameters, no significant differences were found except for MCH and MCHC between PRO vs. AMA. Schumacher et al. found hematological values in elite cyclists from the German national team (blood samples collected between November and January) and the values were similar to ours in Hb (~ 15.5 g/dL), Hct ($\sim 45.0\%$) and RBC ($\sim 5.0 \times 10^6/\text{mm}^3$) in PRO [49]. In addition, other studies have found hematological values of approximately 15.0 g/dL of Hb and 45% of Hct in professional cyclists [50–52]. Well-trained cyclists have found values of 14.3 g/dL in Hb and 43.1% in Hct, values lower than PRO [53]. However, Bejder et al. [54] observed amateur competitive cyclist values of 14.8 g/dL Hb, 42.8% Hct, $4.92 \times 10^6 \cdot \mu\text{L}^{-1}$ RBC, 87.1 fl MCV, 30.1 pg MCH and 34.6 g/dL MCHC, lower than those reported in PRO.

MCH indicates the amount of hemoglobin contained in an erythrocyte and MCHC is the average hemoglobin concentration [55]. Therefore, the red blood cells of PROs will have a higher oxygen transport capacity due to the higher levels of MCH and MCHC. Currently, no study on cyclists has examined the differences in MCH and MCHC, so we cannot draw many conclusions in this regard. These hematological parameters have mainly been used as markers of anemia both in athletes and in the general population [56], but so far, they are not associated with an athlete's performance level in this study.

5. Limitations

Our study had limitations with regards to the sample number, since it was more difficult to recruit PRO athletes than lower-level athletes (AMA).

Differences in endogenous antioxidant marker between this study and previous works may be influenced by the instrumentation and methodology used, the timing of the season at which the measurements were made, and the training status of the cyclists.

6. Conclusions

Regarding the endogenous antioxidants profile, PRO had higher values of CAT, GSSG and GSSG/GSH compared to AMA. An inverse correlation was found for the first time between W_{VT1} and W_{VT2} with GSSG/GSH at rest only in PRO. This indicates better antioxidant status that allow for higher performance with regard to power output. Future studies should examine how training adaptations affect the studied variables and how antioxidant enzymes evolve during a race stage (e.g. Tour de France), in order to

see their association with performance, recovery and fatigue, thereby helping to develop monitoring tools for medical doctors, nutritionists and coaches.

Author Contributions: Conceptualization, F.J.M.-N., C.M.-P., R.O.-R. and P.E.A.; methodology, F.J.M.-N., C.M.-P., R.O.-R. and P.E.A.; formal analysis, F.J.M.-N. and C.M.-P.; investigation, F.J.M.-N., C.M.-P. and R.O.-R.; resources, F.J.M.-N. and C.M.-P.; data curation, F.J.M.-N., C.M.-P. and R.O.-R.; writing—original draft preparation, F.J.M.-N.; writing—review and editing, F.J.M.-N., C.M.-P. and S.P.D.; visualization, F.J.M.-N. and C.M.-P.; supervision, C.M.-P., S.P.D. and P.E.A.; project administration, F.J.M.-N. and C.M.-P. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Catholic University of Murcia (CE091802).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data is contained within the article.

Acknowledgments: This study was supported by the Research Center in High-Performance Sport of the Catholic University of Murcia (Murcia, Spain). We would like to acknowledge Linda H. Chung for her help in this project.

Conflicts of Interest: The authors declare no conflict of interest.

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Bone

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Full Length Article

Professional cyclists have lower levels of bone markers than amateurs. Is there a risk of osteoporosis in cyclist?

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ARTICLE INFO

Keywords:
Bone
Muscle mass
Endurance sport
Osteoporosis
Power output
Oxygen consumption

ABSTRACT

Currently, there is a greater number of amateurs that practice cycling. However, there is no clear evidence regarding bone health in amateur cyclists compared to professional cyclists, as the latter has shown to have lower bone mineral content and density. Therefore, the aim of this study was to identify the differences in bone variables between professional (PRO) and amateur (AMA) road cyclists, and to see if these differences were related to differences in cycling performance. A parallel trial was carried out with 15 AMA and 10 PRO cyclists. All cyclists visited the laboratory twice: 1) in a fasted state, body composition measured by dual-energy X-ray absorptiometry (DXA) and 2) physiological variables measured using an incremental test until exhaustion. Significantly lower values were found in bone mineral density, bone mineral content and fat free mass in PRO compared to AMA ($p < 0.05$). In addition, significantly higher power was produced in ventilatory thresholds 1 and 2 (VT1 and VT2) and $\dot{V}O_{2\text{MAX}}$ in PRO compared to AMA ($p < 0.05$). Overall, PRO cyclists had lower values in bone health and muscle mass but better results in performance compared to AMA.

1. Introduction

There are several differences between professional (PRO) and amateur competitive (AMA) cyclists, among them are the large volumes of training during the season (~32,500 km) with ~100 days of competition in PRO [1], however, AMA perform between 3 and 7 days of training per week (60–120 min per training) participating about 20 competitions per year [2].

Several physiological parameters have been used as performance predictors in top-level cyclists that include: a) maximum oxygen uptake ($\dot{V}O_{2\text{MAX}}$), maximum (W_{MAX}) and relative (WR_{MAX}) power output [1,3–7]; b) oxygen uptake ($\dot{V}O_{2\text{VT2}}$) and absolute (W_{VT2}) and relative (WR_{VT2}) power output at ventilatory thresholds 1 and 2 (VT1 and VT2) [4,6,7]; and c) cycling efficiency [8,9]. It has been shown that PRO record high $\dot{V}O_{2\text{MAX}}$ (70–80 mL·kg⁻¹·min⁻¹) and W_{MAX} (>500 W or 6–7.5 W/kg) in a maximal incremental test, as well as an optimized VT2 (~90% $\dot{V}O_{2\text{MAX}}$), however, lower $\dot{V}O_{2\text{MAX}}$ (70–75 mL·kg⁻¹·min⁻¹), W_{MAX} (300–450 W or 5.0–6.0 W·kg⁻¹) have been shown in AMA

compared to PRO [1,2]. The values of $\dot{V}O_2$ and power output at VT1 (PRO: 3.20 L·min⁻¹; 262 W vs AMA: 2.40 L·min⁻¹; 186 W) and VT2 (PRO: 4.30 L·min⁻¹; 386 W vs AMA: 3.56 L·min⁻¹; 299 W) are also higher in PRO than in AMA (VT1 = 25%; 29% and VT2 = 17.2%; 22.5%, respectively) [7,10].

In addition, PRO develop a high level of cycling efficiency compared to AMA (~24.5% vs 21.7% at 80% $\dot{V}O_{2\text{MAX}}$, respectively; gross efficiency) that enables them to maintain extremely high workloads for long durations [8,9], [11]. Furthermore, it has been reported that PRO have lower percentage (8–10%) of body fat (BF) [12], an average fat-free mass (FFM) of 62.5 kg (58.3–68.3 kg) and bone mineral density (BMD) of 1.145 g/cm² [13]. Average values of 11% in BF and 60 kg in fat free mass (FFM) have been found in AMA, with a BMD of 1.187 g/cm² [14]. Therefore, PRO have lower BF (–22.2%) and BMD (–3.7%) but higher FFM (4.0%) compared to AMA.

In relation to bone metabolism, dietary behavior can modulate both acute bone turnover and long-term bone health, moreover nutrition can have an impact on the diurnal rhythm of markers of bone turnover at

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<https://doi.org/10.1016/j.bone.2021.116102>

Received 7 February 2021; Received in revised form 2 June 2021; Accepted 30 June 2021

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rest [15]. It has been shown that eating a mixed diet with a meal of mixed nutrients (glucose, fat, protein and calcium) suppresses all markers of bone turnover [16]. Previous research has studied the effects of nutrient intake on sedentary and resting subjects. However, it has been reported that after an exhaustive running (performed treadmill running at 75% $\text{VO}_{2\text{MAX}}$), the immediate intake of carbohydrates + protein increases the bone formation markers and decreases the concentrations of bone resorption markers, establishing a positive balance in bone turnover [17]. On the contrary, prolonged and intense exercise has been seen to produce an increase in mass resorption, with an increase in the C-terminal telopeptide of type I collagen [18], although the markers of bone formation, such as N-terminal pro-peptides of type I procollagen, have less response to acute exercise [19].

Cycling is a sport where some low carbohydrate and energy dietary strategies are used [20] which, combined with the high volumes and intensity in training and competition and influenced by non-impact physical activity [21], can promote bone resorption mechanisms that exceed those of synthesis. Due to these factors that influence cycling, we believe that research should be carried out into whether any bone pathology (osteopenia or osteoporosis) really exists in different cohorts of cyclists (PRO and AMA). Therefore, the main objective of this research was to determine the differences in bone and muscle mass markers (body composition) between PRO and AMA, and whether these might be related to differences in performance (PO at VT1, VT2 and $\text{VO}_{2\text{MAX}}$) in an incremental test.

2. Methodology

2.1. Selection of participants

Twenty-six cyclists (11 PRO; 15 AMA) completed this study. The AMA selected for this study were from southeast Spain and had to meet the following criteria: i) 20 and 40 years of age, ii) had at least 3 years of cycling experience and iii) cycling training of 6–12 h per week. PRO competed in *Union Cycliste Internationale* (UCI) PRO TOUR races and participated in UCI major stage races (*Vuelta a España*, *Giro d'Italia*, *Tour de France*). PRO riders were selected based on the following criteria: i) 20 to 40 years of age, ii) enrolled in a licensed professional team, and iii) competed in at least one major 3-week stage races in the last years. All subjects signed their informed consent before their participation. The study was performed following the guidelines of the Helsinki Declaration for Human Research [22] and was approved by the Ethics Committee of Catholic University of Murcia (CE091802).

2.2. Study protocol

The experimental design of the study required each cyclist to visit the laboratory twice during the post-season period. Visit 1 consisted of a medical exam, blood analysis and dual-energy X-ray absorptiometry (DXA). After 48 h, cyclists returned to the lab (visit 2) to perform a maximal incremental test. Two hours before performing this latter test, cyclists consumed a breakfast relative to their body weight (557.7 kcal), which was composed of 95.2 g of carbohydrates (68%), 19.0 g of protein (14%) and 11.3 g of lipids (18%), established by a sports nutritionist. All subjects were previously instructed to refrain from high-intensity training for 48 h before each visit.

2.3. Incremental test

An incremental step test with final ramp until exhaustion was performed on a cycle ergometer (Cyclus 2TM, REM elektronik-automation GmbH, Germany) using a metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine VT1, VT2 and $\text{VO}_{2\text{MAX}}$ as well as the associated levels of power output. The testing protocol started with 35 W and increased by 35 W every 2 min until $\text{RER} > 1.05$ was reached, from which the final ramp (+35 W·min⁻¹) until exhaustion was initiated

[23]. To ensure $\text{VO}_{2\text{MAX}}$ was achieved, at least 2 of the following criteria had to be met: plateau in the final VO_2 values (increase $\leq 2.0 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in the two last loads), reaching maximal theoretical HR ($220 - \text{age}$)·0.95 [24], $\text{RER} \geq 1.15$ and lactate $\geq 8.0 \text{ mmol}\cdot\text{L}^{-1}$ [25,26]. Ventilatory thresholds (VT1 and VT2) were obtained using the ventilatory equivalents method described by Wasserman [27]. The variables analyzed in this test were: VO_2 , VO_2 relative to weight in $\text{VO}_{2\text{MAX}}$ ($\text{VO}_2/\text{R}_{\text{MAX}}$), absolute power output (W), power output relative to weight (WR), heart rate (HR) and respiratory exchange ratio (RER).

2.4. Blood analysis

From an antecubital vein for general analyses, was withdrawn one 3.0 mL tube with ethylenediaminetetraacetic acid (EDTA) for hemogram and another 3.5 mL tube with polyethylene terephthalate (PET) for biochemical parameters, was immediately centrifuged at 3500 rpm at 4 °C for 10 min and sent to the reference laboratory for analysis. Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls.

2.5. Dual-energy X-ray absorptiometry (DXA)

Body composition was evaluated by DXA of the whole body (XR-46; Norland Corp., Fort Atkinson, WI). BMD (g/cm^2), mineral content (BMC) (g), bone area (BA) (cm^2) and FFM (g) were assessed in the morning in fasted conditions. In the prior 5 years, there were no deviations detected in the calibration and there were no firmware or software updates. The measuring device had been checked by the manufacturer. During the measurements, all patients wore underwear with no metal accessories. The scans and analysis were performed by an experienced and certified technician.

2.6. Statistical analyses

Levene and Shapiro-Wilks tests were performed to check for homogeneity and normality of the data, respectively. A Student's *t*-test for unpaired data was used to evaluate differences between groups. Analysis of covariance (ANCOVA) was used to test for mean differences in BMD, BMC and bone area between groups, adjusting for the effect of components with lean mass. The different correlations between the parameters were evaluated using Pearson's correlation (*r*). Linear regression analysis was performed to assess the association within body composition variables and the association between performance and body composition variables. Significance level was set at $p \leq 0.05$. The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 21.0, International Business Machines Chicago, IL, USA). Descriptive statistics are presented as mean \pm standard deviation (SD). Additionally, the standardized mean differences were calculated using Cohen's effect size (ES) with a 95% confidence interval (CI) for all comparisons. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large [28].

3. Results

3.1. Subject characteristics

The general characteristics, DXA and hemogram results are presented in Table 1. Age, body mass and height were not significantly different between PRO and AMA groups. In addition, PRO had higher mean corpuscular hemoglobin (MCH) (4.8%, $p \leq 0.001$) and mean corpuscular hemoglobin concentration (MCHC) (3.6%, $p \leq 0.001$) compared to AMA (Table 1).

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Table 1
Baseline general characteristics, densitometry (DXA) and hemogram variables of professional and amateur cyclists.

	PRO	AMA	p-value	Cohen's d	Effect Size
Age (years)	28.3 (4.65)	29.3 (6.54)	0.671	0.17	Trivial
Body mass (kg)	68.5 (4.43)	69.9 (5.50)	0.488	0.28	Small
Height (cm)	178.0 (6.93)	175.0 (6.71)	0.274	0.44	Small
Hemogram					
RBC ($10^5 \mu\text{l}^{-1}$)	5.06 (0.281)	5.15 (0.260)	0.441	0.08	Trivial
Hb (g dl^{-1})	15.6 (0.827)	15.1 (0.676)	0.107	0.49	Small
HCT (%)	44.5 (2.28)	44.6 (1.57)	0.866	0.13	Trivial
MCV (fl)	87.9 (2.19)	86.8 (2.92)	0.305	1.10	Large
MCH (pg)	30.8 (0.35)	29.4 (1.03)	<0.001	1.44	Large
MCHC (%)	35.0 (0.74)	33.8 (0.60)	<0.001	1.19	Large

Values are expressed as mean (SD). Abbreviations: BMD bone mineral density; BMC bone mineral content; BA bone area; FFM fat-free mass; RBC red blood cell; Hb hemoglobin; HCT hematocrit; MCV mean corpuscular volume; MCH mean corpuscular hemoglobin; MCHC mean corpuscular hemoglobin concentration and SD standard deviation.

3.2. Bone and muscle mass parameters

Interestingly, PRO had significantly lower BMD (0.911 vs 1.052 g/cm^2 , $p \leq 0.001$, $ES = 1.97$), BMC (1709 vs 2993 g , $p \leq 0.001$, $ES = 4.74$), BA (1877 vs 2842 cm^2 , $p \leq 0.001$, $ES = 6.05$) and FFM ($43,758$ vs $55,943$ g , $p \leq 0.001$, $ES = 3.17$) compared to AMA (Fig. 1). All of the PRO and seven AMA had a BMD value below 1033 g/cm^2 , which is the normal BMD cutoff value for men that was established by the North American Health Survey (NHANES III) (Fig. 2). However, when we performed ANCOVA to analyse the differences in the bone variables using lean mass as a covariate, we found significant differences between

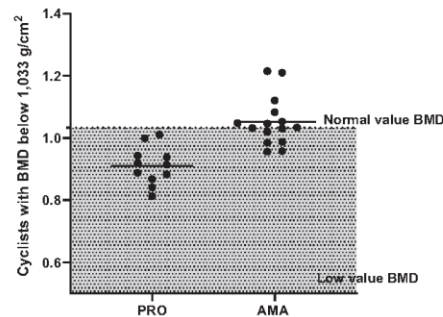


Fig. 2. Cyclists with BMD values below 1033 g/cm^2 . NHANES III has established that BMD values above this threshold are normal.

groups in BMC ($p = 0.002$; $\eta^2 = 0.334$) and bone area ($p \leq 0.001$; $\eta^2 = 0.448$), with no differences between groups in BMD but with a moderate effect size ($p = 0.159$; $\eta^2 = 0.084$).

In the lower limbs, when performing the independent sample t -test, no significant differences were observed between PRO vs AMA in BMD (1140 vs 1200 g/cm^2 , $p = 0.574$, $ES = 0.49$), BMC (1038 vs 1125 g , $p = 0.190$, $ES = 0.54$), BA (911 vs 935 cm^2 , $p = 0.359$, $ES = 0.37$) and FFM ($21,530$ vs $20,846$ g , $p = 0.553$, $ES = 0.24$) (Fig. 3). But when we performed ANCOVA using FFM as a covariate, significant differences were found in BMD ($p \leq 0.001$; $\eta^2 = 0.450$), BMC ($p \leq 0.001$; $\eta^2 = 0.657$) and bone area ($p \leq 0.001$; $\eta^2 = 0.461$), in PRO compared to AMA.

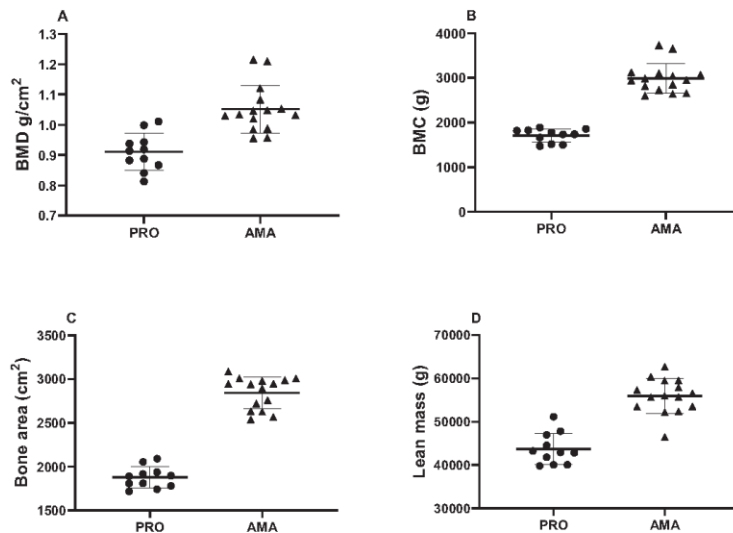


Fig. 1. Bone and muscle differences in PRO compared to AMA, measured by densitometry.

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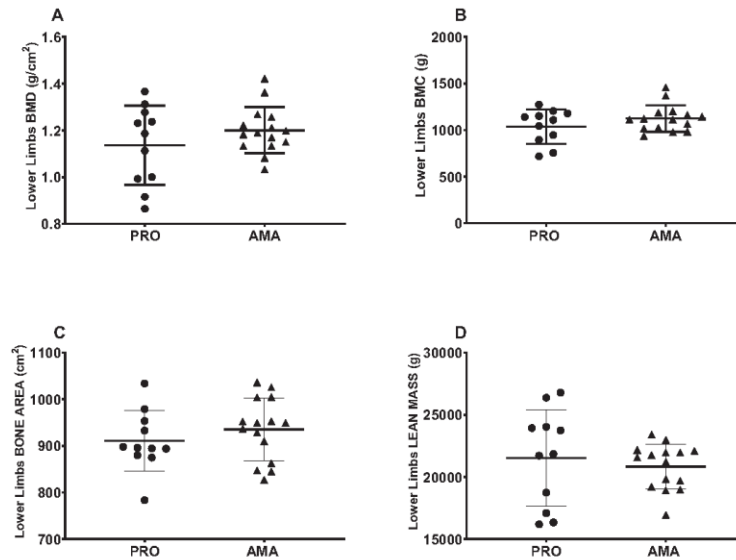


Fig. 3. Differences in bone and muscle mass in the lower limbs in PRO compared to AMA, measured by densitometry.

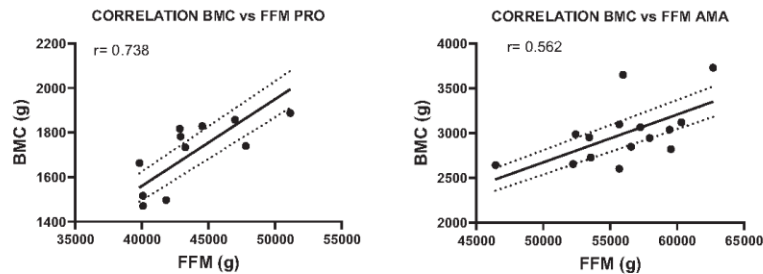


Fig. 4. Correlations between bone mineral content and fat-free mass in PRO and AMA.

After the analysis of the correlations between body composition variables, PRO and AMA showed a positive correlation between BMC and FFM ($r \geq 0.562$, $p < 0.05$) (Fig. 4), where linear regression showed that 55% of the variance in BMC could be due to FFM in PRO and 32% in AMA. Similar correlations were also observed in PRO and AMA between BMC and BMD and BA ($r \geq 0.686$, $p \leq 0.05$) (Table 2). However, the linear regression model only attributed 27% of the variance in BMD to FFM in PRO and 3% in AMA. When correlation analysis was performed for lower limbs, in PRO a significant positive correlation was observed between BMD, BMC, BA and FFM of the legs ($r \geq 0.630$, $p < 0.05$) (Table 3). Although, in AMA, significant positive correlations were seen between BMC with BMD, BA and FFM ($r \geq 0.652$, $p < 0.01$) of the lower limbs, it was not seen between FFM and BMD in the legs. In both PRO and AMA, the linear regression model was improved by comparing the

body composition variables measured between the whole body and the legs (Table 3).

3.3. Physiological and metabolic parameters at VT1

Significant group differences were observed in VO_{2VT1} (3593 vs 2041 mL·min⁻¹, $p \leq 0.001$, ES = 4.40), W_{VT1} (299 vs 157, $p \leq 0.001$, 4.07), WR_{VT1} (4.37 vs 2.27 W·kg⁻¹, $p \leq 0.001$, ES = 4.14), $\%VO_{2MAXVT1}$ (76.2 vs 49.7, $p \leq 0.001$, ES = 5.36) and HR_{VT1} (149 vs 132 beats·min⁻¹, $p = 0.004$, ES = 1.25), but not for RER_{VT1} (0.906 vs 0.899, $p = 0.707$, ES = 0.15) in PRO compared to AMA. Correlations between performance and body composition variables were also evaluated. In the PRO group, significant positive correlations were found between W_{VT1} and FFM ($r = 0.611$, $p \leq 0.05$) (Table 4) (Fig. 5). The linear regression model

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Table 2
Correlation and linear regression between body composition variables (whole body) from professional and amateur cyclists.

		BMD	BMC	BOHE AREA	FFM
PRO (n=11)					
BMD	r	Perfect line	0.690	-0.052	0.516
	p-value		0.02	0.88	0.10
	R ²		0.477	0.003	0.266
BMC	r	0.690	Perfect line	0.686	0.738
	p-value	0.02		0.02	0.01
	R ²	0.477		0.471	0.545
FFM	r	0.516	0.738	0.505	Perfect line
	p-value	0.10	0.01	0.11	
	R ²	0.266	0.545	0.113	
AMA (n=15)					
BMD	r	Perfect line	0.818	0.205	0.179
	p-value		<0.001	0.46	0.52
	R ²		0.669	0.042	0.032
BMC	r	0.818	Perfect line	0.730	0.562
	p-value	<0.001		<0.01	0.03
	R ²	0.669		0.532	0.316
FFM	r	0.179	0.562	0.748	Perfect line
	p-value	0.52	0.029	0.001	
	R ²	0.032	0.316	0.560	

Values are expressed as mean (SD). Abbreviations: BMD bone mineral density; BMC bone mineral content; BA bone area; FFM fat-free mass.
Values p <0.05 are in bold.

Table 3
Correlation and linear regression between body composition variables in the legs of professional and amateur cyclists.

		BMD Legs	BMC Legs	BOHE AREA Legs	FFM Legs
PRO (n=11)					
BMD Legs	r	Perfect line	0.937	0.320	0.807
	p-value		<0.01	0.34	<0.05
	R ²		0.877	0.102	0.651
BMC Legs	r	0.937	Perfect line	0.630	0.925
	p-value	<0.01		<0.05	<0.01
	R ²	0.877		0.397	0.855
FFM Legs	r	0.807	0.925	0.698	Perfect line
	p-value	<0.05	<0.01	<0.05	
	R ²	0.651	0.855	0.488	
AMA (n=15)					
BMD Legs	r	Perfect line	0.848	0.288	0.329
	p-value		<0.01	0.30	0.23
	R ²		0.719	0.083	0.108
BMC Legs	r	0.848	Perfect line	0.750	0.652
	p-value	<0.01		<0.01	<0.01
	R ²	0.719		0.563	0.426
FFM Legs	r	0.329	0.652	0.782	Perfect line
	p-value	0.23	<0.01	<0.01	
	R ²	0.108	0.426	0.611	

Values are expressed as mean (SD). Abbreviations: BMD bone mineral density; BMC bone mineral content; BA bone area; FFM fat-free mass.
Values p <0.05 are in bold.

showed that 11% of the variance of BMC could be explained by the power generated in VT1 (WVT1), and when including FFM as a covariate, the percentage of the variance increased to 46% in PRO. The linear regression model also improved in AMA when comparing WVT1 and BA and adjusting for FFM (6% to 55%).

3.4. Physiological and metabolic parameters at VT2

Significant group differences were observed in VO_{2VT2} (4259 vs 3389 mL·min⁻¹, p <0.001, ES = 2.10), W_{VT2} (379 vs 286, p <0.001, ES = 2.28), WR_{VT2} (5.54 vs 4.13 W·kg⁻¹, p <0.001, ES = 2.28) and % VO_{2MAXVT2} (90.3 vs 84.7, p = 0.005, ES = 1.24), however, no differences

Table 4
Correlation and linear regression between of body composition and performance variables from professional and amateur cyclists.

		BMD	BMC	BOHE AREA	FFM
PRO (n=11)					
W _{VT1}	r	0.441	0.326	0.011	0.611
	p-value	0.17	0.33	0.97	<0.05
	R ²	0.194	0.106	<0.001	0.373
W _{VT2}	r	0.114	0.462	0.244	-
	p-value	0.563	0.603	0.268	0.775
	R ²	0.07	<0.05	0.43	0.01
W _{MAX}	r	0.317	0.364	0.072	0.601
	p-value	0.166	0.434	0.116	-
	R ²	0.684	0.585	0.123	0.586
AMA (n=15)	r	0.027	0.149	0.235	0.010
	p-value	0.92	0.60	0.40	0.97
	R ²	<0.001	0.022	0.055	<0.001
W _{VT1}	r	-0.129	0.226	0.547	-
	p-value	0.119	0.174	0.168	0.107
	R ²	0.67	0.54	0.55	0.70
W _{VT2}	r	0.141	0.030	0.028	0.012
	p-value	-0.118	0.217	0.496	-
	R ²	0.216	0.338	0.336	0.251
W _{MAX}	r	0.44	0.22	0.22	0.37
	p-value	0.047	0.114	0.113	0.063
	R ²	-0.093	0.250	0.514	-

Values are expressed as mean (SD). Abbreviations: BMD bone mineral density; BMC bone mineral content; BA bone area; FFM fat-free mass. R²a Linear regression for BMD, BMC and BA for each of the exercise zones (WVT1, WVT2 and WMAX) was fitted with FFM from each group. For the linear regression of FFM with the different exercise zones no covariates were used.
Values p <0.05, and trend (p 0.06) with r>0.500 are in bold.

were found in HR_{VT2} (168 vs 171 beats·min⁻¹, p = 0.467, ES = 0.29) y RER_{VT2} (1.01 vs 1.03, p = 0.323, ES = 0.40) in PRO compared to AMA. In the PRO group, significant positive correlations were found between W_{VT2} and BMC and FFM (r ≥ 0.603, p ≤ 0.05) (Table 4) (Fig. 5). The linear regression model showed that 36% of the variance in BMC could explained by the power generated in VT2 (WVT2), but when adjusting for FFM (covariate) the model improved to 43% in PRO. In AMA, the model also improved when comparing WVT2 and BA and adjusting for FFM (3% to 50%).

3.5. Physiological and metabolic parameters at VO_{2max}

Significant group differences were found in VO_{2MAX} (4714 vs 4066 mL·min⁻¹, p = 0.002, ES = 1.38), VO_{2/RMAX} (69.0 vs 58.7 mL·kg⁻¹·min⁻¹, p = 0.003, ES = 1.34), W_{MAX} (474 vs 383, p <0.001, ES = 2.13), WR_{MAX} (6.93 vs 5.51 W·kg⁻¹, p <0.001, ES = 2.09), and RER_{MAX} (1.22 vs 1.14, p = 0.001, ES = 1.49), but not for HR_{MAX} (186 vs 186 beats·min⁻¹, p = 0.966, ES = 0.02). In the PRO group, significant positive correlations were found between W_{MAX} and BMD (r = 0.684, p ≤ 0.05) (Fig. 5). We also observed a moderate correlation with trend between W_{MAX} levels and BMC (r = 0.585, p = 0.059) and FFM (r = 0.586, p = 0.058) in PRO (Table 4). The linear regression model results showed that 34% of the variance could be due to W_{MAX}, and when covarying with FFM, the variance increased to 48% in PRO. However, in PRO, a worsening of the model was found when comparing BMD and W_{MAX} and covarying FFM (47% to 36%). In AMA, the model improved when comparing WVT2 and BA and adjusting for FFM (11% to 51%).

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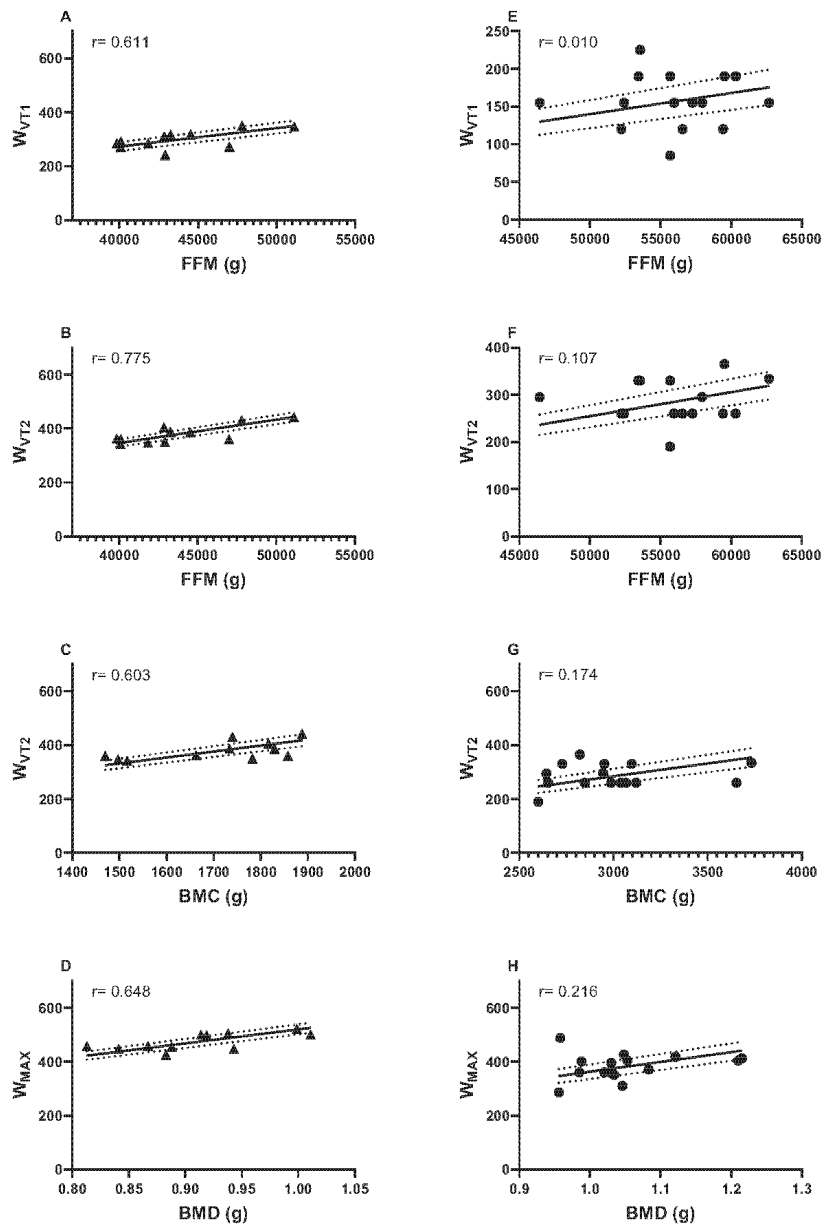


Fig. 5. A-D correlations between body composition markers (bone and muscle mass) and performance in PRO. E-H correlations between body composition markers (bone and muscle mass) and performance in AMA.

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4. Discussion

This research was aimed at finding out differences in body composition and their relationship to performance. Our results demonstrate that: i) lower levels of bone (BMD, BMC and BA) and muscle mass (FFM) variables in PRO vs AMA; ii) positive correlations between BMD, BMC and FFM in PRO; iii) linear regression models were better at comparing body composition and performance variables in the legs than in the whole body in both groups; iv) improvement of the linear regression model when comparing FFM-adjusted performance and body composition variables in both groups, except for WMAX and BMD relationship and v) PRO have higher levels of absolute and relative power output and VO_2 in all intensity zones (VT1, VT2 and $\text{VO}_{2\text{MAX}}$) than in AMA, with the largest differences found at VT1.

4.1. Differences in subject characteristics and body composition

Overall, the data shown in this study report lower values of bone health markers (BMD, BMC, BA) in PRO compared to AMA. According to the NHANES III, the normal BMD total values in men is 1.033 g/cm^2 [29]. We found that all our PRO cyclists were below this value ($0.911 \pm 0.061 \text{ g/cm}^2$, -2.1 to -21.3% with respect to normal values), and in AMA, there were 7 out of the 15 cyclists below the normal value for BMD ($1.052 \pm 0.079 \text{ g/cm}^2$, -9.1 to $+15.2\%$ with respect to normal values). These inferior values concur with the low BMD values observed in elite and master cyclists (highly trained males) [21,30] and are most likely explained by the nature of cycling which elicits low impact forces on the bone. Many authors have studied the relationship between cycling and bone health and have found lower BMD of the lumbar spine in PRO compared to a control group [31], as well as lower BMD of the femoral neck and lumbar spine compared to healthy males [30]. González-Aguero et al. [32] found that young cyclists had lower levels of BMC and volumetric BMD in some sites of the tibia and radius than sedentary young people. This indicates that cycling induces modifications in bone status from an early age, and this is aggravated when cyclists reach higher levels of competition (professional cyclists). Previously, Olmedillas et al. [33] also described a negative impact on bone health in adolescent cyclists, BMD for pelvis, where hip, leg and whole body and leg bone area were lower but higher in the hip area (all, $P < 0.05$) after adjusting for lean mass and height (adolescent cyclists vs sedentary).

Peak BMC is an important parameter in monitoring bone health, particularly during aging [13]. However, anthropometric characteristics (fat and muscle mass) have also been shown to have an effect on bone mass [13]. Medelli et al. [13] showed 2.8 kg of BMC in PRO, which are higher than those obtained in our PRO cohort (1.7 kg). The variations in BMC data between the two studies may be due to differences in the instrumentation used or FFM.

In amateur competitive cyclists it has been seen that a reduction in energy availability leads to a significant reduction of 2.3% in lumbar BMD over a 6 month interval, however a significant increase of 2.2% in lumbar BMD was detected for those who had improved energy availability [34]. On the other hand, cyclists who increased skeletal load showed a significant increase of 1.4% in lumbar BMD, compared to a significant decrease of 2.5% in lumbar BMD for those who had reduced skeletal load [34]. An increase in calcium lost through sweat during 2 h of moderate cycling has also been observed and is associated with a decrease in serum calcium accompanied by an increase in plasma parathyroid hormone concentration, which can promote bone resorption [35]. Although this response was mitigated, when a calcium supplement was taken immediately before or during cycling, the parathyroid response to exercise [36]. Cortisol is another factor influencing bone metabolism, as it has been seen to increase after completing an ultra-marathon (endurance sport) and is negatively correlated with bone formation markers [37]. However, a recent study reported that serum cortisol concentration increased steadily post-exercise in relation

to pre-exercise after 4 days of cycling training (3h daily day), conversely, a decrease in pre-exercise cortisol was found on days 2 to 4 compared to pre-exercise on day 1 [20].

Osteocalcin is another bone marker that responds to metabolic stress and intervenes secondarily in the regulation of energy metabolism. Osteocalcin is a marker of bone formation (in its carboxylated form) and when there is low energy or high energy demand it is excreted in its non-carboxylated form, acting as a secondary messenger [38]. It has been shown that a professional cycling competition (Giro d'Italia, duration 3 weeks) increased the carboxylic form of osteocalcin in the mornings (before the competition) on the 12th and 22nd [39], indicating an increase in metabolic stress and not bone synthesis. Therefore, this continuous state of a decrease in bone synthesis processes together with an increase in bone resorption processes supported by situations of low energy availability that would lead to hormonal modulation, would predispose the professional cyclist to a low BMD. But this increased response of bone turnover can be positive in non-professional athletes, however in professional athletes who train more than once a day with a short recovery time between training sessions and rest between days are more likely to have a continuous increase in bone remodeling (accumulation of micro-damage in the bones), which can lead to negative effects on bone health with an increased risk of stress fractures [20,40,41]. This hypothesis could be in line with our results where we found a low BMD in all PRO but only in around half of the AMA. Future studies should clarify this pathological situation in the PRO and AMA, suggesting new prevention strategies in this sense.

Interestingly, Medelli et al. [42] showed abnormally low BMD values in two-thirds of PRO and observed positive correlations between FFM, BMC and BMD. In contrast to our findings where we found a moderate but non-significant correlation between FFM and BMD in PRO only ($r = 0.516$) but significant between BMC and FFM ($r = 0.738$) in AMA. In addition, the linear regression model showed that 55% in PRO and 32% in AMA of the variation in BMC could be due to FFM. Previous studies have detected a significant positive correlation between FFM and whole-body BMD in AMA ($r = 0.634$) and in PRO ($r = 0.420$) [13,14]. In contrast, we found lower correlations in AMA ($r = 0.179$) and higher correlations in PRO ($r = 0.516$) in whole body measurements. However, Medelli et al. [13] found no significant correlation between total BMC and whole-body FFM in PRO, although, right leg and left leg BMC were positively correlated with FFM ($r = 0.44$; $p < 0.05$ and $r = 0.83$; $p < 0.001$), respectively. In line with these previous results, we observed a significant positive correlation between BMC and FFM in PRO ($r = 0.925$; $p < 0.01$; $R^2 0.855$) and AMA ($r = 0.652$; $p < 0.01$; $R^2 0.652$) in the legs, suggesting that there might be some effect of FFM on the lower limbs. In fact, our PRO showed -21.8% in FFM compared to AMA, together with decreased BMD, BMC and BA. An effect of FFM on bone tissue has been suggested to be exert specific loading on the bones, which involve loading force, frequency and location [43]. This was confirmed by our linear regression models both when assessing whole body and lower limb body composition, with the PRO model being superior to AMA. Overall, PRO have lower values in all bone health markers, which indicate a higher risk of fractures compared to AMA. These differences are not found when comparing the lower limbs. However, the correlations found between BMC and FFM in both PRO and AMA suggest that a larger muscle mass may have a protective effect on bone tissue.

4.2. Performance and metabolic differences

4.2.1. Differences in VT1

At VT1, PRO had higher values in VO_2 (76.0%), W_{VT1} (90.4%), WR_{VT1} (92.5%), $\% \text{VO}_{2\text{MAXVT1}}$ (53.3%) and HR_{VT1} (12.9%) compared to AMA. Compared to our study, Lucia et al. [7] found lower values in $\text{VO}_{2\text{VT1}}$ (3190 mL), W_{VT1} (262 W), WR_{VT1} ($3.8 \text{ W}\cdot\text{kg}^{-1}$), $\% \text{VO}_{2\text{MAXVT1}}$ (65%), HR_{VT1} ($138 \text{ beats}\cdot\text{min}^{-1}$) and RER_{VT1} (0.86) in PRO at VT1. Interestingly, another study in PRO by the same authors observed higher

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values than ours in W_{VT1} (321 W) and WR_{VT1} (4.55 W·kg⁻¹) and HR_{VT1} (154 beats·min⁻¹) [4]. These tests were carried out using a ramp test (continuous workload increases of 5 W·12 s⁻¹ or 25 W·min⁻¹) [4], which may explain the discrepancy between studies.

A novelty of the present study is that the differences between PRO and AMA in power produced and VO_2 were larger at VT1 (90.4% and 76.0%; respectively) than at VT2 (32.5% and 25.6%) or VO_{2MAX} (23.8% and 15.9%). However, Lucia et al. [7] found better values for power output (11.5%) and VO_2 (5.6%) at VT1 in PRO compared to elite riders, these lower differences may be mainly due to the fact that elite riders had a higher competitive level than our AMA sample. These differences could be explained by the fact that PRO cyclists have a high amount of type I muscle fibers, which is associated with a lower sub-maximum oxygen cost (greater gross efficiency) [9], possibly due to a lower turnover of ATP during the contraction [44]. Moreover, the number of years spent performing endurance training is also related to the percentage of type I fibers in skeletal muscle (i.e., higher utilization of fat metabolism in PRO) [12].

We also found significant correlations between W_{VT1} and VO_{2VT1} , % $VO_{2MAXVT1}$, VO_{2VT2} and VO_{2MAX} in both groups ($r \leq 0.683$). These findings suggest that cyclists who generated more power in VT1 had higher VO_2 in the measured metabolic zones. These correlations found in both groups indicate that cyclists who are able to generate more power at VT1 have a higher VO_2 at VT1, VT2 and VO_{2MAX} . There is no literature that studied similar parameters in VT1 to compare with our study. Moreover, we did not find any significant correlation in W_{VT1} between performance and bone variables but with FFM in PRO. Overall, we can establish that an essential requirement for being PRO is the ability to generate high levels of PO at low-moderate intensities (VT1), coupled with high levels of VO_2 and % VO_{2MAX} .

4.2.2. Differences in VT2

VT2 represents high work intensity where there is a considerable accumulation of lactate in blood (production exceeds clearance), associated to a marked increase in ventilation in an effort to buffer acidosis [45,46]. At VT2, we have found higher VO_2 , W_{VT2} , WR_{VT2} and % VO_{2MAX} in PRO vs AMA. Lucia et al. [7] observed similar findings at VT2 in PRO cyclists during an incremental test (VO_2 : 5100 mL, oxygen uptake relative to weight (VO_2/R): 62.2 mL·kg⁻¹·min⁻¹, W: 385 W, WR: 5.5 W·kg⁻¹, % VO_{2MAX} : 87%, HR: 172 beats·min⁻¹ and RER: 0.99).

Some studies have found higher % VO_{2MAX} in VT2 or lactate threshold in AMA compared to lower fitness level participants [12,47]. In PRO, high values of VT2 (~90% of VO_{2MAX}) have been documented, similar to those found in our study [7]. Therefore, these findings indicate that high VT2 values may provide an advantage to cyclists, particularly during climbing stages where cyclists find themselves working close to VT2 for prolonged durations (~30–60 min) [5]. The ability to work for long periods and at high % VO_{2MAX} at VT2 seems to be an important factor for success in professional competitions that are of long duration (> 4h).

On the other hand, previous studies have found a strong correlation between average power output (approximately generated in VT2) and flat cycle TT of 40 km ($r = -0.99$) [48] and during a mountain climb (10-km up-hill cycling) (-0.61 to -0.85), the latter being positively affected by lower weight [49]. It has been reported that absolute power output at VT2 correlates with the 40 km cycle TT ($r = -0.81$) [48] and that sub-maximum power output with various methods to identify ventilatory thresholds was significantly associated with 40 km cycle TT ($r = 0.73$ to 0.81) [50] in competitive cyclists. We have found significant correlations between W_{VT2} and the different VO_2 levels (VT1, VT2 and VO_{2MAX}) in both groups. Therefore, high power production at VT2 might be linked to higher oxygen uptake in PRO and AMA. Also, in PRO, we found a significant positive correlation between W_{VT2} with BMC and FFM ($r \leq 0.603$), which had not been described before. Furthermore, the linear regression model showed that 36% of the changes in BMC could be due to W_{VT2} in PRO, and when adjusted for FFM, the model improved

to 43%. This could originate from different mechanisms. For example, W_{VT2} might correlate with BMC because of the link between W_{VT2} and FFM, due to the fact that the generation of higher tension forces at the muscle-tendon-bone level, plays a key role in the remodeling and/or prevention of bone loss. This indicates a link between muscle mass, bone mass and VT2 performance.

Therefore, we can say that there are great differences between PRO and AMA at the performance and metabolic level in VT2, with an important relationship between muscle, bone, and VT2 power in PRO. Although there is no study in the current literature that has found a cause-effect relationship between higher FFM and BMD and VT2 performance. It should also be mentioned that a greater power generated at VT2 in absolute values does not always guarantee success in a competition, because in a mountain stage the power to weight ratio is very important. Hence, cyclists, who have VT2 values close to VO_{2MAX} , would have greater performance capability in longer races.

4.2.3. Differences in VO_{2MAX} and maximum power output

VO_{2MAX} is one of the best predictor of performance in endurance sports [8]. We found higher values in VO_{2MAX} , VO_2/R_{MAX} , W_{MAX} , WR_{MAX} and RER_{MAX} in PRO compared to AMA. Other studies have also reported higher VO_{2MAX} values (73.9–84.0 mL·kg⁻¹·min⁻¹) in PRO compared to the data found in our PRO cohort [4,7,51,52]. Besides, we showed that the PRO reached average W_{MAX} of 474 W and WR of 6.9 W·kg⁻¹.

Padilla et al. [5] found W_{MAX} ranging from 400 to 500 W (6.5 to 7.5 W·kg⁻¹) in a 4-min incremental test in PRO, which is consistent with our results (474 W). Interestingly, other studies have shown that PRO can reach these power outputs using shorter increments (1-min increments of 25 W) [4,6,7,53]. In the same line, Chicharro et al. [54] found significantly higher VO_{2MAX} (12.7%; 72.0 vs 63.9 mL·kg⁻¹·min⁻¹) and W_{MAX} (30.3%; 499.8 vs 383.7 W) in PRO vs AMA (respectively). The greater difference for W_{MAX} than for VO_{2MAX} suggests that the O₂ cost of cycling is likely better in PRO vs AMA. However, the same authors showed similar RER (-0.9% ; PRO: 1.16 vs AMA: 1.17) and HR (1.6%; PRO: 194 vs AMA: 191 beats·min⁻¹) at exhaustion in both groups after an incremental test (increments of 25 W·min⁻¹).

Although high levels of VO_{2MAX} are needed to be competitive in road cycling races at world level, it has been proposed that this parameter is not a good predictor of performance for PRO [7], whereas W_{MAX} is [3]. Hawley and Noakes [3] reported a significant correlation ($r = -0.91$) between W_{MAX} in a graded exercise test (25 W every 150 s) and 20 km TT in trained cyclists. We found significant correlations between W_{MAX} and VO_{2VT1} , VO_{2VT2} and VO_{2MAX} in PRO and AMA. This suggests that cyclists who generate more power have a higher VO_2 , and this likely contributes to higher performance in competition. Additionally, we showed significant positive correlations between W_{MAX} and BMD ($r = 0.684$, $p = 0.02$), and a trend with BMC ($r = 0.585$, $p = 0.06$) and FFM ($r = 0.586$, $p = 0.06$), only in PRO, which have not been previously demonstrated before. Furthermore, the linear regression model showed that 47% and 34% of the changes in BMD and BMC, respectively, could be due to W_{MAX} in PRO, and when adjusting for FFM, the model improved to 48% for BMC and worsened to 36% for BMD. Based on our results and taking into account the relationship between lower W_{MAX} and BMD in PROs, special attention should be paid to bone health in PROs with lower sports performance (W_{MAX}), in order to establish some type of treatment. Overall all of the PROs had low BMD values (NHANES), so a system for monitoring bone status in professional cycling teams should be put into place. On the other hand, Izquierdo et al. [55] observed a significant correlation between muscle cross-sectional area (at the lower third portion between the greater trochanter and lateral joint line of the knee) and W_{MAX} in amateur cyclists during an incremental test. Previous studies and the present findings question the link between muscle mass/strength and road cycling performance. González-Aguero et al. [32] that cycling may negatively impact bone health of cyclists at an early age, which could

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transcend into further complications later when they become professional cyclists. Furthermore, a literature review concluded that professional road cycling may be more detrimental to bone mass compared to recreational cycling, or worse than other disciplines (e.g., cross-country cycling or cycling/running combinations), hypothesizing that differences in BMD between cyclists and controls or other sport practitioners become greater from the age of 17 years and on [56]. Thus, achieving high power output for long durations is important in cycling performance and the role of muscle strength in cycling performance has been repeatedly reported, especially with studies investigating the effects of concurrent aerobic and heavy strength training [57–61].

5. Limitations

Our study had limitations with regards to the sample, since it is more difficult to recruit PRO athletes than lower-level athletes (AMA).

Differences in body composition markers between this study and previous works may be influenced by the instrumentation and methodology used, the timing of the season at which the measurements were made, and the training status of the cyclists.

6. Conclusions

In our study, 100% of PRO had BMD values below the normal values (1033 g/cm³) proposed by NHANES III in men [29]. In addition, in the physiological-metabolic profile, PRO had lower values of BMD, BMC and FFM, but superior W and VO₂ at VT1, VT2 and VO_{2MAX} compared to AMA. Interestingly, we found positive correlations in PRO between FFM with BMC and BMD, which indicates that muscle mass can promote higher levels of bone markers. Future studies should explore the physiological-metabolic profile at different times of the season in both PRO and AMA.

CRedit authorship contribution statement

Conceptualization, F.J.M.N., C.M.P., R.O.R. and P.E.A.; methodology, F.J.M.N., C.M.P., R.O.R. and P.E.A.; formal analysis, F.J.M.N. and C.M.P.; investigation, F.J.M.N., C.M.P. and R.O.R.; resources, F.J.M.N. and C.M.P.; data curation, F.J.M.N., C.M.P. and R.O.R.; writing—original draft preparation, F.J.M.N.; writing—review and editing, F.J.M.N., C.M.P. and S.D.; visualization, F.J.M.N. and C.M.P.; supervision, C.M.P., S.D. and P.E.A.; project administration, F.J.M.N. and C.M.P. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

This study was supported by the Research Center for High Performance Sport of the Catholic University of Murcia (Murcia, Spain). We would like to acknowledge Linda H. Chung for her help in this project.

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Article

Acute Effects of Hesperidin in Oxidant/Antioxidant State Markers and Performance in Amateur Cyclists

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Received: 16 July 2019; Accepted: 13 August 2019; Published: 14 August 2019



Abstract: Human and animal studies have shown that Hesperidin has the ability to modulate antioxidant and inflammatory state and to improve aerobic performance. The main objective of this study was to assess whether the acute intake of 500 mg of 2S-Hesperidin (Cardiose[®]) improves antioxidant status, metabolism, and athletic performance, during and after a rectangular test (aerobic and anaerobic effort). For this, a crossover design was used in 15 cyclists (>1 year of training), with one week of washout between placebo and Cardiose[®] supplementation. After the intervention, significant differences in average power (+2.27%, $p = 0.023$), maximum speed (+3.23%, $p = 0.043$) and total energy (Σ 4 sprint test) (+2.64%, $p = 0.028$) between Cardiose[®] and placebo were found in the best data of the repeated sprint test. Small changes were also observed in the activity of catalase, superoxide dismutase, reduced glutathione concentration and oxidized/reduced glutathione (GSSG/GSH) ratio, as well as the lipoperoxidation products (thiobarbituric acid reactive substances; TBARS), at different points of the rectangular test, although not significant. Our findings showed improvements in anaerobic performance after Cardiose[®] intake, but not in placebo, suggesting the potential benefits of using Cardiose[®] in sports with a high anaerobic component.

Keywords: hesperidin; hesperitin; antioxidants; catalase; superoxide dismutase; reduced glutathione; oxidized glutathione; performance and exercise

1. Introduction

The use of ergogenic aids in sports has increased considerably in recent years [1]. This growing interest is driven by the emergence of studies that have shown how ergogenic aid intake can contribute to improvement in athletic performance, post-exercise recovery, or antioxidant capacity enhancement, as well as changes in body composition (e.g., body fat loss or increase in lean muscle mass), by a stimulation of fatty acids mobilization [2–6]. Within the ergogenic aids, there exist several categories based on the degrees of evidence shown in scientific literature. For instance, category A refers to the highest level of evidence and includes proteins, amino acids, creatine, beta-alanine, carbohydrates, etc. [7]

One potentially promising group that can serve as an ergogenic aid is polyphenols [8]. Polyphenols are bioactive compounds which are widely distributed in plant and plant-based foods, such as vegetables, fruit, cocoa, tea, coffee, and wine [9]. Polyphenols are a very diverse group of compounds, with over 500 different molecules identified in foods, which can be divided into four main categories, according to their chemical structure: flavonoids (e.g., hesperidin, hesperitin, etc.), phenolic acids, stilbenes, and lignans [10]. In addition, polyphenols are of the most studied compounds for their

positive effects on human health [11]. Specifically, these products are often used for chronic disease, delaying the ageing process, improving body composition, and increasing life expectancy. Moreover, polyphenols have been proposed to be beneficial in exercise and exercise performance [12–15]. In fact, some polyphenols, such as quercetin [8,16–18] or cocoa flavanols [19–21] have been extensively used for this purpose.

For example, polyphenols have been proposed to improve performance by increasing mitochondrial biogenesis in two different ways. Firstly, they stimulate stress-related cell signalling pathways that increase the expression of genes encoding cytoprotective proteins, such as nuclear respiratory factor 2 (NRF2) [22]. Secondly, it has been reported that these phytochemicals may modulate muscle function and mitochondrial biogenesis by activating sirtuins (SIRT1) and increasing PGC-1 α activity. [23–25]. Moreover, polyphenols have been shown to work effectively against exercise induced oxidative stress [26,27], since as seen in many investigations, exercise increases reactive oxygen species (ROS) production, which may result in oxidative stress, and lead to muscle fibre damage, which eventually results in muscle fatigue [28–31]. Within the large family of biomolecules that are polyphenols, the most studied in the field of sports performance is quercetin [16,17], although new molecules of this family such as luteolin, mangiferin [32], and hesperidin [33] are being investigated.

Hesperidin is a polyphenol, specifically a flavonoid, that is mostly found in citrus fruits [34]. Hesperidin is the most relevant flavonoid in some citrus species, such as sweet orange (*Citrus sinensis*), finding high concentration of hesperidin in orange juice (up to 513 mg/L) [35]. Hesperidin is a chiral molecule, and can be found in two isomeric forms, as 2S- and 2R-Hesperidin. However, the 2S-Hesperidin form is naturally predominant in citrus fruits [36], being present in fresh sweet orange juice with an 2S isomer/2R isomer ratio of 15.4:1 in favour of the 2S-epimer (92% 2S-Hesperidin) [37]. However, during the industrial extraction and isolation of Hesperidin, part of this 2S-epimer naturally present in the citrus fruits is transformed into the 2R-epimer. In commercial hesperidin samples, the 2S isomer/2R isomer ratio is close to 1.5:1 in favour of the 2S-epimer (about 60% 2S-Hesperidin). Cardiose® is a natural orange extract, produced by HealthTech BioActives (Murcia, Spain), that due to its unique manufacturing process maintains most of the natural hesperidin isomeric form (NLT 85% 2S-Hesperidin).

Hesperidin antioxidant [38], anti-inflammatory [39], and health promoting [40] properties have been extensively described. Moreover, the intake of hesperidin improves the nitric oxide (NO) synthesis through the activation of phosphorylation of proto-oncogene tyrosine-protein kinase (Src), protein kinase B (Akt), adenosine monophosphate-activated protein kinase (AMPK), and endothelial NO synthase, leading to an increased flow-mediated dilation [41]. An increased NO production leads to an improved endothelial function, allowing an enhanced O₂ transport to working muscles during acute exercise and prolonged exercise [42]. In humans, the intake of flavanone-rich foods (a type of flavonoids including Hesperidin) has been linked to an increase in NO production, increased endothelium-dependent microvascular reactivity, as well as a reduction in diastolic blood pressure [43–47]. Endothelial function and different cardiovascular parameters were also improved after hesperidin supplementation in individuals with metabolic syndrome [41] and in overweight individuals [48].

Hesperetin, hesperidin, and its main metabolite have shown to boost mitochondrial energy production (spare capacity by 25%), to increase intracellular ATP by 33%, to reduce oxidative stress in a human skeletal muscle cell model [49]. Moreover, this same study also reported that the intake of hesperidin in aged mice (50 mg/kg/day) reverted the age-related muscle loss, improving its running performance. In the same direction, the effects of hesperidin on biochemical parameters and physical performance have been also studied in humans. Pittaluga et al. (2013) [50] investigated the effect of supplementation with a self-administrated amount of 250 mL of fresh red orange juice (ROJ) (natural source of hesperidin), thrice a day and 1 h before each meal during 4 weeks and after a single bout of exercise until exhaustion in healthy trained elderly women. The working capacity expressed as metabolic equivalents (METs) was significantly higher after ROJ supplementation (+9.0%) than

in placebo (−1.5%), while there was no significant increase of maximal oxygen uptake (VO_{2max}) in any group.

Previous studies have observed effects in biochemical markers after the intake of hesperidin. De Oliveira et al. (2013), after supplementation with hesperidin (glucosyl hesperidin; 100 mg/kg body mass) led to a decline of serum glucose with combined beneficial effect on swimming. Continuous or intermittent swimming with hesperidin supplementation lowered total cholesterol (−16%), low-density lipoprotein C (LDL-C) (−50%) and triglycerides (−19%), and increased high density lipoprotein (HDL-C) (48%) [51] in rats. Previous research has shown that daily consumption of 500 mL of orange juice for 3 months decreases lactic acidosis generated by the incremental exercise. The decrease in plasma lactate concentration was higher in the trained and hesperidin supplemented group (−27%) than in the control trained group (−17%) of overweight middle-aged women subjected to aerobic training [52].

Another ability of hesperidin is its capability to modulate the antioxidant state. For instance, De Oliveira et al. (2013) found that the consumption of hesperidin enhanced the antioxidant capacity on the continuous swimming group (183%) and decreased the lipid peroxidation (TBARS) on the interval swimming group (−45%) in rats [51]. Similarly, Estruel-Amades et al. (2019) observed an impact of hesperidin on the oxidant/antioxidant status of lymphoid tissues after an intensive training program was evaluated on rats. Supplementation with hesperidin, enriched in its 2S-isomer, led to a prevention of the increased ROS production and the decrease in superoxide dismutase (SOD) and catalase activities after an exhaustion test. These antioxidant effects of hesperidin were associated with a higher performance in the assessed training model [53].

In trained male athletes, supplementation for 4 weeks with 2S-Hesperidin (500 mg/day) improved cycling time-trial performance with a significant increase in power output during the exercise test [33]. Gelabert-Relato et al. (2019) compared acute and chronic effect (48 h and 15 days of supplementation) with high- and low-dose intake of polyphenols (luteolin and mangiferin), on sprint test and endurance exercise in physically active men [32]. The results showed a significant improvement in the sprint test (sprint 15 s after ischemia) in peak power output (PPO) and mean power output, after the polyphenol supplementation. Also, in the Wingate test, the experimental group improved by 4%. Also, Davis et al. (2010) conducted a crossover study examining quercetin's ability to increase endurance capacity and maximal oxygen uptake (VO_{2max}) in healthy untrained volunteers. They showed that a 7-day quercetin supplementation (1000 mg/day) produced improvements in time-to-fatigue and VO_{2max} by 13.2% and 3.9%, respectively, during a cycling test [17].

Previous studies, which have used different types of polyphenols and dosages, have observed enhancements in performance. Although the exercise protocols used were different, all of them showed improvements in exercise performance with high aerobic component [17,54–57]. However, to our knowledge, only one study reported improvements in the anaerobic component of exercise [32]. Thus, it is not clear if there is a beneficial effect of polyphenols on anaerobic exercise. Furthermore, only one study has examined the effect of hesperidin on exercise performance, showing an increase of absolute power output by 5% [33]. Moreover, given the importance of anaerobic component on performance in endurance sport and the limited evidence found related to the intake of hesperidin in anaerobic performance and its mechanisms, it seems necessary to conduct more research in order to clarify this relationship.

Therefore, the aims of the present study were (1) to examine the effects of acute intake of Cardiose® (500 mg of 2S-hesperidin) on anaerobic performance (peak power, power average, time to peak power, max speed and total energy (Σ 4 sprint test)), (2) to determine the metabolic markers during exercise in ventilatory threshold 1 (VT1), and (3) to compare oxidative/antioxidant state during a rectangular test and after 24 h recovery in amateur cyclists. The recommendations from this experimental study will have the potential to inform about the optimal supplementation guidelines to optimise the performance and the recovery practices in athletes and provide sports nutritionist key information regarding the effects of polyphenol intake on sports performance and markers of oxidative stress.

2. Methodology

Fifteen healthy male amateur cyclists (Table 1) completed the study. All participants had to meet the following inclusion criteria: aged 18–50 years, normal BMI ($19\text{--}25\text{ kg m}^{-2}$), at least 1-year of cycling experience, undergoing 6–12 h/week of training and being regular citrus consumers. The exclusion criteria were: (a) smokers or alcohol drinkers, (b) metabolic or cardiorespiratory pathologies or anomalies, (c) acute or chronic digestive pathologies that may interfere with the capacity to absorb nutrients, (d) injury in the last 6 months, (e) intake any type of supplementation or drug in the last 2 weeks, (f) no normal values in some parameter of the previous blood analysis and regular consumer of citrus fruits (≥ 5 oranges and derivatives/week). All subjects signed their informed consent before participating in the study. The study was performed in accordance with the guidelines of the Helsinki Declaration for Human Research [58] and was approved by the Ethics Committee of the Catholic University of Murcia.

Table 1. Baseline general characteristics of the study participants.

Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)	BF (%)	$\text{VO}_{2\text{max}}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	VT1 (%)	VT2 (%)
33.3 ± 7.9	174.9 ± 4.2	69.4 ± 4.5	22.7 ± 1.2	11.2 ± 2.2	61.6 ± 7.4	53.0 ± 6.1	86.0 ± 4.7

BMI = Body mass index; BF = Body fat; $\text{VO}_{2\text{max}}$ = Maximum oxygen consumption; VT1 = Ventilatory threshold 1 (aerobic); VT2 = Ventilatory threshold 2 (anaerobic).

2.1. Experimental Design

A randomized, single-blinded cross-over design was performed (Figure 1). Participants completed a total of 2 exercise sessions. Five hours before the exercise sessions, they ingested Cardiose[®] (500 mg of 2S-hesperidin) or placebo (500 mg of microcellulose), supplied by HealthTech BioActives (Murcia, Spain). Cardiose[®] contains standardized hesperidin (90% hesperidin, being at least 85% as 2S-Hesperidin isomer) from sweet orange (*Citrus Sinensis*).

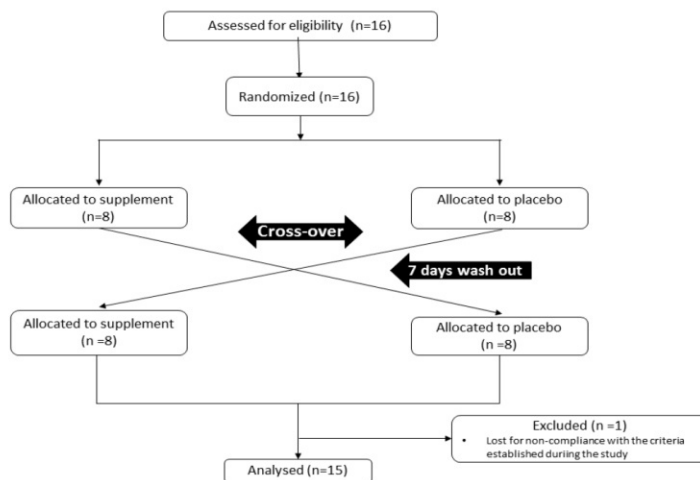


Figure 1. Consolidated Standards of Reporting Trials flow chart of participants during the study intervention.

2.2. Procedures

Participants visited the Research Center for High Performance Sport at the Catholic University of Murcia at six different times. Visit 1 consisted of a medical examination, blood extraction, and anthropometry. Visit 2 consisted of a 24 h diet questionnaire before testing and an incremental test until exhaustion on a bike. Five hours prior to visit 3, participants were supplemented with Cardiose[®] or placebo, according to the treatment arm. During Visit 3, participants underwent another 24 h diet questionnaire before testing, a 20 min test at ventilatory threshold 1 (VT1) intensity on a bike before and after a repeated all-out sprints test on a cycle ergometer, and four blood extractions. Visit 4 (24 h following visit 3) consisted of blood extraction and obtaining 24 h urine collection from the participant. Visits 5, 6, and 7 involved the same procedures performed as in visit 2, 3 and 4, respectively, but five hours prior to visit 6, participants were supplemented the other ingredient (Cardiose[®] or placebo). There were no significant differences between the 24-h diet questionnaire made by the subjects. A standardized breakfast was consumed 2.5 h prior to each testing session (visits 2 and 3). The breakfast contained 95.16 gr of carbohydrates (68%), 18.86 g of protein (14%) and 11.30 g of lipids (18%), prescribed by a sports nutritionist.

2.3. Tests

2.3.1. Medical Exam

The medical exam included a medical history, resting electrocardiogram, and medical examination (auscultation, blood pressure reading, etc.), so as to confirm that the participant was healthy and without any risk to be enrolled in the study.

2.3.2. Anthropometry

The same researcher (International Society for the advancement of the Kinanthropometry Level-1 certified) performed the anthropometric measurements in both pre- and post-test. Height and body weight were measured using a digital scale for clinical use with a stadiometer (SECA 780; Vogel & Halke GmbH & Co., Hamburg, Germany). The skinfold thickness was assessed in accordance with ISAK guidelines [59], using Holtain Skinfold Calipers (Holtain Ltd., Crymych Pembrokeshire, UK). Percentage of body fat was determined with the Faulkner Equation [60] and the percentage of muscle mass with the modified Matiegka equation [61]. The sum of the eight skinfolds was also calculated.

2.3.3. Maximal Test

An incremental step test was performed with the metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine the ventilatory threshold 1 and 2 (VT1 and VT2) and $\dot{V}O_{2max}$. The test started at 35 W and increased 35 W every 2 min until exhaustion. To verify $\dot{V}O_{2max}$, the following criteria were assumed: plateau in the final $\dot{V}O_2$ values (increase $\leq 2.0 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in the 2 last loads), maximal theoretical heart rate (HR) ($220 - \text{age}$), respiratory exchange ratio (RER) ≥ 1.15 and a lactate value $\geq 8.0 \text{ mmol}\cdot\text{L}^{-1}$ [62,63]. The ventilatory threshold was obtained using the ventilatory equivalents method described by Wasserman [64].

2.3.4. Rectangular Test

A constant effort was carried out on the bike at VT1 intensity during 20 min before and after the repeated all-out sprints test. The main objective of this test was to determine cardiorespiratory variables ($\dot{V}O_2$, CO_2 , RER, HR, and exercise economy) during a steady effort at low-intensity (Figure 2). This test was conducted before and after the repeated sprints test.

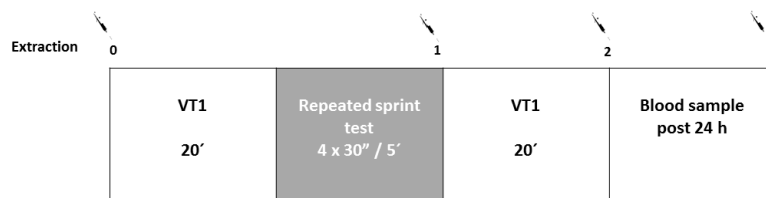


Figure 2. Exercise protocol and blood sampling plan during Visit 3/6. VT1 = ventilatory threshold 1; syringe = blood sample.

2.3.5. Repeated Sprints Test

The exercise protocol consisted of 4×30 sec all-out sprints (Wingate test; WAnT) performed on a cycle ergometer (Monark Ergonomic 894E Peak Bike, Vansbro, Sweden) with 5 min of rest between sprints. For every sprint, the breaking resistance was constant (7.5% of body mass) and individualized for each participant (Figure 2). All subjects were verbally encouraged to continue to pedal as fast as they could for the entire 30 s. Peak power and anaerobic capacity were calculated and recorded in watts (W) and watts per kilogram body weight (W/kg^{-1}). The total energy was calculated as the energy produced during the four test sprints in joules (J).

2.3.6. Blood and Urine Analysis

Venous blood samples were taken for general analytics, in one tube of 3 mL ethylenediaminetetraacetic acid (EDTA) for hemogram, and in another tube of 3.5 mL with polyethylene terephthalate (PET) for biochemical parameters. Red blood cells count (RBC) was carried out in an automated Cell-Dyn 3700 analyzer (Abbott Diagnostics, Lake Forest IL, USA), using internal (Cell-Dyn 22, Abbott Diagnostics, IL, USA) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, hemoglobin, haematocrit, and hematimetric indexes (mean cell volume, MCV; mean cell haemoglobin, MCH; mean corpuscular hemoglobin concentration, MCHC; and red cell distribution width, RDW) were estimated.

Additionally, venous blood samples were collected pre VT1 test, post repeated sprint test, post second VT1 test, and 24 h after the end of the testing session for the measurement of antioxidant parameters (Figure 2). At each of the extraction points, 6 tubes of 3 mL of EDTA were obtained and one of them was centrifuged at 3500 rpm at 4 °C during 10 min and sent to the laboratory for later analysis. Urine samples, corresponding to 24 h urine collection from each participant after the supplementation, were frozen in liquid nitrogen after collection and thawed for its analysis.

2.3.7. Hesperidin Metabolites Urine

Fifty μ L of urine were mixed with 100 μ L of water with 1% formic acid containing the internal standard (rac-Hesperetin-d3). Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series Agilent Technologies, Sta. Clara, CA, USA). The method was validated using a pool of samples by determining the limit of detection (MDL) and quantification (MQL), repeatability (expressed as relative standard deviation RSD), and accuracy (%). Metabolites were quantified by external standard calibration using rac-Hesperetin-d3 as the internal standard.

2.4. Markers of Oxidative Stress and Antioxidant Status

2.4.1. TBARS

Thiobarbituric acid reactive substances (TBARS) are a by-product of the oxidative degradation of lipids by reactive oxygen species (lipid peroxidation), a commonly used marker of oxidative stress [65]. The principle of the method consists of isolating the lipid fraction of the plasma by precipitation of the

lipids with phosphotungstic acid, followed by a reaction with thiobarbituric acid (TBA) that forms an adduct that allows detection by UV-VIS spectrophotometer at a wavelength of 532 nm. The assay involves the reaction of malondialdehyde (MDA), a product of lipid peroxidation, with thiobarbituric acid (TBA) under high temperature and acidic conditions to form an MDA–TBA complex that can be measured colorimetrically [66].

2.4.2. Catalase

Catalase (CAT) activity was determined using a UV-VIS spectrophotometer. The principle of the method is that the absorbance of H_2O_2 decreases at 240 nm proportional to its decomposition, so that the concentration of H_2O_2 is critical in this determination. The decrease in absorbance per unit time is the measure of catalase activity. This is expressed in sec^{-1} per gram of hemoglobin [67]. The coefficient of variation between replicas must be less than or equal to 4.9%.

2.4.3. SOD

Superoxide dismutase (SOD) activity was measured using a SD125 Ransod kit (Randox Ltd., Crumlin, United Kingdom). This method consists of the use of xanthine and xanthine oxidase to produce superoxide anion (O_2^-), which responds with the 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) reactive and forms a red complex detectable at 420 nm. The activity of SOD was measured through the inhibition of this reaction. The SOD activity is then quantified by measuring the degree of inhibition of this reaction [68]. The coefficient of variation between replicas must be less than or equal to 5.1%.

2.4.4. Glutathion

Glutathion (GSH) was analyzed by the glutathione-S-transferase assay described by Akerboom and Sies [69]. The GSH was determined from whole blood, which was treated with perchloric acid to a final concentration of 6%, obtaining the supernatant after vortexing and centrifuging at 10,000 rpm for 10 min. After collecting the supernatants in vials, it was quantified by high performance liquid chromatography (HPLC) using a Waters ODS S5 NH2 Column (0.052, 25 cm) for separation purposes. Glutathion oxidized form, glutathione disulphide (GSSG), was determined in a similar way to GSH as shown above, as described by Asensi [70].

2.5. Statistical Analyses

The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 21.0, International Business Machines Chicago, IL, USA). Descriptive statistics are presented as mean \pm SD. The assumption of normality was verified using the Shapiro–Wilks test. Paired sample t-test was used to evaluate differences between groups (Cardiose[®] or placebo supplement). Moreover, a General Linear Model (repeated measures, analysis of variance, ANOVA) was performed for analyzing the within-group effects of the intake of the Cardiose[®] or placebo supplement (4 time points). Additionally, the standardized mean differences (Cohen's d (ES)) between groups was calculated together with the 95% confidence intervals and η^2 to analyze the size between groups. Finally, the relationships between levels of excreted hesperidin metabolites in urine and total energy and catalase activity were analyzed using Spearman correlation analysis (r). For all procedures, a level of $p \leq 0.05$ was selected to indicate statistical significance.

3. Results

3.1. Repeated Sprint Test

Results for each variable were analyzed in two different ways: taking the best data of each of the four sprint test included in the series, and considering all the sprints as a unique exercise, using for each variable the average of all sprints results.

On one hand, taking into account the data corresponding to the best sprint, significant positive changes were observed in Cardiose[®] compared to placebo in average power, maximum speed, and total energy (Σ 4 sprint test). However, no significant changes were found in peak power and time-to-peak power comparing Cardiose[®] versus placebo group using these data (Table 2) (Figure 3).

Considering the average values of the four sprints trials, positive changes were observed in the peak power, time-to-peak, and total energy in the Cardiose[®] group compared to placebo, but not reaching the statistically significance (Table 2) (Figure 3). In addition, there was a positive significant correlation ($r = 0.547$; $p = 0.043$) between the levels of excreted hesperidin metabolites in urine and the difference in total energy (Σ 4 sprint test) between the placebo and supplemented group.

Table 2. Repeated sprint test outcomes.

Parameters	Best Sprint Data		Average (All Sprints)	
	Cardiose [®] Mean \pm SD	Placebo Mean \pm SD	Cardiose [®] Mean \pm SD	Placebo Mean \pm SD
PeakPower (w)	835.50 \pm 96.08	803.79 \pm 110.43	740.16 \pm 74.52	729.55 \pm 91.36
Poweraverage (w)	567.84 \pm 55.44 *	555.25 \pm 51.81 *	511.71 \pm 52.68	510.78 \pm 52.99
Time to peakpower (ms)	2840.69 \pm 715.99	3235.85 \pm 1516.06	3003.13 \pm 950.28	3476.14 \pm 1546.57
Max speed(rpm)	132.86 \pm 9.59 *	128.70 \pm 9.24 *	120.83 \pm 7.79	119.92 \pm 9.79
Totalenergy (J)	16246.29 \pm 1600.37 *	15827.79 \pm 1505.86 *	14874.79 \pm 1570.83	14818.36 \pm 1608.24

Δ = percentage of pre-post change; W = watts; ms = millisecond; J = joules; Max speed = maximum speed; rpm = revolutions per minute SD = standard deviation. * = between-group significant changes ($p < 0.05$).

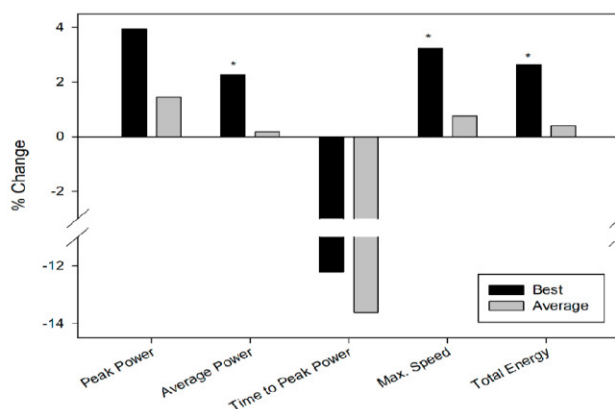


Figure 3. Changes in the repeated sprint test results after supplementation with Cardiose[®] using best data of each of the four sprint tests and the average of all sprints. * = between-group significant changes ($p < 0.05$).

3.2. Metabolic Parameters

Metabolic parameters were evaluated and compared for the rectangular tests (20 min at VT1) carried out before and after the repeated all-out sprints test. During the VT1 testing period, no significant differences were observed in pre-post changes between Cardiose[®] and placebo group (Table 3).

Table 3. Metabolic parameters in VT1 stage before and after the repeated sprint test.

Metabolic parameters		Cardiose [®]	Placebo
		Mean ± SD	Mean ± SD
VO ₂ (L/min)	Pre	2.11 ± 0.39	2.06 ± 0.39
	Post	2.15 ± 0.45	2.13 ± 0.51
VCO ₂ (L/min)	Pre	1.93 ± 0.41	1.86 ± 0.35
	Post	1.83 ± 0.43	1.77 ± 0.43
RER	Pre	0.91 ± 0.03	0.90 ± 0.02
	Post	0.85 ± 0.03	0.83 ± 0.02
Efficiency (mL/Kg/W)	Pre	3.97 ± 0.48	3.85 ± 0.39
	Post	4.02 ± 0.49	3.94 ± 0.51
HR (pul/min)	Pre	128.55 ± 9.53	128.04 ± 8.95
	Post	144.15 ± 12.50	144.74 ± 11.31
Carbohydrates	Pre	105.5 ± 33.3	97.6 ± 19.7
	Post	72.9 ± 28.5	63.6 ± 17.7
Fat	Pre	14.1 ± 6.0	15.9 ± 6.1
	Post	28.5 ± 4.0	31.8 ± 8.7

Δ = percentage of pre-post change; heart rate (beats·min⁻¹); SD = standard deviation; VO₂ = oxygen uptake (L·min⁻¹); VCO₂ = carbon dioxide production (L·min⁻¹); W = watts; HR = Heart rate; RER = Respiratory exchange ratio.

In relation to the cardiorespiratory parameters, a nonsignificant increase in oxygen consumption and a significant decrease in CO₂ production were observed in both groups. However, no significant differences between groups were observed for these two parameters. In the same way, respiratory exchange ratio (RER), which is the ratio between the amount of CO₂ produced and oxygen used in metabolism, significantly decreased from the first to the second rectangular test in both groups, but without significant differences between them. Exercise economy is defined as the oxygen uptake relative to body mass used at the VT1 workload. After the repeated sprint test, there was a non-significant increase in exercise economy in both groups. Also, there were no significant differences between groups. Moreover, the high-intensity repeated sprint test caused a significant increase in heart rate during the second VT1 stage of rectangular test in both groups. However, there were no relevant differences between supplements (Table 3).

Regarding energy substrates, a significant decrease in carbohydrates consumption and a significant increase in fat energy contribution from the first to the second rectangular test was observed in both groups, as expected after an intense physical activity such as repeated sprint test. Despite no significant differences observed between the Cardiose[®] and placebo groups, this modulation of the energy substrates was slightly different in both supplements. Cardiose[®] led to a higher increase (+15.1%) in the fat energy contribution (Figure 4).

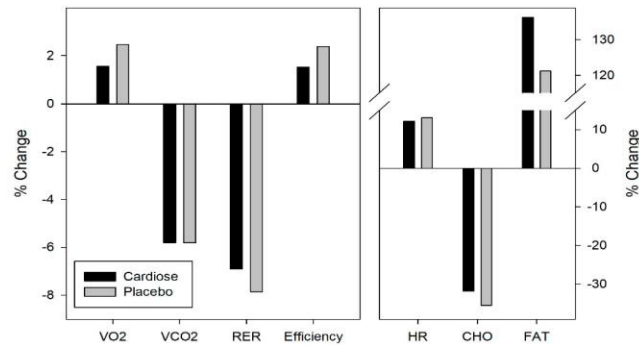


Figure 4. Changes in cardiorespiratory and metabolic parameters between the rectangular test (VT1 intensity during 20 min) carried out before and after the repeated all-out sprints test.

3.3. Antioxidant Parameters

Effects of each treatment on the modulation of oxidative status were evaluated by the activity of the endogenous antioxidant system, such as SOD, CAT, GSH, GSSG, and TBARS, as biomarker of lipid peroxidation. These markers were evaluated prior to the testing session (E1: pre VT1 test, and 5 h after supplementation), post repeated sprint test (E2), post second VT1 test (E3), and 24 h after the end of the testing session (E4).

Regarding the activity of the CAT enzyme, the exercise protocol included in this study led to significant differences in CAT activity at the different evaluated points in both groups. For instance, a significant decrease was observed from the end of the repeated sprint test to the end of rectangular test in both groups. Regarding differences between the Cardiose[®] and placebo groups, catalase activity was slightly increased in the Cardiose[®] group after supplementation with this product—prior to the testing session, after the repeated sprint test, and at the end of the testing session—despite catalase activity being almost the same in both groups 24 h after the end of the testing session. However, these differences between groups were not statistically significant (Table 4). An inverse significant correlation between the levels of hesperidin metabolites excreted in urine and the percentage variations in the catalase activity at points E1 and E3 ($r = -0.625$; $p = 0.013$) between the placebo and supplemented group was observed.

Regarding the activity of SOD, repeated sprint test increased SOD activity both in Cardiose[®] and placebo groups. However, a completely different trend was observed after this strenuous exercise in SOD activity of both groups. At the end of the rectangular test, SOD activity decreased in the Cardiose[®] group, while it slightly increased in the placebo group. In contrast, both groups experienced a decrease in SOD activity from the end of the physical test to 24 h after exercise. However, these differences were not statistically significant (Table 4) (Figure 5).

Physical activity included in the exercise protocol increased TBARS levels in both groups. However, a greater attenuation of lipid peroxidation, identified by a decrease in TBARS, was observed in the Cardiose[®] group from after repeated sprint test to the end of rectangular test. However, no significant changes were observed between interventions and between blood extraction points (Table 4) (Figure 5).

Table 4. Antioxidants and TBARS measured in repeated sprint test. Antioxidant markers were evaluated prior to the testing session (pre VT1 test, and 5 h after supplementation) [E0], post repeated sprint test [E1], post second VT1 test [E2], and 24 h after the end of the testing session [E3].

Antioxidant/Oxidant Status Markers	Cardiose®				Placebo			
	E0	E1	E2	E3	E0	E1	E2	E3
CAT (U/g Hb)	25.66 ± 4.74	53.93 ± 13.41 *	27.53 ± 6.54 *	24.66 ± 4.27	24.02 ± 3.13	51.41 ± 16.41 *	27.07 ± 4.63 *	24.53 ± 4.18
SOD (U/g Hb)	1298.00 ± 261.75	1349.13 ± 225.31	1269.27 ± 271.13	1228.33 ± 229.77	1319.00 ± 145.54	1352.13 ± 201.31	1364.80 ± 272.74	1337.67 ± 193.97
GSH (nmol/mg protein)	25.02 ± 2.80	24.89 ± 2.90	23.73 ± 2.10	24.36 ± 2.75	24.36 ± 2.24	23.59 ± 3.37	23.62 ± 3.19	24.60 ± 1.72
GSSG (nmol/mg protein)	0.351 ± 0.073	0.334 ± 0.075	0.315 ± 0.067	0.378 ± 0.152	0.325 ± 0.073	0.316 ± 0.078	0.336 ± 0.068	0.388 ± 0.130
% GSSG/GSH	1.42 ± 0.32	1.35 ± 0.31	1.34 ± 0.31	1.54 ± 0.54	1.34 ± 0.28	1.37 ± 0.41	1.45 ± 0.37	1.57 ± 0.48
TBARS (nmol/mg protein)	2.49 ± 0.34	2.71 ± 0.45	2.56 ± 0.44	2.63 ± 0.26	2.43 ± 0.22	2.63 ± 0.36	2.57 ± 0.38	2.58 ± 0.32

Abbreviations: CAT = catalase; SOD = superoxide dismutase; GSH = Reduced glutathione; GSSG = oxidized glutathione; % GSSG/GSH = % oxidized glutathione/ Reduced glutathione; TBARS = Thiobarbituric acid reactive substances; SD = standard deviation. * = intra-group significant changes ($p < 0.05$)

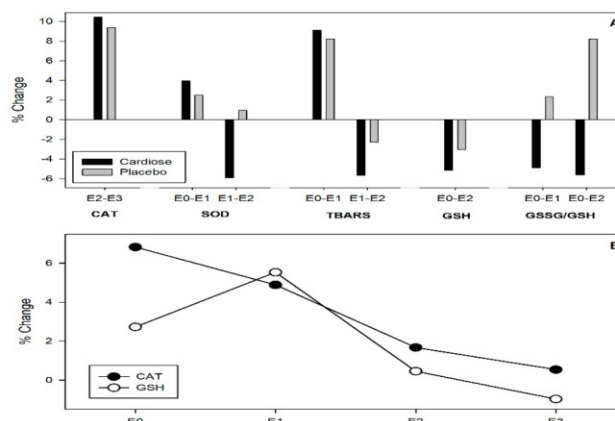


Figure 5. Changes in antioxidants and TBARS measured in repeated sprint test. (A) Changes between different time points. (B) Changes between treatments. Antioxidant markers were evaluated prior to the testing session (pre VT1 test, and 5 h after supplementation) [E0], post repeated sprint test [E1], post second VT1 test [E2], and 24 h after the end of the testing session [E3].

No significant decrease in GSH levels during the exercise protocol was observed in both groups. Despite levels of this antioxidant peptide being higher after Cardioise[®] supplementation at baseline and after the repeated sprint test, differences between treatments were not significant (Figure 5). A positive significant correlation between the levels of hesperidin metabolites in urine and the percentage variations in the levels GSH $\% \Delta$ 01-02 ($r = 0.551$; $p = 0.033$) between the placebo and supplemented group was observed.

A completely different trend in GSSG/GSH ratio was observed in both groups during the testing session. In the Cardioise[®] group, GSSG/GSH ratio decreased during the testing session: after the repeated sprint test, and after the end of rectangular test. In the placebo group, GSSG/GSH ratio increased during the physical exercise: after the repeated sprint test and after the end of the testing session. Despite this different behavior in the GSSG/GSH ratio during the test, differences in the GSSG/GSH ratio were not statistically significant (Table 4) (Figure 5).

3.4. Hesperidin Metabolites Urine

Different hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analyzed in urine of the participants after the intake of Cardioise[®]. The main metabolite was Hesperetin-3-glucuronide, representing $78.9 \pm 5.0\%$ ($n = 15$) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate were $6.9 \pm 2.9\%$ ($n = 15$) and $14.7 \pm 4.1\%$ ($n = 15$) of the excreted metabolites, respectively. Despite the similarities in the excreted metabolites profile, a large interindividual variability was observed in the amount of hesperidin metabolites excreted, ranging from 2.3 to 37.5 μmol . These wide differences between subjects in the absorption and excretion of hesperidin have been already reported [71].

4. Discussion

The main objective of this study was to assess the acute effects of 500 mg of 2S-hesperitin on physical performance, specifically in exercise with high anaerobic component [32], and secondary in metabolism and antioxidant status in amateur cyclists. The results showed that a single supplementation with 500 mg amount of 2s-Hesperidin may improve anaerobic parameters in a repeated sprint test for the

Cardiose[®] group. In addition, an improvement in antioxidant capacity and energy metabolism were observed after Cardiose[®] supplementation during the exercise protocol.

A significant improvement in average power (+2.27%), maximum speed (+3.23%), and total energy (+2.64%) was observed after Cardiose[®] supplementation when the best data of the sprint test series were considered. However, no significant improvements in anaerobic performance parameters were found for Cardiose[®] group when average values of the repeated sprint test were evaluated. In addition, a positive correlation between excreted hesperidin metabolites in urine and the differences in total energy (Σ 4 test) between placebo and Cardiose[®] was also found. Therefore, these data show that supplementation with Cardiose[®] improves physical performance in an anaerobic trial such as the repeated sprint test. These results are in line with the improvement in physical performance observed in trained rats after 2S-Hesperidin supplementation [53] or in cycling time-trial performance in trained male athletes after supplementation with 2S-Hesperidin (500 mg/day) for 4 weeks [33]. These studies reported improvements of 58% in the time the exhaustion test and 5% in absolute power output a 10 min time-trial, respectively. Since anaerobic power is a key factor in sport performance [72], but sometimes difficult to improve, achieving small improvements in anaerobic performance as those described in this study may be very important for athletes, especially in high sports performance.

Antioxidant status and endogenous antioxidant capacity are key factors for the athlete's performance [73]. Especially during high-intensity and short-duration or low-intensity and high-duration exercises which provoke high production of free radicals (ROS), these may be mediated through a variety of pathways [74]. Our study showed small changes in different antioxidant enzymes (CAT and SOD), peptides with antioxidant activity (GSSG/GSH), and oxidation markers (MDA-TBARS) between the Cardiose[®] and placebo groups. Exercise-induced ROS production causes lipid peroxidation [31], superoxide anion generation through xanthine oxidase (XO) activation, and the increase in oxidized/reduced glutathione (GSSG/GSH) ratio [28,29]. Enzymes like SOD and glutathione are important antioxidant defences that protect cells from ROS-induced oxidative stress [75]. Oxidative stress may cause cellular damage through modifications to macromolecules, including proteins, lipids, and nucleic acids, and can occur as a result of high-intensity or moderate- to long-duration exercise [30]. In our study, the intense physical exercise causes an increase in CAT activity, which was observed in both experimental groups. An increase in the activity of CAT, versus placebo, was observed following the acute supplementation with Cardiose[®]. Also, an inverse correlation between the excreted levels of hesperidin metabolites in urine, and the percentage variations in the activity of CAT $\% \Delta$ 01–03 ($p = 0.013$) was observed. These results suggest that the acute intake of Cardiose[®] might promote the activity of this antioxidant enzyme. In rats submitted to intense exercise, 2S-hesperidin supplementation contributed to maintain catalase activity, and avoid changes induced by physical activity [53]. An increase in catalase activity during exercise may offer an advantage in high intensity efforts (e.g., sprint), where there is a high rate of ROS production, decreasing damage to the muscle cell.

Hesperidin has been also described to increase the activity of this antioxidant enzyme during senescence [76] or modulate its activity when it is impaired by different conditions [77,78]. In general, intense physical activity increases SOD activity [79]. However, a decrease in SOD activity after repeated sprint test to the end of rectangular test was observed in Cardiose[®] (−5.9%) but not in placebo (+0.9%). This decrease was maintained 24 h after the end of exercise session. Cardiose[®] seems to reduce the overexpression of SOD induced by physical exercise. In previous studies, supplementation with 2S-Hesperidin decreased SOD activity in trained rats [53]. Due to its scavenging activity hesperidin neutralizes reactive oxygen species—such as superoxide anion—generated during conditions of oxidative stress, as intense physical exercise. The decrease in SOD activity may be related to the reduced need for this endogenous enzyme when an exogenous antioxidant, such as hesperidin or other flavonoids, is provided [80,81]. Therefore, this decrease in SOD activation would indicate a lower production of free radicals, which leads to less damage to muscle cell structures and a better post-exercise recovery.

As we have already mentioned, intense physical activity increases ROS production and consequently lipid peroxidation, producing an increase of malondialdehyde and TBARS [82]. ROS produced during physical activity may react with unsaturated fatty acids comprising cellular membrane, leading to lipid peroxidation, a chain reaction that oxidizes fatty acids and produces more ROS [83]. In our exercise protocol, high-intensity exercise increased TBARS in both groups. However, a greater attenuation of lipid peroxidation (TBARS) was observed in Cardioise® (−5.7 %) from after repeated sprint test to the end of rectangular test versus placebo (−2.3%). In previous studies, in rats subjected to interval swimming, the intake of hesperidin lowered (−45%) the lipid peroxidation [51]. Flavonoids such as hesperidin play a key role as free radical scavengers *in vivo*, preventing the increase in lipid peroxidation associated with high-intensity exercise. Furthermore, the antioxidant activity of citrus flavanones is not only related to their radical scavenging activity, but also to their ability to increase cellular defences via the Nrf2-ARE pathway, which regulates the expression of antioxidant genes including SOD, CAT, HO-1, GPX, and TXN, decreases intracellular pro-oxidants, and enhances antioxidant enzymes [76].

On the other hand, glutathione is a widespread peptide with antioxidant properties that may be found in plasma either as glutathione (GSH) or as glutathione disulfide (GSSG), its oxidized form [84]. Cardioise® supplementation led to no significant increase in glutathione (GSH) levels, and additionally a significant correlation (GSH % Δ 01–02, $r = 0.551$; $p = 0.033$) between the levels of hesperidin metabolites in urine and the percentage variations in the GSH levels was observed. The ratio between the oxidized (GSSG) and reduced (GSH) glutathione form is also evaluated as an antioxidant status marker [85]. A different trend in this ratio was observed according to the supplementation. Placebo led to an increase in the GSSG/GSH ratio during the exercise protocol, while Cardioise® supplementation decreased the GSSG/GSH ratio. These results suggest that Cardioise® promotes glutathione antioxidant role, indicating a better antioxidant status in the experimental group. The reason of these modifications in GSH and GSSG could be caused by low concentrations of lipoperoxides and hydrogen peroxide, which are metabolized by glutathione peroxidase (GPX), generating an increase in GSSG [86]. This increase is neutralized by the increase in the activity of glutathione reductase (GR) [87]. This effect can explain the finding from our study with GSH and GSSG/GSH ratio. In previous works [88,89], hesperidin supplementation has been shown to minimize the impairment of glutathione antioxidant system induced by different alterations, restoring the usual levels of this body's antioxidant peptide. All these changes in the endogenous antioxidant system (CAT, SOD, GSH, and GSSG) generate an ideal muscular environment to improve performance and recovery. Taken together, these results suggest that the intake of Cardioise® affects the body's own antioxidant capacity, even after an acute single intake.

Improvements in endurance sports' performance may be due to metabolic adaptations, which could be explained by the activation of PGC-1 α (a key regulator of energy metabolism that increases biogenesis and mitochondrial working capacity). Finally, in terms of the cardiorespiratory parameters analyzed, no significant differences were found between the Cardioise® group and placebo. Furthermore, no significant differences between treatments were found regarding the consumption of energy substrates, carbohydrates and fats, but Cardioise® supplementation promoted the use of fats as energy substrates (+15.1% versus placebo). Polyphenols induce changes in PGC-1 α activity via increased activation of the intracellular signalling pathways AMP-activated protein kinase (AMPK). Another factor that promotes metabolic adaptations induced by exercise is NRF2, a member of the Cap-N-Collar family of transcription factors, plays an important role in mitochondrial biogenesis, and variants of the NRF2 gene have been associated with endurance performance [90,91]. *In vitro* studies have shown that hesperidin also activates AMPK stimulating its phosphorylation [41]; besides this, in animal tissue an increase in NRF-2 expression was also observed [76]. These metabolic changes generate muscular level adaptations that prioritize the oxidation of fatty acids versus glucose, leading to higher energy efficiency [92–96], and therefore may predispose the athlete to a better sports performance. However, the small differences in oxygen consumption at the same intensity indicates a better exercise economy and, consequently, an improvement in performance.

As shown previously, Cardiose[®] supplementation seems to improve physical performance during a complete exercise protocol, modulating athlete's oxidative status during the physical activity in semi-professional cyclists. The absence of human studies with hesperidin including anaerobic power tests, makes difficult any comparison. It is important to highlight that these results were obtained after an acute and single intake of 500 mg of Cardiose[®] and placebo. The small changes observed after this single intake may be increased after a chronic consumption of this product. The main limitation of this study was the size of the sample. A larger sample could improve our results' power and the lack of previous research studies on the topic. Future research should be conducted to evaluate the chronic effect of 2S-hesperidin supplementation on sports performance and oxidative stress, as well as to clarify if hesperidin can improve physical performance during high-intensity exercise.

5. Conclusions

A single acute intake of Cardiose[®] (500 mg of 2S-hesperidin) improves performance in maximum anaerobic effort in semi-professional cyclists. In addition, oxidative status and antioxidant defenses were slightly modulated. These findings could help improve performance in high-intensity exercises for both amateur and high-performance athletes.

Author Contributions: Conceptualization, F.J.M.-N., C.M.-P. and P.E.A.; methodology, F.J.M.-N., C.M.-P. and P.E.A.; formal analysis, F.J.M.-N., C.M.-P. and J.C.-V.; investigation, F.J.M.-N., C.M.-P. and J.C.-V.; resources, F.J.M.-N., C.M.-P., J.A.R.-A. and J.C.-V.; data curation, F.J.M.-N., C.M.-P., J.A.R.-A. and J.C.-V.; writing—original draft preparation, F.J.M.-N.; writing—review and editing, F.J.M.-N., C.M.-P., J.A.R.-A. and J.C.-V.; visualization, C.M.-P.; supervision, C.M.-P. and P.E.A.; project administration, C.M.-P. and P.E.A.; funding acquisition, P.E.A.

Funding: This research was funded by HealthTech BioActives (Murcia, Spain).

Acknowledgments: This study was supported by Research Center in High Performance Sport of the Catholic University of Murcia and HealthTech BioActives (Murcia, Spain). We would like to acknowledge Linda H. Chung for her help in this project. We thank Iris Samarra, Antoni del Pino, and Núria Canela from the Metabolomics facility of the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat, for their contribution to urine analysis.

Conflicts of Interest: The authors declare that this study has been financed by HealthTech BioActives (Murcia, Spain), who kindly provided the product Cardiose[®], but they did not participate in the experimental design, data collection, data analysis, interpretation of the data, writing of the manuscript, or in the decision to publish the results.

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Article

Effects of 8 Weeks of 2S-Hesperidin Supplementation on Performance in Amateur Cyclists

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Received: 9 November 2020; Accepted: 18 December 2020; Published: 21 December 2020



Abstract: 2S-Hesperidin is a flavanone (flavonoid) found in high concentrations in citrus fruits. It has an antioxidant and anti-inflammatory effects, improving performance in animals. This study investigated the effects of chronic intake of an orange extract (2S-hesperidin) or placebo on non-oxidative/glycolytic and oxidative metabolism markers and performance markers in amateur cyclists. A double-blind, randomized, placebo-controlled trial was carried out between late September and December 2018. Forty amateur cyclists were randomized into two groups: one taking 500 mg/day 2S-hesperidin and the other taking 500 mg/day placebo (microcellulose) for eight weeks. All participants completed the study. An incremental test was used to evaluate performance, and a step test was used to measure oxygen consumption, carbon dioxide, efficiency and oxidation of carbohydrates and fat by indirect calorimetry. The anaerobic power (non-oxidative) was determined using Wingate tests (30 s). After eight weeks supplementation, there was an increase in the incremental test in estimated functional threshold power (FTP) (3.2%; $p \leq 0.05$) and maximum power (2.7%; $p \leq 0.05$) with 2S-hesperidin compared to placebo. In the step test, there was a decrease in VO_2 (L/min) (−8.3%; $p \leq 0.01$) and VO_2R (mL/kg/min) (−8.9%; $p \leq 0.01$) at VT2 in placebo. However, there were no differences between groups. In the Wingate test, there was a significant increase ($p \leq 0.05$) in peak and relative power in both groups, but without differences between groups. Supplementation with an orange extract (2S-hesperidin) 500 mg/day improves estimated FTP and maximum power performance in amateur cyclists.

Keywords: flavonoid; polyphenols; orange extract; performance; endurance; aerobic; anaerobic; nutrigenomic and sport nutrition

1. Introduction

Hesperidin is a flavonoid found mainly in citrus fruits [1], reaching high concentration in sweet orange (*Citrus sinensis*) [2]. Due to its chemical structure, including a chiral carbon (C-2), hesperidin can be present as S or R isomer (Figure 1). 2S-Hesperidin is the predominant natural form in citrus fruits [3], but industrial processing leads to the transformation of the natural S isomer into the R isomer (Figure 1) [4]. The bioavailability of the two isomers is different, for instance a 5.2-fold higher efficiency in the glucuronidation has been observed for S-hesperetin compared to R-hesperetin in vitro, without any significant change in the sulfonation kinetics [5]. Clinical trials have demonstrated the therapeutic effects of hesperidin and its metabolites in various diseases

(e.g., neurological and psychiatric disorders, cardiovascular diseases, etc.) due to its anti-inflammatory properties, antioxidants, inhibition of fat accumulation, improvement in glucose homeostasis and insulin sensitivity [6–9]. In view of its effects, the pharmaceutical and nutritional industries have extensively marketed hesperidin. However, little attention has been paid to the effects of hesperidin on exercise performance.

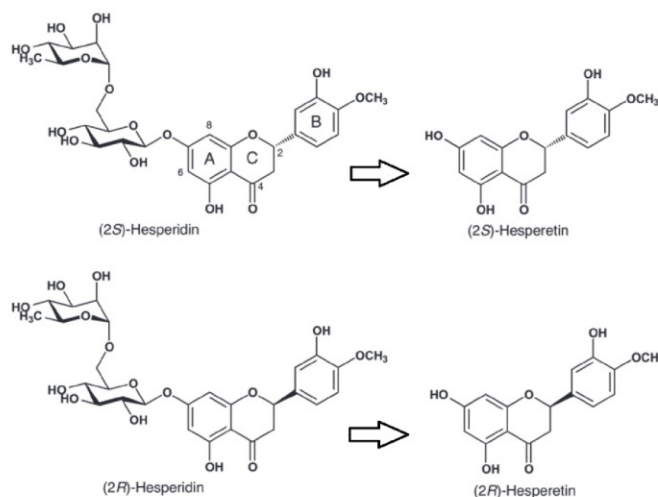


Figure 1. Structure of hesperidin enantiomers S and R and their metabolites hesperetin, produced by the intestinal microbiota. Modified from Li et al. [10].

Regarding performance, only one acute effects study in humans has investigated 2S-hesperidin [11]. This study showed that after ingesting one single 500 mg dose of either 2S-hesperidin 5 h before the test, trained cyclists significantly improved average power (2.3%), maximum speed (3.2%) and total energy (Σ 4 sprint test; total work) (2.6%) with 500 mg hesperidin supplementation in the best sprint out the four repeated sprint test (4×30 s all-out sprints with 5 min of rest between sprints). No significant changes were observed in any of these variables with placebo.

In humans, chronic supplementation of hesperidin has also been studied. Pittaluga et al. [12] investigated the effect of 250 mL of red-orange juice, which has a high content of hesperidin, on exercise performance (incremental test) in healthy, trained older women. Following four weeks of consumption of ROJ (3 per day), these older women significantly increased their work capacity by 9.0% compared to placebo (−1.5%). Another chronic study evaluated the effect of a four-week supplementation of 2S-hesperidin (500 mg/day) in trained cyclists and observed significant increases in average power output (14.9 W = 5.0%) in a 10 min time-trial test on a cycle ergometer, whereas those that consumed placebo had a non-significant increase in average power output (3.8 W = 1.3%), moreover, differences were found when comparing the groups [13]. In addition, another performance-enhancing mechanism has been observed in other substances, such as menthol or capsaicin (polyphenols) through taste, but this pathway has not been explored with 2S-hesperidin [14].

The effect of long-term intake of hesperidin has also been investigated in animal studies. Biesemann et al. [15] observed that six weeks of hesperetin supplementation (main metabolite of hesperidin) (50 mg·kg^{−1}·d^{−1}) improved running performance by 28.8% (exercise time until exhaustion) compared to placebo in aged mice. This study also found an improvement in endogenous antioxidant enzymes, such as reduced glutathione (GSH), oxidized glutathione (GSSG) and GSH:GSSG ratio.

De Oliveira et al. [16] found that four weeks of hesperidin consumption (100 mg/kg body mass) enhanced the antioxidant capacity in the continuous swimming group (183%) and decreased the lipid peroxidation (TBARS) in the interval swimming group (−45%) compared to placebo in rats. In the same line, a recent study in trained animals reported that intake of hesperidin for four weeks improved performance and prevented immune alterations induced by exhausting exercise compared to placebo [17]. Recently, one parallel-group study has shown improvements in the time until exhaustion (58%) on maximal exercise test at 3 weeks of a 5-week chronic supplementation of 2S-hesperidin (200 mg/kg), but not in placebo group (with differences between groups) [18]. In the same study, an enhancement of the antioxidant state was observed (superoxide dismutase (SOD), glutathione peroxidase (GPx)) in the lymphoid and hepatic tissue after the test until exhaustion in the rats that consumed 2S-hesperidin compared to placebo.

Hesperidin strongly increases intracellular ATP compared to the AMP-activated protein kinase (AMPK) activator 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), even when AICAR concentration has been increased by 10-fold (100 μ M) [15]. In addition, hesperetin (10 μ M) has been shown to increase intracellular ATP by 33% and mitochondrial spare capacity by 25%, as well as establish an antioxidant state [15]. Based on the understanding behind the mechanism of hesperidin *in vitro*, as well as the evidence presented above, hesperidin is a good candidate for improving performance.

Currently, some animal trials have shown that chronic hesperidin supplementation can improve performance, but in humans the evidence is weak and more research is needed. We hypothesised that chronic intake of 2S-hesperidin would improve performance at submaximal and maximal exercise intensities. Therefore, the main aims of this study were to examine the chronic effects of 2S-hesperidin (500 mg, Cardioise[®]) supplementation on: (1) power production at FatMax, ventilatory threshold 1 and 2 (VT1 and VT2) and maximum power in an incremental test (high aerobic component), and (2) maximum absolute and relative power during a Wingate test (high anaerobic component). The secondary objective was to evaluate whether hesperidin supplementation modified metabolic (O_2 and CO_2) and energy substrate (carbohydrates and fats) markers during a step test that could explain a possible enhancement in performance.

2. Methodology

2.1. Participants

Forty healthy, male amateur cyclists participated and completed the study. All the participants had to meet the following inclusion criteria: 18–55 years, BMI of 19–25.5 $kg \cdot m^{-2}$, at least 3 years of cycling experience and training for 6–12 $h \cdot wk^{-1}$. Volunteers were excluded if they: (a) were smokers or regular alcohol drinkers, (b) had a metabolic, cardiorespiratory, or digestive pathology or anomaly, (c) had an injury in the prior 6 months, (d) were supplementing or medicating in the prior 2 weeks and/or (e) had non-normal values in the blood analysis parameters. First, participants were informed about the procedures, and a signed informed consent was obtained. The study was conducted according to the guidelines of the Helsinki Declaration for Human Research [19] and was approved by the Ethics Committee of the Catholic University of Murcia (CE091802).

2.2. Study Design

A double-blind, parallel, and randomized experimental design was performed. Randomization was performed using computer software (Randomizer) to assign codes to the groups established in this study [20]. Participants were divided into two groups: experimental (2S-hesperidin; $n = 20$) and control (Placebo; $n = 20$). Total distance of usual training was balanced to make it similar between groups (Table 1). Participants consumed two capsules of 250 mg at the same time of either 2S-hesperidin (500 mg) (Cardioise[®], produced by HTBA (HealthTech BioActives—Murcia, Spain)) or placebo (microcellulose) for 8 weeks. Specifically, Cardioise[®] is a natural orange extract that, due to its unique manufacturing process, maintains most of the natural hesperidin isomeric form (NLT 85%

2S-hesperidin). The placebo supplements were also in capsulated form and similar in appearance to the 2S-hesperidin capsule. Cyclists were instructed to take the supplement along with breakfast and to continue their usual diet and training schedule. Subjects in both groups were instructed not to consume foods high in citrus flavonoids (grapefruit, lemons, or oranges) for 5 days prior to and during the study. This was verified by diet recalls records.

Table 1. Baseline general characteristics and training variables of participants.

	2S-Hesperidin	Placebo	p-Value
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Body mass (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI (kg·m ⁻²)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
VO _{2MAX} (L·min ⁻¹)	3.99 (0.36)	3.98 (0.63)	0.971
VO _{2MAX} (mL·kg ⁻¹ ·min ⁻¹)	57.5 (6.97)	57.9 (9.53)	0.880
HR _{MAX} (bpm)	184.9 (11.11)	183.2 (8.68)	0.593
VT1 (%)	50.9 (5.63)	50.0 (4.78)	0.610
VT2 (%)	84.9 (5.85)	84.1 (5.70)	0.644
Training variables	2S-Hesperidin	Placebo	p-value
Total distance (km)	1121.12 (534.99)	1082.43 (810.46)	0.868
HR _{AVG} (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
W _{AVG} (W)	174.9 (15.79)	163.5 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are expressed as mean (SD). BMI = body mass index; BF = body fat; VO_{2max} = maximum oxygen volume; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); Total distance = of all the training sessions carried out during the study period; HR_{avg} = average heart rate of all the training sessions carried out during the study period; W_{avg} = average power output of all training sessions during the study period.

2.3. Procedures

Participants visited the laboratory on seven occasions. Visit 1 consisted of a medical examination, blood extraction to determine health status and a familiarisation session with the Wingate test. When urine samples were collected on visit 2 in the fasted state, both groups consumed the supplements under the supervision of an investigator, which was followed by a standardized breakfast. On visits 2 and 5, a 24-h diet recall and a Wingate test were performed. On visits 3 and 6, another 24-h diet recall was conducted, followed by an incremental test until exhaustion on a cycle ergometer. On visits 4 and 7, the 24-h diet recall was repeated, and participants performed a step test on the cycle ergometer (Figure 2 and Table 2). Prior to each testing session (visits 2, 3, 4, 5, 6, and 7), a standardized breakfast (557.7 kcal) composed of 95.2 g of carbohydrates (68%), 18.9 g of protein (14%) and 11.3 g of lipids (18%) was prescribed by the sport nutritionist.

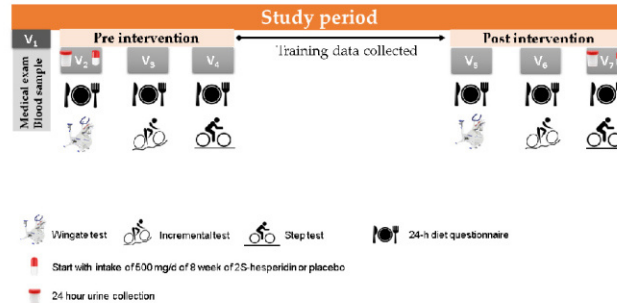


Figure 2. Study planning with explanation of the different visits (V 1–7).

Table 2. Between-group comparisons in dietary intake of cyclists.

	Pre-Intervention			Post-Intervention		
	2S-Hesperidin	Placebo	<i>p</i> -Value	2S-Hesperidin	Placebo	<i>p</i> -Value
Kcal	2163.6 (519.02)	2100.2 (515.77)	0.708	1974.1 (377.97)	2133.5 (437.98)	0.237
Kcal/BM	31.1 (9.34)	30.2 (8.71)	0.768	27.9 (6.53)	30.3 (6.46)	0.249
CHO (g)	245.7 (73.46)	222.0 (69.68)	0.312	216.6 (63.47)	248.3 (58.15)	0.117
CHO/BM	3.5 (1.31)	3.2 (1.14)	0.416	3.1 (1.08)	3.5 (0.94)	0.173
PRO (g)	113.5 (25.21)	115.2 (25.37)	0.837	109.0 (23.05)	101.5 (23.67)	0.332
PRO/BM	1.6 (0.41)	1.7 (0.48)	0.778	1.5 (0.35)	1.5 (0.42)	0.596
LP (g)	80.8 (27.24)	83.5 (23.65)	0.739	71.5 (17.61)	71.6 (18.89)	0.985
LP/BM	1.2 (0.45)	1.2 (0.37)	0.758	1.0 (0.27)	1.0 (0.29)	0.823

Values are expressed as mean (SD). Kcal = kilocalories; CHO = carbohydrates; PRO = protein; LP = lipids; BM = body mass. The mean values correspond to the average of all 24-h diet recall data collected at pre-intervention (visits 2, 3 and 4) and post-intervention (visits 5, 6 and 7).

2.4. Testing

2.4.1. Medical Exam

A medical examination, performed by the research centre's medical doctor and including health history, resting electrocardiogram and examination (auscultation, blood pressure, etc.), was used to confirm that the volunteer was healthy enough to be enrolled in the study.

2.4.2. Incremental Test

An incremental step with a final ramp test was performed on a cycle ergometer (Cyclus 2, RBM Elektronik-Automation GmbH, Leipzig, Alemania) using a metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine maximal fat oxidation zone (FatMax), VT1 and VT2 and maximal oxygen consumption (VO_{2max}). Participants began cycling at 35 W for 2 min, increasing then by 35 W every 2 min upon attainment of RER > 1.05, participants completed a final ramp 35 W/min until volitional exhaustion. To ensure VO_{2max} , at least 2 of the following criteria had to be achieved: plateau in the final VO_2 values (increase $\leq 2.0 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in the 2 last loads), reaching maximal

theoretical HR $((220 - \text{age}) \cdot 0.95)$, $\text{RER} \geq 1.15$ and lactate $\geq 8.0 \text{ mmol} \cdot \text{L}^{-1}$. VT1 was determined using the criteria of an increase in $\text{VE} \cdot \text{VO}_2^{-1}$ (VE = pulmonary ventilation) without further increase in $\text{VE} \cdot \text{VCO}_2^{-1}$ and departure from the linearity of VE , whereas VT2 corresponded to an increase in both $\text{VE} \cdot \text{VO}_2^{-1}$ and $\text{VE} \cdot \text{VCO}_2^{-1}$ [21,22]. All VT1 and VT2 assessments were made by visual inspection of graphs in which were time-plotted against each relevant respiratory variable measured during testing. Ventilatory thresholds were obtained using the ventilatory equivalents method described by Wasserman [23]. FTP was defined as the highest average power output (PO) that can be maintained for 1 h [24]. The estimated functional threshold power (FTP) was calculated using the following equation [25]:

$$\text{FTP (W)} = \text{Pmax (W)} \times 0.865 - 56.484$$

2.4.3. Step Test

Step test was performed on a cycle ergometer (Cyclus 2, RBM Elektronik-Automation GmbH, Leipzig, Alemania) using a metabolic cart (Metalyzer 3B, Leipzig, Germany) (maximal error: 2%; in power values $<100 \text{ W}$) and applying the power output values resulting from the incremental test (FatMax, VT1 and VT2). Participants exercised continuously from FatMax (W) to VT1 (W) and to VT2 (W) for 10 min at each step without rest between them. Cardiorespiratory variables (oxygen consumption (VO_2), relative oxygen consumption to body mass (VO_2R), carbohydrate oxidation (CHO), fat oxidation (FAT) and cycling efficiency = $(\text{work}/\text{energy expenditure}) \times 100$) [26] were determined for each metabolic zones.

2.4.4. Wingate Test

In visit 1 a familiarisation session was performed for this test. Prior to the Wingate test (WAnT), participants warmed up on a cycle ergometer for 10 min at 50 W. The WAnT consisted of an all-out, 30-s sprint on a cycloergometer (Monark Ergonomic 894E Peak Bike, Vansbro, Sweden). Breaking resistance was held constant at 7.5% of each individual's body mass [27]. All participants were verbally encouraged to pedal as fast as possible during the entire sprint. The anaerobic capacity (non-oxidative) was determined by obtaining the absolute and relative (i.e., to body mass) peak power, initial absolute and relative power, power at maximum speed, time at peak power and time at maximum speed. Participants were familiarized with the WAnT on the same day as the medical exam.

2.4.5. Blood Samples

For blood analytics, two samples were taken, namely one in a 3-mL tube with ethylenediaminetetraacetic acid (EDTA) and another in a 3.5-mL tube with polyethylene terephthalate (PET). Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, haematocrit, and haematimetry indexes were determined. These data were used to verify the health status of the subjects and were not included in the study.

2.4.6. Urine Samples

Main hesperidin metabolites were analysed in participants' urine. Urine samples, corresponding to the collection of urine 24 h before (V2) and after (V7) the supplementation in both groups for each participant, were frozen in liquid nitrogen after collection and thawed for its analysis. For analysis, 50 μL of urine were mixed with 100 μL of water with 1% formic acid containing the internal standard. Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series Agilent Technologies, Sta. Clara, CA, USA). Metabolites were quantified by external standard calibration, using rac-Hesperetin-d3 as the internal standard).

2.5. Statistical Analysis

Statistical analysis was carried out using IBM Social Sciences software (SPSS, v.21.0, Chicago, IL, USA). Data are presented as mean \pm SD. Levene and Shapiro–Wilk tests were performed in order to check for homogeneity and normality of the data, respectively. Depending on the normality and homogeneity outcomes obtained, paired T-test or Wilcoxon signed-rank test were carried out to examine within-group pre-post differences. Likewise, between-group comparison was calculated using ANCOVA test or Mann–Whitney U test, using pre-test values as covariates (to eliminate any possible bias caused by the initial level of each group in the different dependent variables). Partial eta squared (η^2) was calculated as effect size for between-group comparisons. Partial eta square thresholds were used as follow: <0.01 , irrelevant; ≥ 0.01 , small; ≥ 0.059 , moderate; ≥ 0.138 , large [28]. Furthermore, the step test data analysis was done using repeated measures T-test to obtain within-group differences when comparing the different time points. Relationships between levels of excreted hesperidin metabolites in urine and other evaluated parameters were analysed using Pearson correlation analysis (r). Significance level was set at $p \leq 0.05$. Cohen’s d effect sizes (ES) (95% confidence interval) were calculated for comparisons between groups. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large [28].

3. Results

3.1. Hesperidin Metabolites Urine

Different hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analyzed in the urine of the participants after 2S-hesperidin intake. The main metabolite detected was hesperetin-3-glucuronide, representing $78.9 \pm 5.0\%$ ($n = 20$) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate made up $6.9 \pm 2.9\%$ ($n = 20$) and $14.7 \pm 4.1\%$ ($n = 20$) of the excreted metabolites. Despite the similarities in the excreted metabolites profile, a large interindividual variability was observed in the excreted amount, with hesperidin metabolites ranging from 2.3 to $37.5 \mu\text{mol}$. These differences between subjects indicate differences in the absorption and excretion of hesperidin, which have been previously reported [29].

3.2. Incremental Test

Figure 3 shows the pre- and post-intervention values and changes in VT1 and VT2 power, estimated FTP and maximum power achieved during the incremental test.

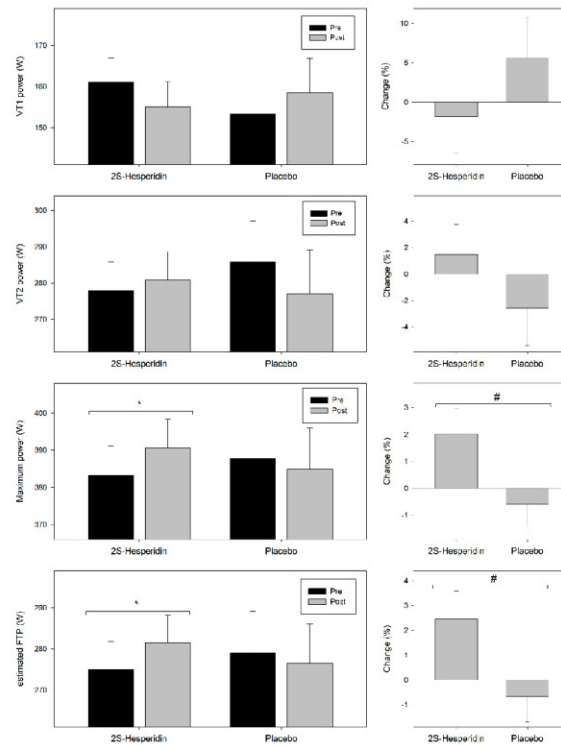


Figure 3. Changes in ventilatory 1 (VT1) power, ventilatory threshold 2 (VT2) power, estimated functional threshold power (FTP) and maximum power during the incremental test. Values are mean \pm SE. * Within-group significant changes ($p \leq 0.05$). # Between group significant changes ($p \leq 0.05$).

At VT1, there was no differences in pre-post power neither in 2S-hesperidin group ($-3.7\% = -6.0$ W; $p = 0.437$) nor in Placebo group ($3.4\% = 5.3$ W; $p = 0.453$), without differences in VT1 power changes between groups ($p = 0.423$; $\eta^2 = 0.017$; ES = 0.35). At VT2, there was no differences pre-post in power output in Placebo ($-3.1\% = -8.9$ W; $p = 0.264$), and no changes were observed in 2S-hesperidin group ($1.0\% = 2.9$ W; $p = 0.642$). Comparison between groups showed no effect ($p = 0.299$; $\eta^2 = 0.029$; ES = 0.38).

Interestingly, there were pre-post increases in maximum power ($1.9\% = 7.4$ W; $p = 0.049$) and estimated FTP ($2.3\% = 6.4$ W; $p = 0.049$) in 2S-hesperidin group. However, there were no changes in estimated FTP ($-0.9\% = -2.51$ W; $p = 0.387$) and maximum power ($-0.8\% = -2.9$ W; $p = 0.388$) after the intervention in the placebo group. Between-group comparisons revealed an effect with the increase in estimated FTP ($3.2\% = 8.9$ W; $p = 0.042$; $\eta^2 = 0.107$; ES = 0.68) and maximum power ($2.7\% = 10.3$ W; $p = 0.042$; $\eta^2 = 0.107$; ES = 0.68) in 2S-hesperidin group versus placebo.

Additionally, there was a positive correlation between the levels of excreted hesperidin metabolites in urine and the difference in maximum power ($r = 0.701$; $p < 0.001$) and estimated FTP ($r = 0.725$; $p < 0.001$) in the supplemented group.

3.3. Step Test

At FatMax, there was a pre-post decrease in fat oxidation (FAT) ($p = 0.007$) and efficiency ($p = 0.010$) in the Placebo group, whereas the 2S-hesperidin supplemented group showed no changes in these parameters (Table 3). No differences were found for between-group comparisons in FAT ($p = 0.125$; $\eta^2 = 0.084$; ES = 0.59).

Table 3. Changes in metabolism, energy substrate, energy and energy efficiency in FatMax, ventilatory threshold 1 (VT1) and ventilatory threshold 2 (VT2) during the step test.

	2S-Hesperidin			Placebo			η^2	ES
	Pre-Intervention	Post-Intervention	<i>p</i> -Value	Pre-Intervention	Post-Intervention	<i>p</i> -Value		
FatMax								
VO ₂ (L·min ⁻¹)	2.23 (0.50)	2.02 (0.37)	0.063	2.27 (0.48)	2.10 (0.57)	0.151	0.005	0.08
VO ₂ R (ml·kg ⁻¹ ·min ⁻¹)	31.45 (6.17)	28.54 (5.43)	0.060	32.40 (6.82)	29.51 (6.99)	0.100	0.003	0.00
CHO (g·min ⁻¹)	2.20 (0.58)	2.01 (0.37)	0.169	2.20 (0.50)	2.27 (0.56)	0.521	0.090	0.47
FAT (g·min ⁻¹)	0.29 (0.90)	0.26 (0.14)	0.247	0.32 (0.14)	0.21 (0.14)	0.007	0.064	0.59
Efficiency (%)	26.68 (2.95)	26.05 (3.90)	0.411	26.94 (2.79)	24.62 (2.27)	0.010	0.064	0.49
VT1								
VO ₂ (L·min ⁻¹)	2.19 (0.39)	2.10 (0.35)	0.396	2.10 (0.41)	2.09 (0.47)	0.961	0.001	0.17
VO ₂ R (ml·kg ⁻¹ ·min ⁻¹)	31.05 (5.34)	29.62 (5.20)	0.357	29.96 (5.84)	29.64 (6.37)	0.824	0.001	0.17
CHO (g·min ⁻¹)	2.08 (0.47)	2.07 (0.30)	0.974	1.86 (0.47)	2.19 (0.49)	0.020	0.028	0.57
FAT (g·min ⁻¹)	0.31 (0.10)	0.27 (0.15)	0.184	0.35 (0.12)	0.23 (0.14)	0.003	0.044	0.53
Efficiency (%)	26.55 (2.62)	25.25 (5.38)	0.250	27.49 (3.25)	25.86 (5.85)	0.282	<0.001	0.77
VT2								
VO ₂ (L·min ⁻¹)	3.49 (0.43)	3.36 (0.41)	0.135	3.63 (0.52)	3.33 (0.54)	0.002	0.039	0.49
VO ₂ R (ml·kg ⁻¹ ·min ⁻¹)	49.48 (6.83)	48.25 (6.84)	0.211	51.90 (8.17)	47.29 (7.76)	0.002 †	0.084	0.67
CHO (g·min ⁻¹)	5.11 (1.18)	5.42 (1.37)	0.349	5.53 (1.45)	5.25 (1.13)	0.369	0.022	0.43
FAT (g·min ⁻¹)	0.04 (0.08)	0.04 (0.09)	1.000	0.02 (0.06)	0.01 (0.03)	0.334	0.048	0.03
Efficiency (%)	20.58 (3.09)	19.65 (3.37)	0.272	20.15 (2.25)	20.20 (4.30)	0.965	0.009	0.24

Values are mean (SE). VO₂ = volume of oxygen uptake; VO₂R = body mass oxygen consumption; FatMax = intensity at which maximum fat oxidation is given; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); CHO = carbohydrate oxidation; FAT = fat oxidation; efficiency = percentage. The *p*-values refer to intra-group comparisons. There were no significant changes when comparing the groups. The trend towards significance between groups is indicated by a †.

At VT1, there was a pre-post increase in carbohydrate oxidation (CHO) ($p = 0.020$) and a decrease pre-post in fat oxidation ($p = 0.003$) in Placebo group, but no changes were observed in 2S-hesperidin (Table 3). No changes were found between groups in CHO ($p = 0.314$; $\eta^2 = 0.028$; ES = 0.57) and FAT ($p = 0.205$; $\eta^2 = 0.044$; ES = 0.53).

After the supplementation period, there was a decrease in VO₂ (L/min) (-8.3% ; $p = 0.002$) and VO₂R (ml/kg/min) (-8.9% ; $p = 0.002$) at VT2 in Placebo group, in contrast to 2S-hesperidin, which showed no changes (Table 3). Between-group comparison showed a trend towards a decrease ($p = 0.074$; $\eta^2 = 0.084$; ES = 0.67) in VO₂R (ml/kg/min) for placebo versus 2S-hesperidin group.

3.4. Wingate Test

Table 4 shows the results of the parameters evaluated during the Wingate test prior and after supplementation, which are also summarized in Figure 4.

Table 4. Changes in performance parameters in the Wingate test.

	2S-Hesperidin			Placebo			ηp^2	ES
	Pre-Intervention	Post-Intervention	p-Value	Pre-Intervention	Post-Intervention	p-Value		
Initial power absolute (W)	718.8 (143.05)	754.3 (143.09)	0.001 *	712.5 (103.46)	743.0 (101.78)	0.084	0.003	0.08
Initial power relative (W)	10.2 (1.82)	10.6 (1.78)	0.004 *	10.1 (1.38)	10.6 (1.29)	0.078	<0.001	0.01
Absolute peak power (W)	810.8 (160.26)	860.6 (170.37)	<0.001 *	792.0 (100.96)	840.2 (118.93)	0.016 *	<0.001	0.02
Relative peak power (W)	11.5 (2.04)	12.1 (2.27)	0.001 *	11.3 (1.37)	11.9 (1.49)	0.014 *	<0.001	0.02
Power at maximum speed (W)	760.0 (156.45)	793.5 (132.23)	0.051 †	746.3 (110.30)	754.3 (96.14)	0.709	0.044	0.30
Time at peak power (ms)	3541.4 (1722.52)	2900.2 (923.99)	0.052 †	3193.4 (1218.48)	2816.9 (1013.54)	0.138	0.001	0.82
Time at maximum speed (ms)	7208.7 (1098.24)	7157.9 (2005.11)	0.888	7024.4 (1347.65)	6095.2 (957.33)	0.001 *	0.119	0.73

Values are mean (SE). * Within-group significant changes ($p \leq 0.05$). † Within-group trend to significant changes ($p = 0.05-0.10$).

In the 2S-hesperidin group, there were increases in absolute (4.9% = 35.5 W; $p = 0.001$) and relative (4.3% = 0.44 W·kg⁻¹; $p = 0.004$) initial power (first five seconds of the test), but no differences between groups. In the experimental group, there was an increase in absolute (6.1% = 49.8 W; $p < 0.001$) and relative (5.6% = 0.64 W·kg⁻¹; $p = 0.001$) peak power. Also, there was a trend towards an increase in power at maximum speed (4.4% = 34.0 W; $p = 0.051$) and a descending trend in time at peak power (−18.1% = −641.2 ms; $p = 0.052$) after the supplementation with 2S-hesperidin. No changes were observed in time at maximum speed.

Placebo group showed an increase in absolute (6.1% = 47.2 W; $p = 0.016$) and relative peak power (5.6% = 0.64 W·kg⁻¹; $p = 0.014$), and a decrease in time at maximum speed (−13.2% = −929.2 ms; $p = 0.001$). No changes were observed in absolute and relative initial power, power at maximum speed and time at peak power for placebo.

Between-group comparison only reported a trend to decrease in time at maximum speed (−12.5% = −878.4 ms; $p = 0.059$) in Placebo compared with 2S-hesperidin.

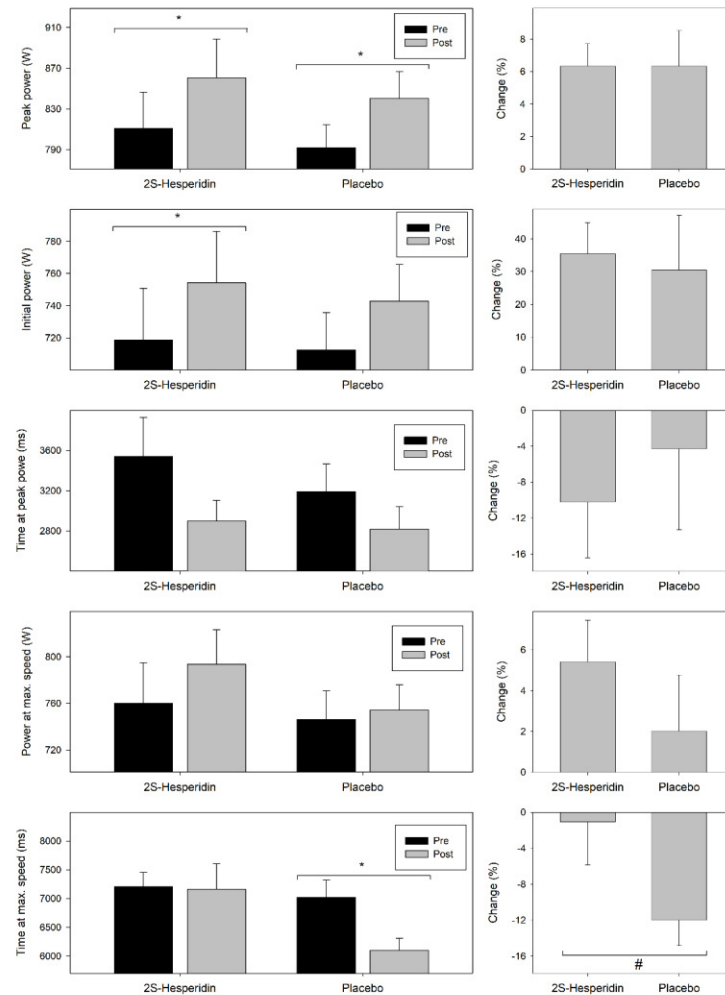


Figure 4. Changes in parameters evaluated during the Wingate test prior and after supplementation. Values are mean \pm SE. * Within-group significant changes ($p \leq 0.05$). # Between group trend t significant changes ($p = 0.05$ – 0.010).

4. Discussion

The main objective of this study was to evaluate the effects of chronic intake of 2S-hesperidin on non-oxidative/glycolytic and oxidative metabolism and performance markers in amateur cyclists. For this purpose, participants were supplemented for eight weeks with 500 mg 2S-hesperidin, a natural extract of sweet orange (*Citrus sinensis*) which contains hesperidin in its natural 2S form (NLT 85% 2S-hesperidin). Following the eight-week intervention, 2S-hesperidin supplementation led to significant improvements in submaximal and maximal intensity exercise performance in the incremental tests

versus placebo. There was a significant decrease in VO_2R (mL/kg/min) at VT2 in placebo compared with 2S-hesperidin, in the step test.

The bioavailability of hesperidin is a factor that must be taken into account when examining its effectiveness, since the average maximum peak blood plasma concentration occurs after 5–7 h of its ingestion and is almost eliminated post-24 h [30]. However, the excreted metabolites in urine has been shown to reach at maximum levels at post-24 h with continued remnants after 48 h [30]. It is interesting to mention that the area under the curve was more than doubled (0.5 L orange juice; 4.19 $\mu\text{mol h/L}$ vs 1 l orange juice; 9.28 $\mu\text{mol h/L}$) at 24 h when high doses of hesperidin were consumed (1L orange juice = 444 mg hesperidin) [30]. This indicates that high doses increase exposure to the body of 2S-hesperidin metabolites than low doses (222 mg/L). The dose that the cyclists in our study consumed was equivalent to more than one liter of orange juice, with the high carbohydrate load that it entails. The metabolites of hesperidin that appear mainly in the blood are glucuronides (87%) and sulfoglucuronides (13%) [30]. These results are very similar to those found in this study.

Another key factor in the metabolism and absorption of 2S-hesperidin is the intestinal microbiota. In particular, Amaretti et al. [31] established that the species *Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum* had the ability to hydrolyze hesperidin, because in their genome they have the gene encoding for the enzyme α -L-rhamnose (limiting enzyme), which contributes to the release of aglycone from certain routine-conjugated polyphenols, such as hesperidin. A recent study suggests that the contradictory finding regarding the intake of hesperidin in humans may be due, in part, to the interindividual variability in its bioavailability, which highly depends on the α -rhamnosidase activity and the composition of the gut microbiota [32]. On the other hand, hesperidin has shown to have a probiotic effect by promoting the growth of some beneficial bacterial species in the colon, the key role being the production of short-chain fatty acids (SCFA) (*Bifidobacterium spp.*, *Lactobacillus spp.*, or *Akkermansia muciniphila*) [33].

4.1. Incremental Test

The results of this study showed an improved performance in eFTP and maximum power (\uparrow generated power) after chronic intake of 2S-hesperidin compared to placebo in incremental test. These changes are supported by a significant positive correlation between excretion of urinary 2S-hesperidin metabolites and maximum power ($r = 0.701$) and estimated FTP ($r = 0.725$). Regarding flavonoid supplementation, a previous study reported a 5% increase in absolute power output in a 10-min time trial (TT) after four weeks of 2S-hesperidin intake (500 mg) in cyclists [13]. Other authors have also reported performance improvements (time until exhaustion ~58%) in animals [17,18]. Currently there are no other studies that analyzing the effects of chronic hesperidin intake on performance. Several authors have reported that hesperidin exerts an antioxidative effect and promotes nitric oxide synthesis in different pathological study models [18,34–39]. In a rat model with pleurisy, the antioxidant activity of hesperidin reduced the production of ROS in the liver and increased the liver activities of CAT and SOD [35]. Estruel-Amades et al. [18] observed that five weeks of supplementation with 2S-hesperidin (200 mg/kg three days per week) prevented an increase in ROS and decline in SOD and CAT activity after a test until exhaustion in the thymus and spleen of mice with an intensive training plan. This scavenging activity hesperidin neutralizes reactive oxygen species, such as superoxide anion, generated during conditions of oxidative stress, like intense physical exercise [36]. In particular, citrus flavanones (such as hesperidin and hesperetin) have the ability to modulate cellular antioxidant defenses through the Nrf2-ARE pathway, which regulates gene expression of antioxidant enzymes, such as SOD, CAT, HO-1 and GPx, decreasing intracellular pro-oxidants [40]. In addition, several authors have described a stimulating effect of nitric oxide production after hesperidin supplementation [34,37–39], by an increase in endothelial activity NO synthase and gene expression of endothelium nitric oxide synthase. NO can relax human vascular cells (vasodilatation), which leads to improved blood flow during rest and exercise. Vasodilation is a physiological mechanism used not only for the supply of oxygenated blood, but also for the

delivery of glucose, lipids, and other nutrients to a variety of tissues [41]. Theoretically, increased blood flow would increase the delivery of O₂ and nutrients (e.g., amino acids and glucose) to exercising skeletal muscle, thus aiding exercise performance during high intensity (conditions of hypoxia) [42]. These mechanisms may be responsible for performance improvement in eFTP and maximum power in the incremental test in the group that consumed 2S-hesperidin.

Other flavonoids such as quercetin, has also demonstrated to improve the 5 km running performance time (−11.3% quercetin group; −3.9% control group) after its 14 day supplementation (250 mg/d) by trained triathletes [43]. However, a systematic review that included 13 randomized controlled trials found that cocoa-derived flavonoid (epicatechin and catechin, and oligomeric procyanidin) supplementation did not affect performance [44]. Thus, there may be some specificity regarding the type of flavonoid that affects physical performance.

It should be noted that this study was carried out during a period when cyclists are reducing their training and competitions (late September–mid December) which involves training misadaptations (physiological and metabolic changes) [45,46]. These changes may justify the drop in the performance at sub-maximal and maximum intensities for placebo in our study. However, the intake of 2S-hesperidin was not able to prevent the loss of performance at VT1, although it was not significant, but it did maintain performance at VT2 and improve it at eFTP and maximum power. This supports our hypothesis, that the chronic intake of 2S-hesperidin could help generate or maintain adaptations at the mitochondrial level and of the endogenous antioxidant system in a period where the volume and intensity of training is decreasing, as in the conducted study (late September–mid December), maintaining performance levels in high-intensity exercise in amateur cyclists. The fact that 2S-hesperidin has an effect on different physiological mechanisms [6–9] may be the reason why it cannot maintain performance at low but in high exercise intensities. In line with our hypothesis, the intake of hesperetin in elderly rats (hesperidin metabolite) has been shown to prevent loss of performance by improving mitochondrial and endogenous antioxidant status [15]. The improvements in training adaptations of cyclists who ingested 2S-hesperidin may be due to the ability of this molecule to increase gene expression of the peroxisome proliferator-activated receptor-gamma coactivator 1- α (PGC-1 α) and nuclear factor respiratory 2 (NRF2), also, it increased the level of proteins of PGC-1 α and of complexes I, III, and IV of the electron transport chain in the mitochondria, in muscle cells (in vitro) [15]. In addition, hesperetin has shown increased activation of AMPK in liver cells [47] and fibroblasts [48]. AMPK is a sensor of cellular energy status that plays a central role in skeletal muscle metabolism, regulating muscle exercise capacity, mitochondrial function and contraction-stimulated glucose uptake [49]. PGC-1 α and AMPK are an important transcriptional masters regulators of mitochondrial biogenesis (\uparrow biogenesis mitochondrial and oxidative capacity) [49,50] and NRF2 which is an essential regulator in the control of cellular redox homeostasis and controls glutathione synthesis (reactive oxygen species (ROS) scavenging) [51]. Modifications in these transcription factors have shown performance improvements in endurance athletes [52]. Therefore, 2S-hesperidin has the ability to promote muscle-level adaptations of endurance athletes, which could improve their performance in competitions.

It has been hypothesized that some molecules with anti-inflammatory and antioxidant activity may interfere with exercise-generated adaptations causing a decline in performance when ingested chronically [53]. Although, there is controversy on this issue, since supplementation of polyphenols, such as quercetin, has been shown to improve performance [54]. With the results obtained in the incremental test, we can say that the chronic intake of 2S-hesperidin improves the power generated in eFTP and maximum power that would enhance the performance of endurance athletes for competition, avoiding the loss of performance (eFTP and maximum power) observed in the placebo group due to the loss of adaptations achieved during the cycling post-season. In addition, our results were strengthened by the positive correlations found between performance improvements at eFTP and maximum power with the excretion of metabolites in urine after 2S-hesperidin intake. Therefore, an increase in power production at high intensity is a key factor in cycling performance, which can increase your success in endurance competitions. However, at low intensity exercise levels there were no differences between

groups. This could be because, at high intensities, the antioxidant action of 2S-hesperidin could improve performance [16,18], but this capacity does not influence exercises at low intensities where oxidative stress is lower.

4.2. Step Test

In the step test, the differences found between 2S-hesperidin and placebo indicate mismatches mediated by the reduction in training volume and intensity over the period of the study [45,46], identified as a decrease in FAT (FatMax and VT1) and a decrease in VO_2R (mL/kg/min) (VT2) in placebo. These findings were in line with those found in the incremental test, where mismatches to training (\downarrow generated power at eFTP and maximum power) were also found. In endurance athletes, a 7% ($p < 0.05$) and 16% ($p < 0.05$) decrease in $\text{VO}_{2\text{MAX}}$ after 21 and 56 days of inactivity respectively has already been described in scientific literature, related to a decrease in systolic volume and decrease in citrate synthase and succinate dehydrogenase in muscle activities [55]. Moreover, a decrease in oxygen consumption values in the ventilatory thresholds and in maximum exercise has been associated with a decrease in power outputs in professional cyclists after three weeks of cycling competition [56]. In the detraining process could also be involved the loss of oxidative capacity mediated by the reduction of PGC-1 α (\downarrow mitochondrial content) [15]. Therefore, it is normal that after a period of detraining there are changes in different physiological-biochemical markers that lead to a loss of performance in athletes.

However, the 2S-hesperidin group maintained the oxidation of fats at FatMax and VT1, without decreasing the oxygen consumption in VT2. Similarly, a treatment with low doses of (-)-epicatechin (flavonoid) has shown an attenuation of training losses (14 d of detraining) in skeletal muscle capillarity and bioenergetics achieved after five weeks of resistance training [46]. This suggests a similar effect of both molecules in preventing the physiological changes produced by detraining. In addition, hesperidin (0.5 mmol·kg⁻¹ of body mass) intake has been shown to be effective in reducing the accumulation of body fat mass, glucose levels and blood lipids in rats fed a high-fat diet [57]. The possible pathways used by chronic intake of 2S-hesperidin to decrease physiological changes derived from detraining would be related to the modulating gene components, such as AMPK and PGC-1 α [15,47,48], which control energy production, utilization of metabolic substrates (fats and carbohydrates), mitochondrial biogenesis and oxidative capacity [49,50]. Our results suggest that chronic intake of 2S-hesperidin may prevent the decrease in VO_2R (mL/kg/min) (VT2) that is associated with a decrease in the ability to produce power in cyclists, and a drop in FAT (FatMax and VT1), increasing carbohydrate utilization at moderately low intensities, which could anticipate fatigue in subsequent high-intensity work, such as in a cycling competition.

4.3. Wingate Test

The results obtained in Wingate test (high anaerobic component) after intake 2S-hesperidin showed an improvement in both initial power absolute and relative when compared to placebo. On the other hand, both groups improved both power variables for a 30 s sprint (Wingate test), without differences when comparing the groups. Currently, there are no other studies that have evaluated the chronic intake of 2S-hesperidin using a Wingate test. Martínez et al. [11] observed improvements in average power (2.3%) and maximum speed (3.2%) during a repeated 30-s sprint test in amateur cyclists following an acute intake of 2S-hesperidin. However, there are no previous studies that have evaluated the effect of chronic hesperidin intake on maximum anaerobic capacity (non-oxidative). In addition, combined intake of mangiferin and luteolin (polyphenols) for 15 days has also displayed improvements in average power (5.0%) during a Wingate [58].

In the short maximum effort tests, some of the changes can be explained by an initial learning effect, followed by a typical variation within the test(s) [59]. Considering that the significant differences between the two experimental conditions have been small in the measurements evaluated in the Wingate test, it should be taken into account that in this type of trial they may be susceptible to the effects of placebo, nocebo or Hawthorne [60,61]. Intra-individual variability and therefore the probability of

committing a type one error was further reduced by assessing study subjects at approximately the same time of day, thus avoiding effects of the circadian system about physiological, psychological, and molecular mechanisms in the body, resulting in varying physical performance over the day [62]. We consider that in this type of test (Wingate) familiarisation can have an important effect on the final results, therefore, for future research we will introduce a comparison between the values obtained in familiarisation and the placebo, in order to observe variations that can affect the final result or when comparing experimental groups taking into account the variability of the test [63].

One limitation of our study is the lack of having muscle biopsies to examine the possible mechanisms that could explain these improvements due to financial restrictions. They could have provided valuable.

4.4. Practical Applications

The data found in this research shows how chronic intake of 2S-hesperidin enhances performance in FTP and maximum power. Advances in these areas of intensity are crucial for improving results in cycling competitions. Furthermore, as observed in the step test, 2S-hesperidin has the ability to maintain oxygen consumption in VT2 and fatty acid oxidation levels in FatMax and VT1, in periods with a decrease in training exercise volume and intensity (i.e., this study was conducted in the off-season). Given the effects reported by 2S-hesperidin, sports nutritionists would have other ergogenic aids available to improve the performance of their athletes. In this period, cyclists had decreased the volume and intensity of training with respect to other periods of the year. This is an important aspect to consider when comparing our results with other studies, as the outcomes could be different due to the volume and intensity of usual training during the testing time period.

5. Conclusions

Supplementation with 2S-hesperidin for eight weeks promotes an improvement in estimated FTP and maximum power in amateur cyclists during an incremental test. Furthermore, the supplementation with 2S-hesperidin can prevent a drop in VO_2R (VT2) and FAT (FatMax and VT1) in step test on training periods with less volume and load. These findings support the use of 2S-hesperidin as a natural new ergogenic aid, which can help cyclists improve both their aerobic performance.

Author Contributions: Conceptualization, F.J.M.-N., C.M.-P. and P.E.A.; methodology, F.J.M.-N., C.M.-P. and P.E.A.; formal analysis, F.J.M.-N., C.M.-P. and J.C.-V.; investigation, F.J.M.-N., C.M.-P. and J.C.-V.; resources, F.J.M.-N., C.M.-P. and J.C.-V.; data curation, F.J.M.-N., C.M.-P. and J.C.-V.; writing—original draft preparation, F.J.M.-N.; writing—review and editing, F.J.M.-N., C.M.-P. and J.C.-V.; visualization, C.M.-P.; supervision, C.M.-P. and P.E.A.; project administration, C.M.-P. and P.E.A.; funding acquisition, P.E.A. All authors have read and agreed to the published version of the manuscript.

Funding: The authors declare that this study has been financed by HTBA (Murcia, Spain), who kindly provided the product Cardiose[®], but they did not participate in the experimental design, data collection, data analysis, interpretation of the data, writing of the manuscript, or in the decision to publish the results.

Acknowledgments: This study was supported by the Research Center for High Performance Sport of the Catholic University of Murcia and HTBA (Murcia, Spain). We would like to acknowledge Linda H. Chung for her help in this project. We also thank Iris Samarra, Antoni del Pino and Nuria Canela, from the Metabolomics facility of the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat, for their contribution to the urine analysis. The results of the current study do not constitute endorsement of the product by the authors or the journal.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

8-Week Supplementation of 2S-Hesperidin Modulates Antioxidant and Inflammatory Status after Exercise until Exhaustion in Amateur Cyclists

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Abstract: Both acute and chronic ingestion of 2S-hesperidin have shown antioxidant and anti-inflammatory effects in animal studies, but so far, no one has studied this effect of chronic ingestion in humans. The main objective was to evaluate whether an 8-week intake of 2S-hesperidin had the ability to modulate antioxidant-oxidant and inflammatory status in amateur cyclists. A parallel, randomized, double-blind, placebo-controlled trial study was carried out with two groups (500 mg/d 2S-hesperidin; $n = 20$ and 500 mg/d placebo; $n = 20$). An incremental test was performed to determine the working zones in a rectangular test, which was used to analyze for changes in antioxidant and inflammatory biomarkers. After 2S-hesperidin ingestion, we found in the rectangular test: (1) an increase in superoxide dismutase (SOD) after the exercise phase until exhaustion ($p = 0.045$) and the acute recovery phase ($p = 0.004$), (2) a decrease in the area under the oxidized glutathione curve (GSSG) ($p = 0.016$), and (3) a decrease in monocyte chemoattractant protein 1 (MCP1) after the acute recovery phase ($p = 0.004$), post-intervention. Chronic 2S-hesperidin supplementation increased endogenous antioxidant capacity (\uparrow SOD) after maximal effort and decreased oxidative stress (\downarrow AUC-GSSG) during the rectangular test, decreasing inflammation (\downarrow MCP1) after the acute recovery phase.

Keywords: polyphenols; flavonoids; endogenous antioxidant enzymes; reduced glutathione; oxidized glutathione; catalase; superoxide dismutase; interleukin 6; tumor necrosis factor; endurance sports



Citation: Martínez-Noguera, F.J.; Marín-Pagán, C.; Carlos-Vivas, J.; Alcaraz, P.E. 8-Week Supplementation of 2S-Hesperidin Modulates Antioxidant and Inflammatory Status after Exercise until Exhaustion in Amateur Cyclists. *Antioxidants* **2021**, *10*, 432. <https://doi.org/10.3390/antiox10030432>

Academic Editors: Irene Dini and Domenico Montesano

Received: 3 February 2021

Accepted: 8 March 2021

Published: 11 March 2021

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1. Introduction

Flavonoids are bioactive substances found mainly in fruits and vegetables, with more than 15,000 molecules identified within this family [1]. However, one of the most well-known is hesperidin, which is a flavonoid present at high concentrations in citrus fruits, being the main one in sweet orange (*Citrus sinensis*). Hesperidin may be found in two isomeric forms, 2S- and 2R-, where the 2S isomer is predominant in nature [2]. When hesperidin reaches the intestine, bacterial flora converts it into hesperetin (aglycon), which is effectively absorbed, being the main metabolite of hesperidin [3]. Previous studies have shown the positive effects of hesperidin on some diseases (neurological, cardiovascular, insulin sensitization) due to its antioxidant and anti-inflammatory properties [4,5]. Moreover, the intake of hesperidin (in orange juice) has been shown to modulate leukocyte gene expression, boosting its antioxidant and inflammatory profile, and therefore showing a nutrigenomic effect [6]. On the other hand, the ability of 2S-hesperidin to improve performance has been observed [7]. It should be noted that there are other important factors that can modulate the effect of flavonoids like hesperidin, such as intestinal flora transformations, absorption and bioavailability [8].

The antioxidant effect of hesperidin is mainly related to its radical scavenging capabilities, as well as the increase in antioxidant cellular defense catalase (CAT), superoxide

dismutase (SOD), reduced glutathione (GSH) and oxidized glutathione (GSSG) via the nuclear respiratory factor 2 (NRF2) signaling pathway [4]. On the other hand, the hesperidin anti-inflammatory effect is produced by a decrease in inflammatory markers, such as nuclear factor kappa B (NF- κ B), interleukin 6 (IL6), tumor necrosis α (TNF α) and inducible nitric oxide synthase (iNOS) [4].

Regarding the potential of hesperidin on physical performance, a recent study reported that the acute intake of 500 mg of 2S-hesperidin significantly improved anaerobic performance [9]. In the same study, they also found small non-significant changes in CAT, SOD, GSH and the GSSG/GSH ratio compared to a placebo during a rectangular test (with different intensities) in amateur cyclists. Similarly, a study performed in rats observed that 2S-hesperidin (200 mg/kg, three days per week during five weeks) showed a protective effect on the oxidative stress induced by an exhausting exercise [10]. Hesperidin supplementation prevented the increase in reactive oxygen species (ROS) production and avoided a decrease in SOD and catalase activities, while leading to a higher physical performance. In the same way, 6 weeks of hesperetin (main metabolite of hesperidin) supplementation (50 mg·kg⁻¹·d⁻¹) significantly increased the GSH/GSSG ratio and improved running performance (exercise time) in aged mice [11]. In addition, a recent study found that eight weeks' intake of 2S-hesperidin improved performance at the threshold of estimated functional power and maximum power in an incremental test until exhaustion compared to a placebo in amateur cyclists [7]. Other polyphenols have shown hesperidin-like effects. For example, the intake of 100 mL per day for six weeks of acai berry-based juice (\uparrow anthocyanins) increased the levels of GSH and CAT post-exercise and after 1 h of recovery, without changes in SOD and exercise performance (300 m running times) in junior athletes [12].

With regards to exercise, it is known that almost 0.15% of the oxygen consumed is converted into ROS, which can be detrimental to muscle and mitochondrial function [13]. In sports physiology, it is hypothesized that rapid increases in ROS during intensive exercise may be a contributor to fatigue [14]. Based on recent findings, a new theory proposes that antioxidant supplementation (vitamins A, C, E, thiols, ubiquinones and flavonoids) may delay fatigue [15]. However, this mitigation of ROS generation may disrupt cellular signaling involved in training adaptations [16]. ROS are intracellular messengers and activators of transcription factors that promote the expression of genes related to training adaptations and performance improvement [16]. Thus, antioxidant supplementation could decrease ROS production and delay fatigue, but in turn it may slow down the physiological adaptations of training [17,18]. Due to the current controversy on this topic, further investigations are required to evaluate if the intake of antioxidant polyphenols, such as hesperidin, could improve endogenous antioxidant status without negatively affecting performance.

Currently, studies have shown that acute [9] and chronic [7] intake of 2S-hesperidin in amateur cyclists improve anaerobic and aerobic performance, respectively. However, no research has explained the metabolic, biochemical and molecular mechanisms by which 2S-hesperidin intake improves performance. We hypothesized that the chronic intake of 2S-hesperidin would improve amateur cyclists' antioxidant status, evaluated through markers such as CAT, SOD, GSSG, GSH and hemoxygenase 1(HO1), but decrease inflammatory markers, such as IL6, TNF α , monocyte chemoattractant protein-1 (MCP1) and C reactive protein (CRP). However, the implications of long-term or prolonged use are unknown. Therefore, this study aimed to evaluate the effect of eight weeks of 2S-hesperidin supplementation (500 mg/day) on the antioxidant-oxidant (CAT, SOD, GSH, GSSG, HO1 and TBARS) and anti-inflammatory (IL6, TNF α , MCP1 and CRP) state in amateur cyclists before and at the end of the rectangular test and after the resting phase.

2. Methodology

2.1. Study Design

A randomized, double-blind, parallel clinical trial was conducted. Forty subjects were divided into 2 groups: 2S-hesperidin ($n = 20$) and placebo ($n = 20$). Subjects were randomized into groups using the Randomizer software. Participants consumed two 250 mg capsules of either Placebo (microcellulose, 500 mg) or 2S-hesperidin (500 mg Cardiose[®], produced by HealthTech BioActives (HTBA), Murcia, Spain) at breakfast for 8 weeks. The Cardiose[®] supplement consisted of a natural orange extract that, due to its unique manufacturing process, retains most of the natural isomeric form of hesperidin (NLT 85% 2S-hesperidin). The placebo supplements were similar in appearance to the 2S-hesperidin capsule. Cyclists were instructed to continue their usual diet and training program. The usual total training distance was balanced between the groups (Table 1).

Table 1. Baseline general characteristics and training variables of the cyclists.

	2S-Hesperidin	Placebo	<i>p</i> -Value
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Body mass (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI ($\text{kg}\cdot\text{m}^{-2}$)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
VO ₂ MAX ($\text{L}\cdot\text{min}^{-1}$)	3.99 (0.36)	3.98 (0.63)	0.971
VO ₂ MAX ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	57.5 (6.97)	57.9 (9.53)	0.88
HR _{MAX} (bpm)	184.9 (11.11)	183.2 (8.68)	0.593
VT1 (%)	50.9 (5.63)	50.0 (4.78)	0.61
VT2 (%)	84.9 (5.85)	84.1 (5.70)	0.644
Training variables	2S-Hesperidin	Placebo	<i>p</i>-value
Total distance (km)	1121.12 (534.99)	1082.43 (810.46)	0.868
HR _{AVG} (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
W _{AVG} (W)	174.86 (15.79)	163.47 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are expressed as mean (SD). BMI = body mass index; BF = body fat; VO_{2max} = maximum oxygen volume; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); Total distance = of all the training sessions carried out during the study period; HR_{avg} = average heart rate of all the training sessions carried out during the study period; W_{avg} = average power output of all training sessions during the study period and RPE = rating of perceived exertion of all training sessions during the study.

2.2. Participants

Forty healthy male, amateur cyclists completed the study (Table 1). Subjects met the following inclusion criteria: 18–55 years old, BMI of 19–25.5 $\text{kg}\cdot\text{m}^{-2}$, at least 3 years of cycling experience, and training for 6–12 $\text{h}\cdot\text{wk}^{-1}$. Amateur cyclists were excluded if: (a) regular smoking or alcohol drinking, (b) metabolic, cardiorespiratory or digestive pathology or abnormality, (c) injury in the previous 6 months, (d) supplements or medication in the previous 2 weeks and (e) abnormal values in blood test parameters. Before the start of the study, participants were informed about the procedures, and signed informed consent was obtained. The study was conducted following the Declaration of Helsinki guidelines for research on human subjects [19] and was approved by the Ethics Committee of the Catholic University of Murcia (CE091802), registered in [ClinicalTrials.gov](https://clinicaltrials.gov) (Identifier: NCT04597983).

2.3. Procedures

Participants visited the laboratory on five different occasions. Visit 1 consisted of a medical examination and blood extraction to determine health status. On visits 2 and 4, a 24-h diet recall was conducted, followed by an incremental test until exhaustion on a cycle ergometer to estimate the rectangular test zones. On visits 3 and 5, the 24-h diet recall was repeated, and participants performed a rectangular test on the cycle ergometer

(Figure 1) (Table 2). Before each testing session (visits 2, 3, 4 and 5), a standardized breakfast composed of 95.2 g of carbohydrates (68%), 18.9 g of protein (14%) and 11.3 g of lipids (18%) was prescribed by a sports nutritionist. Intake of both treatments began at visit 1 under the supervision of an investigator and finished at visit 5. Subjects in both groups were instructed not to consume foods with a high content of citrus flavonoids (grapefruit, lemons, or oranges) for 5 days prior to and during the study. This was verified by diet recall records.

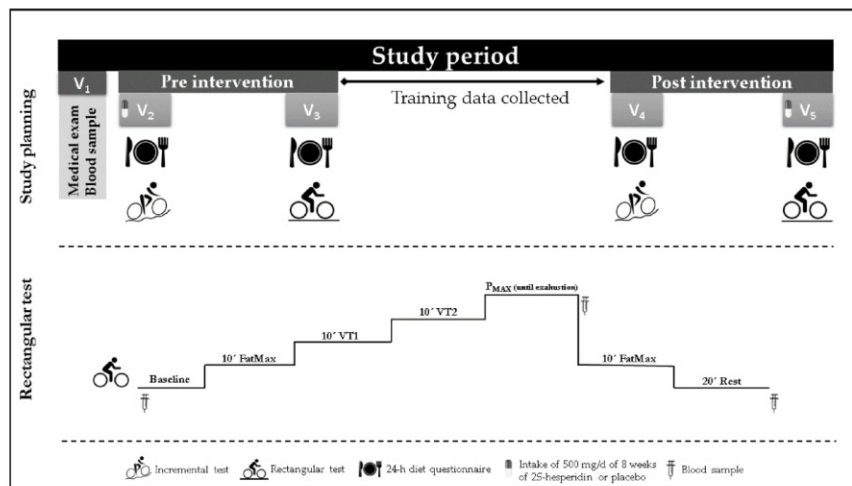


Figure 1. Study planning and rectangular test protocol.

Table 2. Between-group comparisons of dietary intake of cyclists.

	Pre-Intervention			Post-Intervention		
	2S-Hesperidin	Placebo	<i>p</i> -Value	2S-Hesperidin	Placebo	<i>p</i> -Value
Kcal	2163.6 (519.02)	2100.2 (515.77)	0.708	1974.1 (377.97)	2133.5 (437.98)	0.237
Kcal/BM	31.1 (9.34)	30.2 (8.71)	0.768	27.9 (6.53)	30.3 (6.46)	0.249
CHO (g)	245.7 (73.46)	222.0 (69.68)	0.312	216.6 (63.47)	248.3 (58.15)	0.117
CHO/BM	3.5 (1.31)	3.2 (1.14)	0.416	3.1 (1.08)	3.5 (0.94)	0.173
PRO (g)	113.5 (25.21)	115.2 (25.37)	0.837	109.0 (23.05)	101.5 (23.67)	0.332
PRO/BM	1.6 (0.41)	1.7 (0.48)	0.778	1.5 (0.35)	1.5 (0.42)	0.596
LP (g)	80.8 (27.24)	83.5 (23.65)	0.739	71.5 (17.61)	71.6 (18.89)	0.985
LP/BM	1.2 (0.45)	1.2 (0.37)	0.758	1.0 (0.27)	1.0 (0.29)	0.823

Values are expressed as mean (SD). Kcal = kilocalories; CHO = carbohydrates; PRO = protein; LP = lipids; BM = body mass. The mean values correspond to the average of all 24-h diet recall data collected at pre-intervention (visits 2, 3 and 4) and post-intervention (visits 5, 6 and 7).

2.4. Testing

2.4.1. Medical Exam

A medical examination was conducted by the research center's medical doctor and consisted of medical and health history, resting electrocardiogram and examination (auscultation, blood pressure, etc.). These evaluations confirmed that the volunteer was healthy enough to be enrolled in the study.

2.4.2. Maximal Test

Incremental step with final ramp test was performed on a cycle ergometer using a metabolic cart (Metalyzer 3B, Leipzig, Germany) to determine the maximal fat oxidation zone (FatMax), ventilatory thresholds 1 (VT1) and 2 (VT2) and maximal oxygen consumption ($\text{VO}_{2\text{max}}$). Participants started cycling at 35 W for 2 min, increasing by 35 W for every 2 min until $\text{RER} > 1.05$, and then initiating the final ramp ($+ 35 \text{ W}\cdot\text{min}^{-1}$) until exhaustion. To ensure they reached $\text{VO}_{2\text{max}}$, at least 2 of the following criteria had to be fulfilled: plateau in final VO_2 values (increase $\leq 2.0 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ in the 2 last loads), reaching maximal theoretical HR ($(220-\text{age})\cdot 0.95$), $\text{RER} \geq 1.15$ and lactate $\geq 8.0 \text{ mmol/L}$. Ventilatory thresholds were obtained using the ventilatory equivalents method described by Wasserman [20].

2.4.3. Rectangular Test

Rectangular test procedures are shown in Figure 1. This test was performed on a cycle ergometer using power output values achieved during the maximal test at different intensity zones (FatMax, VT1, VT2 and maximum power). Participants exercised continuously as follows: 10 min at FatMax, 10 min at VT1, 10 min at VT2, at maximum power until exhaustion (post- P_{MAX}) and 30 min rest (post-REC). There were no rest periods between phases.

2.4.4. Blood Samples

Venous blood (arm antecubital area) was collected into one 3 mL ethylenediaminetetraacetic acid (EDTA) tube for hemogram and another 3.5 mL polyethylene terephthalate (PET) tube was collected by a nurse for overall health analysis (visit 1). Red blood cell count was carried out in an automated Cell-Dyn 3700 analyzer (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, haematocrit and haematimetric indexes were estimated.

Additionally, venous blood samples were collected in the baseline, after the maximum power stage (post- P_{MAX}) and during the resting phase (post-REC), to measure antioxidant and anti-inflammatory parameters (visits 3 and 5) (Figure 1). During every extraction point, 6 tubes of 3 mL of EDTA were obtained. Blood samples were centrifuged at 3500 rpm in 4°C for 10 min and sent to the laboratory for later analysis.

2.4.5. Urine Samples

The main hesperidin metabolites were analyzed in the urine of participants. Urine samples were collected for 24 h before V2 and V5 visits from each participant, before and after the supplementation, and were frozen in liquid nitrogen after collection and thawed for its analysis. For analysis, 50 μL of urine was mixed with 100 μL of water with 1% formic acid containing the internal standard. Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 Series Agilent Technologies, Sta. Clara, CA, USA). Metabolites were quantified by external standard calibration using *rac*-Hesperetin-d3 as the internal standard.

2.5. Antioxidant and Inflammatory State Markers

The following parameters were selected to measure the antioxidant and inflammatory status.

2.5.1. TBARS (Lipoperoxidation Biomarker)

Thiobarbituric acid reactive substances (TBARS) are a by-product of the oxidative degradation of lipids by reactive oxygen species (lipid peroxidation), which is commonly used as an oxidative stress marker [21]. TBARS assay involves the reaction of malondialdehyde (MDA), a product of lipid peroxidation, with thiobarbituric acid (TBA) under high temperature and acidic conditions to form an MDA-TBA complex that can be measured colorimetrically [22]. The coefficient of variation between replicas had to be less than or equal to 4.6% (Supplementary File 1).

2.5.2. Catalase (CAT)

CAT activity was determined using a UV-VIS spectrophotometer. This was expressed in sec^{-1} per gram of hemoglobin [23]. The coefficient of variation between replicas had to be less than or equal to 4.9% (Supplementary File 1).

2.5.3. Superoxide Dismutase (SOD)

SOD activity was measured using an SD125 Ransod kit (Randox Ltd. Crumlin, United Kingdom) [24]. The coefficient of variation between replicas had to be less than or equal to 5.1% (Supplementary File 1).

2.5.4. Glutathione Reduced (GSH) and Oxidized (GSSG)

GSH was analyzed by the glutathione-S-transferase assay described by Akerboom and Sies [25]. Glutathione oxidized form and glutathione disulfide (GSSG) were determined in a similar way to GSH as shown above, as described by Asensi [26]. The coefficient of variation for GSH between replicas must be less than or equal to 4.1% (Supplementary File 1).

2.5.5. Hemoxygenase 1 (HO1)

A commercial kit was used based on the Enzyme-Linked ImmunoSorbent Assay (ELISA) method (Shanghai BlueGeneBiotech Co., Ltd., Shanghai, China) with a detection limit of 0.1 ng/mL, according to the manufacturer's instructions. The coefficient of variation between replicas must be less than or equal to 4.9% (Supplementary File 1).

2.5.6. Measurement of Cytokines IL6, TNF α and MCP1

These assays employed the quantitative sandwich enzyme immunoassay technique (DRG Instruments GmbH, Marburg, Germany), according to the manufacturer's instructions. A monoclonal antibody specific for IL6, TNF α and MCP1 was precoated onto a microplate. Standards and samples were placed into the wells, and any IL6, TNF α and MCP1 present were bounded by the immobilized antibody. After washing away any unbounded substances, an enzyme-linked polyclonal antibody specific for IL6, TNF α and MCP1 was added to the wells. Following a wash to remove any unbounded antibody-enzyme reagent, a substrate solution was added to the wells, and color developed in proportion to the amount of IL6, TNF α and MCP1 bounded in the initial step. The color development was stopped, and the intensity of the color was measured. The coefficient of variation for IL6, TNF α and MCP1 between replicas must be less than or equal to 4.4, 6.4 and 4.7%, respectively (Supplementary File 1).

2.5.7. C reactive Protein (CRP)

For CRP-ultrasensitive (PCR-Turbidítext, Spinreact, Girona, Spain) detection, a turbidimetric test was used for the quantification of low serum CRP levels, according to the manufacturer's instructions. Latex particles coated with anti-human CRP antibodies were agglutinated by CRP that was present in the subject's sample. The agglutination process caused an absorbance change proportional to the CRP concentration of the sample, and by comparison with a CRP calibrator of known concentration, the CRP content in the analyzed sample was determined. The coefficient of variation between replicas had to be less than or equal to 4.7%.

2.6. Statistical Analyses

Data analysis was conducted using IBM Social Sciences software (SPSS, version 21.0, Chicago, IL, USA). Descriptive statistics are presented as mean and standard deviation (SD). Levene's and Shapiro–Wilk tests were applied to check the homogeneity and normality of the data, respectively. A group \times time \times moment ANOVA was conducted to analyze within-group and between-group differences in all dependent variables and for every time-point of measurement (baseline, post-P_{MAX} and post-REC) and in both moments (pre-test and post-test). In addition, the area under the curve (AUC), resulting from the integration of the three time-points of measurement taken during the rectangular test, was calculated for each variable. The AUC was used to analyze pre-post differences both within groups and between groups. The within-group differences in the AUC were analyzed by repeated-measures *t*-test, and between-group comparisons in the AUC were conducted by applying an independent samples T-test. Cohen's *d* effect size (ES) (95% confidence interval) was calculated for all comparisons. Threshold values for ES statistics were as follows: > 0.2 small, > 0.5 moderate, > 0.8 large [27]. Significant differences were considered for $p \leq 0.05$.

3. Results

3.1. Biomarkers of Antioxidants and Oxidants Endogenous

Obtained values for CAT, SOD, GSSG, GSH, GSSG/GSH and HO1 during the rectangular test, pre- and post-intervention, are presented in Table 3. For each parameter, within group changes at each time point (baseline, Post-P_{MAX} and Post-REC) during supplementation were evaluated. A significant increase in SOD activity was found for the 2S-hesperidin group in post-P_{MAX} (15.5%) and post-REC (16.3%), while the placebo showed a significant increase in SOD at baseline (18.1%), intragroup pre-post-intervention (Figure 2). In addition, a similar increase in SOD in the AUC was found in 2S-hesperidin (14.1%) and placebo (11.9%) in the intragroup statistical analysis, without significant differences between groups (Figure 3).

Additionally, a trend towards a decrease with a moderate size effect in GSSG levels at post-P_{MAX} (−17.7%) was found in 2S-hesperidin in the post-intervention intragroup statistical analysis. In contrast, a significant decrease with a large size effect in GSSG was observed in the placebo at post-P_{MAX} (−15.1%) after the intervention (Figure 2). When comparing baseline post-intervention between groups, 2S-hesperidin had lower GSSG values (−20.1%) than the placebo (Figure 2). After the analysis of the AUC intragroup, there was a decrease in GSSG (−14.6%) only in 2S-hesperidin, without differences between groups (Figure 3).

Table 3. Cont.

	2S-HESPERIDIN				PLACEBO				Between-Group Comparison			
	Baseline	Post- P _{MAX}	Post- REC	AUC	Baseline	Post- P _{MAX}	Post- REC	AUC	ΔBaseline	ΔPost-P _{MAX}	ΔPost-REC	ΔAUC
HO1	Pre-Int	0.539 (0.24)	0.473 (0.20)	0.513 (0.22)	0.998 (0.41)	0.626 (0.23)	0.551 (0.28)	0.589 (0.27)	1.158 (0.51)			
	Post-Int	0.650	0.581	0.585	1.198	0.740	0.678	0.705	1.400			
	P-value	0.135	0.172	0.281	0.081	0.34	0.26	0.30	0.55			
	Effect size	0.44	0.53	0.32	0.47	0.059	0.066	0.027	0.038	0.972	0.849	0.548
TBARS	Pre-Int	2.55 (0.42)	2.80 (0.38)	2.41 (0.48)	5.27 (0.68)	2.64 (0.37)	2.92 (0.54)	2.75 (0.41)	5.62 (0.78)			
	Post-Int	2.79	2.88	2.54	5.54	2.73	2.80	2.57	5.45			
	P-value	0.052	0.519	0.393	0.246	0.50	0.49	0.50	0.551	0.399	0.406	0.134
	Effect size	0.55	0.22	0.25	0.38	0.22	0.21	0.42	0.21	0.27	0.27	0.48

Values are expressed as mean (SD). Abbreviations: AUC = area under curve; CAT = catalase; SOD = superoxide dismutase; GSH = reduced glutathione; GSSG = oxidized glutathione; % GSSG/GSH = oxidized/reduced glutathione ratio; HO1 = hemoxygenase 1; TBARS = thiobarbituric acid reactive substances and SD = standard deviation. Group comparison = *p*-value comparison of Δ pre-post intervention between groups at different times (baseline, post-P_{MAX} and post-REC) of rectangular test.

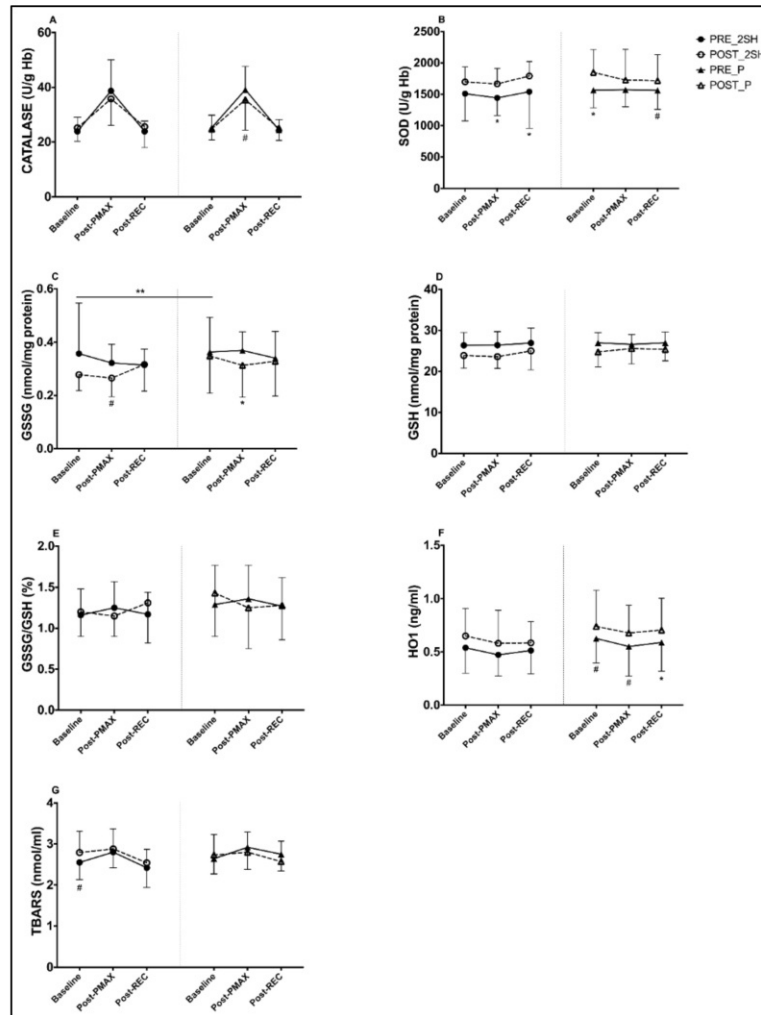


Figure 2. Differences between pre- and post-intervention intragroup in antioxidant and oxidant parameters at different points of the rectangular test (A–G). (C), a significant difference ($p = 0.04$) appears, comparing baseline of the second rectangular test between groups. * $p < 0.05$. # $p = 0.05$ – 0.06 . ** $p < 0.05$ between post-intervention time points of rectangular test between groups (2S-hesperidin vs. placebo).

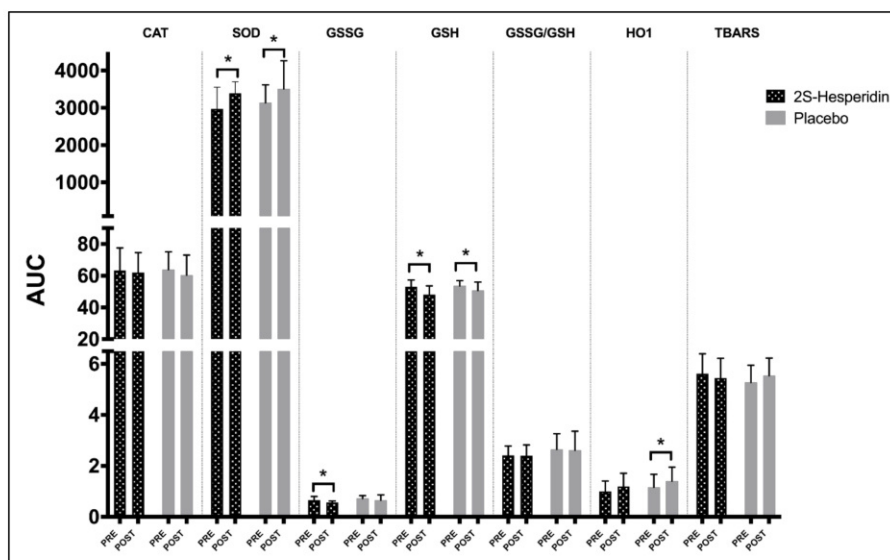


Figure 3. Intragroup differences between AUC (pre- and post-intervention) in antioxidant parameters. * $p < 0.05$. There were no significant differences between groups in AUC.

For GSH, a decrease was reported at baseline (-9.4%) and post- P_{MAX} (-10.7%) in 2S-hesperidin after the intervention. On the other hand, a significant decline was found at baseline (-8.3%) in the placebo (Figure 2). Intragroup AUC analysis of GSH showed a decrease in 2S-hesperidin (-9.5%) and the placebo (5.5%), without differences between groups (Figure 3).

After the intragroup analysis, HO1 significantly increased at post-REC (19.7%) in the placebo, while there was a non-significant increase with a moderate size effect in HO1 at post- P_{MAX} (22.8%) in 2S-hesperidin (Figure 2). Intragroup AUC analysis showed an increase in HO1 in the placebo (20.9%) without any differences between groups (Figure 3).

When we analyzed the intragroup TBARS data, we found a trend towards an increase with a moderate size effect at baseline (9.4%) in 2S-hesperidin, without significant differences between groups.

When results for each parameter at each time point during supplementation were compared between groups, no significant changes were found in any antioxidant-oxidant parameter.

3.2. Inflammatory Biomarkers

Table 4 shows the obtained values for inflammatory biomarkers IL6, TNF α , MCP1 and CRP during the rectangular test, pre- and post-intervention. Within-group changes for each parameter and time point (baseline, Post- P_{MAX} and Post-REC) during supplementation have been evaluated. The placebo group showed a significant decrease in IL6 at Post- P_{MAX} (-35.7%), without significant changes in 2S-hesperidin (Figure 4), in the intra-group comparison pre- and post-intervention. However, after the intragroup analysis, we reported a decline in the AUC of IL6 (-33.0%) in the placebo after the supplementation period, without differences between groups (Figure 5).

Table 4. Changes in inflammatory status markers before, during and after rectangular test comparing pre- and post-intervention.

	2S-Hesperidin						Placebo						Between-Group Comparison				
	Pre	Post-P _{MAX}	Post-REC	AUC	Pre	Post-P _{MAX}	Post-REC	AUC	ΔBaseline	ΔPost-P _{MAX}	ΔPost-REC	ΔAUC					
IL6	Pre-Int	2.46 (3.78)	3.05 (4.92)	4.85 (6.55)	6.71 (9.94)	5.41 (9.26)	7.17 (10.02)	9.44 (10.62)	14.59 (20.67)								
	Post-Int	2.04 (2.32)	2.57 (2.94)	2.01 (2.61)	4.59 (5.01)	4.35 (8.65)	4.61 (7.78)	5.96 (11.98)	9.77 (17.69)								
	p-value	0.537	0.695	0.128	0.255	0.129	0.045	0.065	0.021	0.514	0.243	0.807	0.31				
	Effect size	0.11	0.10	0.42	0.20	0.11	0.42	0.31	0.22	0.21	0.39	0.08	0.33				
TNFα	Pre-Int	8.06 (1.51)	8.43 (2.07)	7.97 (2.08)	16.44 (3.17)	8.71 (1.62)	8.90 (1.83)	7.94 (1.35)	17.22 (2.76)								
	Post-Int	7.58 (1.70)	8.17 (1.98)	7.44 (2.00)	15.68 (3.40)	7.55 (1.89)	7.61 (2.46)	7.41 (1.94)	15.09 (3.6)								
	p-value	0.271	0.466	0.148	0.338	0.015	0.038	0.127	0.021	0.252	0.21	0.999	0.239				
	Effect size	0.31	0.12	0.25	0.23	0.69	0.25	0.38	0.74	0.37	0.4	0	0.38				
MCP1	Pre-Int	568 (177.05)	614 (240.06)	631 (184.77)	1214 (403.32)	714 (324.12)	766 (359.13)	656 (291.75)	1452 (617.47)								
	Post-Int	453 (137.74)	543 (174.45)	466 (131.81)	1002 (299.31)	550 (228.61)	657 (268.48)	571 (231.280)	1218 (484.67)								
	p-value	0.007	0.096	0.004	0.002	<0.001	0.026	0.108	0.021	0.388	0.567	0.275	0.845				
	Effect size	0.63	0.29	0.86	0.5	0.49	0.86	0.28	0.36	0.28	0.18	0.35	0.06				
CRP	Pre-Int	1.200 (2.48)	1.268 (2.47)	1.063 (2.00)	2.399 (4.71)	1.487 (2.56)	1.579 (2.57)	2.049 (3.45)	3.347 (5.12)								
	Post-Int	0.724 (-0.49)	0.798 (-0.54)	0.669 (0.45)	1.494 (1.01)	1.517 (2.50)	1.539 (2.49)	1.404 (2.49)	3.000 (4.98)								
	p-value	0.521	0.523	0.634	0.398	0.981	0.943	0.402	0.832	0.606	0.658	0.819	0.774				
	Effect size	0.18	0.18	0.19	0.18	0.01	0.01	0.18	0.07	0.16	0.14	0.07	0.09				

Values are expressed as mean (SD). Abbreviations: AUC = area under curve; IL-6 = interleukin 6; TNFα = tumor necrosis factor α; MCP-1 = monocytes chemoattractant protein 1 and CRP = C-reactive protein. Group comparison = p-value comparison of Δ pre-post intervention between groups at different times (baseline, post-P_{MAX} and post-REC) of rectangular test.

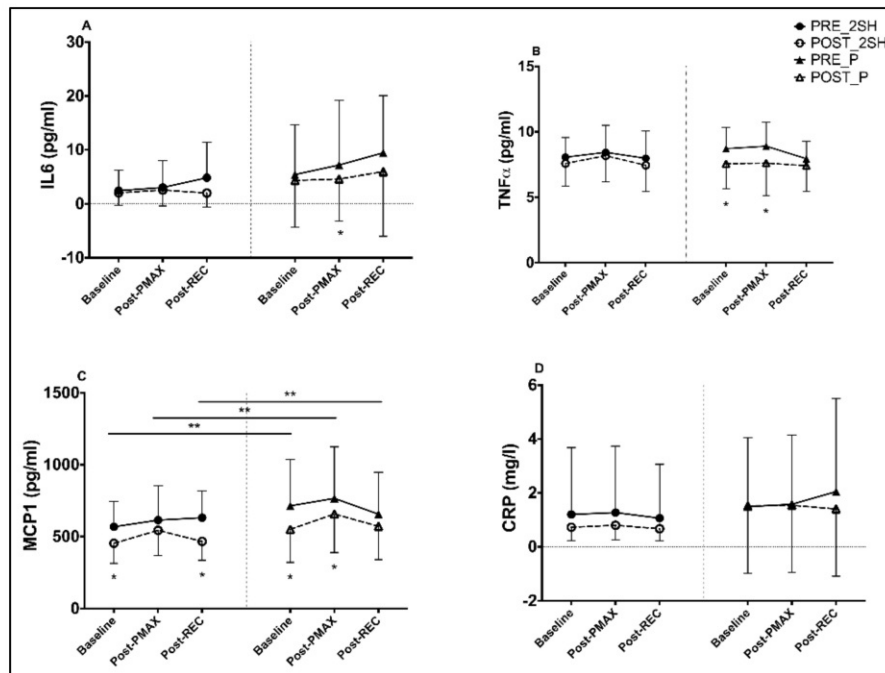


Figure 4. Differences between pre- and post-intervention intragroup and intergroup in inflammatory parameters at different points of the rectangular test (A–D). (C) a significant difference appears, comparing baseline ($p = 0.043$), Post- P_{MAX} ($p = 0.026$) and Post-REC ($p = 0.045$) of the second rectangular test between groups. * $p < 0.05$. ** $p < 0.05$ between post-intervention time points of rectangular test between groups (2S-hesperidin vs. placebo).

Regarding $TNF\alpha$, a significant drop in levels at baseline (-13.3%) and post- P_{MAX} (-14.5%) was found in the placebo (Figure 4). In addition, intragroup AUC analysis of $TNF\alpha$ found a decrease (-12.4%) in the placebo without differences between groups (Figure 5).

Significant decreases were observed in MCP1 at baseline (-20.2%) and post-REC (-26.1%) in 2S-hesperidin. In the placebo, significant decreases were also observed in MCP1 at baseline (-23.0%) and post- P_{MAX} (-14.2%) in the post-intervention intragroup statistical analysis (Figure 4). When comparing MCP1 at different times (baseline, Post- P_{MAX} and Post-REC) of the rectangular test post-intervention between groups, 2S-hesperidin had lower MCP1 values (-17.6% , -17.4% and -18.4% , respectively) than the placebo (Figure 4). In addition, a similar decrease in the AUC was found in 2S-hesperidin (-17.5%) and the placebo (16.1%) in intragroup statistical analysis, but in the case of 2S-hesperidin, a moderate size effect was observed, without significant differences between groups (Figure 5).

No significant within-group changes were reported for CRP in any group (Figure 4).

When results for each parameter at each time point during supplementation were compared between groups, no significant changes were found in any inflammatory parameter.

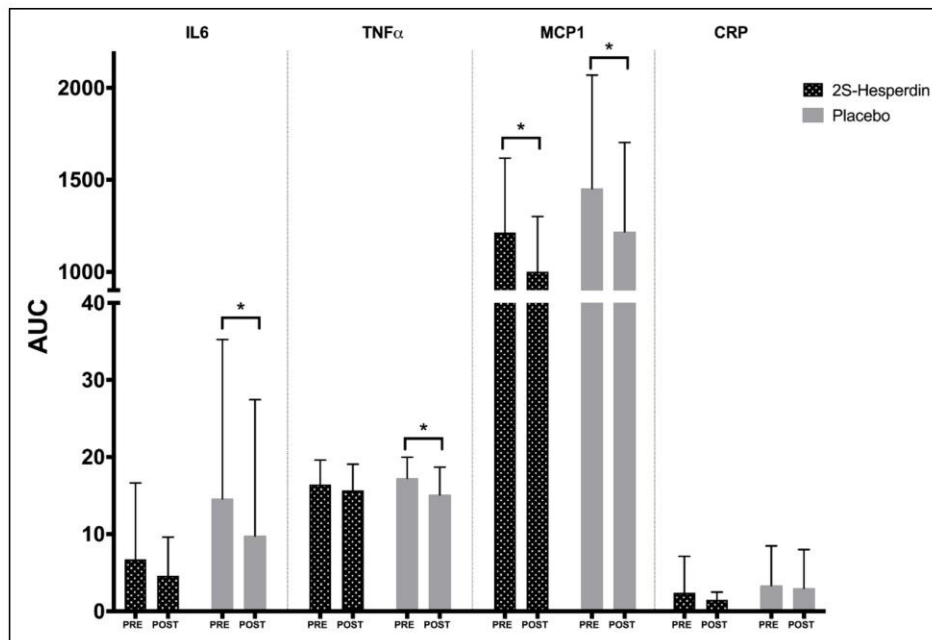


Figure 5. Intragroup differences between AUC (pre- and post-intervention) in inflammatory parameters. * $p < 0.05$. There were no significant differences between groups in AUC.

3.3. Hesperidin Metabolites Urine

Different hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analyzed in urine after Cardiose[®] intake. The main metabolite detected was hesperetin-3-glucuronide, representing $78.9 \pm 5.0\%$ ($n = 20$) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate made up $6.9 \pm 2.9\%$ ($n = 20$) and $14.7 \pm 4.1\%$ ($n = 20$) of the excreted metabolites. Despite the similarities in the excreted metabolites' profiles, a large interindividual variability was observed in the number of excreted hesperidin metabolites ranging from 2.3 to 37.5 μmol .

4. Discussion

This study evaluated the effect of 8-week supplementation with 500 mg/d of 2S-hesperidin or placebo on antioxidant and inflammatory status in amateur cyclists during a rectangular cycle-ergometer test. To the best of our knowledge, this is the first study that examines the effect of chronic 2S-hesperidin intake on the antioxidant and inflammatory status of athletes at baseline, during and after exercise. In the rectangular test, oxidative status improved (\downarrow GSSG AUC) after the 2S-hesperidin intervention, but not with the placebo. In addition, significant improvements in antioxidant capacity (\uparrow SOD) after maximal exercise (Post- P_{MAX}) and inflammatory status after the acute recovery phase (\downarrow MCP1) were found in the 2S-hesperidin group compared to the placebo (baseline and Post-REC).

4.1. Changes in Endogenous Antioxidant Markers

SOD activity is usually increased after training, as an exercise-mediated adaptation [28]. In contrast, a previous study showed no increases in SOD activity in untrained individuals after an 8-week moderate training program (35-min aerobic cycle, 3 times/week) [29]. Conversely, we observed a maintenance of SOD from baseline to Post-P_{MAX} and an increase to Post-REC in the post-intervention rectangular test, with significant increases in Post-P_{MAX} and Post-REC pre-post-intervention in 2S-hesperidin. However, an increase in SOD activity levels, evaluated as the AUC, was observed for both groups during intervention. In amateur cyclists, the acute intake of 2S-hesperidin (single dose; 500 mg) led to no significant decrease in SOD at baseline [9]. In animals, 2S-hesperidin supplementation (200 mg/kg for three days per week), along with a 5-week training program, led to no significant changes in SOD activity in rats after an exhaustive exercise test [10]. Additionally, 2S-hesperidin significantly increased SOD activity in heart tissues, which was attenuated by doxorubicin (induced cardiac toxicity) treatment [30]. The 2S-hesperidin antioxidant capacity enhancement may be explained by the antioxidant characteristics of this molecule, related to hydroxyl groups in its B-ring [31]. In addition, Parhiz et al. found that hesperidin had significant radical scavenging activity and prevented H₂O₂-induced oxidative damage on the cellular membranes of red blood cells, with radical scavenging activities comparable to ascorbic acid and trolox (a vitamin E derivative) [32]. Furthermore, 2S-hesperidin shows a neutralizing effect on non-enzymatic lipid peroxidation and superoxide, hydroxyl, peroxynitrite, and nitric oxide radicals [4,31], leading to a lower depletion of antioxidant enzymes and allowing the maintenance of high antioxidant levels, even after exercise-induced oxidative stress.

Another mechanism that has been reported *in vitro* and in animal models, but has yet to be confirmed in humans, is the improvement of the antioxidant status through a nutrigenetic effect. Hesperidin has shown increased regulation of respiratory nuclear factor 2 (NRF2) [33]. NRF2 is a basic leucine zipper transcription factor that binds and activates the antioxidant response element in the promoters of many antioxidant and detoxification genes encoding proteins, such as SOD, glutathione, thioredoxin and HO1, and thus it promotes the regulation of the intracellular redox environment [34]. Interestingly, flavonoids have been proposed as inducers of the expression of genes related to enzymes of the endogenous antioxidant system through the activation of the NRF2 transcription pathway [35]. The higher SOD activity at the end of maximal effort and after a short recovery period after the intervention indicates that the chronic intake of 2S-hesperidin improves antioxidant capacity at maximal effort and in the acute phase of recovery in amateur cyclists.

The oxidation of GSH to GSSG is a sensitive marker of oxidative stress [36]. In addition, a GSH decrease and GSSG and GSSG/GSH ratio increases have been observed in professional cyclists after competitions [37]. When comparing both groups at baseline after the intervention, the 2S-hesperidin group had lower GSSG values than the placebo, indicating lower levels of oxidative stress. This is in line with the decrease found in the AUC (GSSG) in 2S-hesperidin, indicating a decrease in oxidative stress when considering the whole rectangular test, which may be related to detraining adaptation. In fact, lower training volumes and intensities are associated with lower levels of GSH and GSSG in professional cyclists [38], which was also found Post-P_{MAX} in the placebo. Therefore, this decrease in both groups is due to a lower exposure to high levels of free radicals leading to a maladaptation in the glutathione antioxidant system. The main advantage of incorporating the AUC in this study is that it allows us to precisely define the duration and magnitude of the variable being evaluated, which cannot be done in a point-by-point comparison [39]. Despite the fact that there are no previous studies in humans evaluating the effects of chronic hesperidin intake on GSH and GSSG, instead, non-significant decreases in GSH, GSSH and the GSSG/GSH ratio were observed after a repeated sprint test in amateur cyclists after a single-dose of 2S-hesperidin (500 mg) [9]. In the same way, pathological animal models have shown the positive effect of hesperidin supplementation on these glutathione markers (↑GSH and ↓GSSG) [40,41].

It is known that regulation exists between GSH and GSSG by the enzymes glutathione reductase (GR) and GPx to maintain a balance between both molecules and avoid an increase in ROS [42]. The changes observed in GSSG in the experimental group could be due to the modulation of GPx and GR activity, which was not measured in this study. In addition, another factor that may influence the GSSG/GSH ratio is the levels of nicotinamide adenine dinucleotide phosphate (NADPH), which are used with an indispensable cofactor by the GR and GPx enzymes to synthesize the GSH and GSSG forms [43]. In this context, NADPH donates two electrons to reduce GSSG to GSH by GR; the recycled GSH can then be used to reduce H_2O_2 to water by GPxs [44]. In addition, increased glucose-6-phosphatase dehydrogenase (G6PD) (a major source of cytosolic NADPH) activity by genetic or pharmacological means has been seen to raise cellular stores of NADPH and GSH, promote the detoxification of ROS, and increase cell viability in primary vascular endothelial and smooth muscle cells *in vitro* [45]. Increased G6PD activity is positively correlated with increased GR activity, where hesperidin was capable of restoring the activity of G6PD in rats [46]. In addition, Salvemini et al. [47] reported that a three-fold increase in G6PD activity resulted in a two-fold increase in GSH levels, as well as a very significant increase in resistance to oxidative stress. As we can see, the GSSG/GSH ratio can be modulated by different components involved in the endogenous antioxidant system, which makes it difficult to explain its changes. Therefore, the chronic intake of 2S-hesperidin could decrease GSSG levels (evidenced by \downarrow AUC), indicating a better antioxidant state in the rectangular test, but specifically immediately after exercise. This would facilitate faster post-training recovery or competition for cyclists.

In relation to HO1, in the placebo there was an increase Post-REC with an increasing trend in baseline and Post-P_{MAX} post-intervention; however, after 2S-hesperidin supplementation, no significant change was seen, but there was a moderate effect in Post-P_{MAX}. The high variability in the HO1 data in 2S-hesperidin may have been the consequence of no significant changes being observed. What is clear is that amateur-level cycling for 8 weeks improves HO1 levels.

Although there is no clear pattern of improvement in antioxidant markers in 2S-hesperidin, there is an improvement in certain components (\uparrow SOD, \downarrow GSSG) of the endogenous antioxidant system measured in this study and at key times during recovery. However, further studies are needed to provide clarity on this issue.

4.2. Changes in Inflammatory Markers

The production of ROS at the mitochondria of the working muscle stimulates the production of myokines or pro-inflammatory cytokines [48]. IL6 (inflammatory cytokine) plasma levels can increase up to 100-fold after exercise, and circulating muscle-derived IL6 levels are closely related to the duration and intensity of exercise [49]. To our knowledge, no studies have evaluated the effects of 2S-hesperidin intake on inflammatory markers in humans. In this study, IL6 levels increased during the first and second rectangular test from baseline to Post-P_{MAX} in both groups, but there were different trends from Post-P_{MAX} to Post-REC in the second rectangular test (\downarrow 2S-hesperidin and \uparrow placebo). A significant decrease in IL6 during the recovery stage was observed in the placebo, post-intervention. Other flavonoids, such as cocoa-derived flavanols, have also failed to inhibit the increase in IL6 after intense exercise (75% of peak power output for 30 min) in cyclists [50]. We believe that the high variability in the IL6 data was a factor that did not allow us to find significant intra- and intergroup differences. In addition, IL6 values in the placebo were quantitatively higher than those of 2S-hesperidin, which may favor a significant decrease in IL6 after the reduction in the training load performed by cyclists from post-season to pre-season, as was the period in which the study was conducted (from the end of September to the end of December). As IL6 is known to stimulate the expression of TNF α [51], a decrease in IL6 levels in the placebo would lead to a decrease in TNF α levels (baseline and Post-P_{MAX}). Since there is less training load, there is less induction of oxidative stress [38] and, consequently, less stimulation of the inflammatory system.

However, numerous *in vitro* studies (inflammatory models) have shown the ability of hesperidin to lower IL6 levels and TNF α [52–55]. A recent study in trained animals showed that hesperidin intake (200 mg/kg for three days per week) during 5 weeks prevented an increase in IL6 levels in peritoneal macrophages after an exhausting exercise [56]. Interestingly, in this study, a significant increase in IL6 after an exhausting exercise, from pre-training to post-intervention, was observed in the placebo group. Hesperidin intake has also led to a decrease in IL6 in a rheumatoid arthritis rat model [57]. In rats, the intake of alcohol to induce a gastric ulcer increased the expression of cyclooxygenase-2 mRNA and decreased GPx, SOD, and CAT, but the intake of hesperidin reversed these changes, improving the antioxidant and inflammatory status [58]. In addition, in a model of Alzheimer's disease in mice, treatment with hesperidin (40 mg/kg, 90 days intragastric) increased HO1 and decreased levels of TNF α , CRP, NF- κ B and MCP1, suppressing oxidative stress and inflammation [59].

A hypothesis has been generated at the nutrigenomic level of how the intake of hesperidin can improve the inflammatory state, related to the activation of the Akt/NRF2 axis and the inhibition of NF- κ B [59], with the latter being a transcription factor well known for its role in the innate immune response and a transcriptional activator of inflammatory mediators such as cytokines [60]. On the other hand, NRF2 is not only important for redox signaling, but also for the attenuation of the inflammatory mediator synthesis [59]. In this sense, the impairment of NRF2 signaling by ultraviolet B (UVB) was reversed by the topical application of hesperidin methyl chalcone, which inhibited the production of the cytokines TNF α , IL-1 β , IL6, and IL-10 that had been induced by UVB irradiation in hairless mice [61]. This suggests that there is a connection between the antioxidant and inflammatory status and their signaling pathways. In our case, the group ingesting 2S-hesperidin did not experience a significant decrease in TNF α .

MCP1 is another inflammatory cytokine that increases after exercise in plasma [62]. In our study, lower MPC-1 levels during the whole exercise (AUC) were observed after supplementation in both groups. This decrease was statically significant at baseline and during the recovery phase for the 2S-hesperidin supplemented group. In addition, when comparing between groups at different post-intervention test times, the 2S-hesperidin group had lower levels compared to the placebo. In previous studies with an acute lung damage model, both *in vitro* and *in vivo*, hesperidin has shown immunomodulatory effects, down-regulating the expression of MCP1 as well as other pro-inflammatory cytokines, such as IL6 and TNF α [52,63]. Precisely, treatment with hesperetin-7-O-glucuronide (5 mg kg⁻¹) has been observed to decrease the MCP1 mRNA expression in rat aortic endothelial cells [63]. On the other hand, the oral administration of 100 or 200 mg/kg of hesperidin three times a week for four weeks in rats produced a decrease in the pro-inflammatory cytokines interferon-gamma- γ and MCP1 in the lymphocyte of the mesenteric lymph node [10]. Additionally, polyphenols and hesperidin can modulate gut microbial composition or functionality, which modulate the release of microbial-derived metabolites [64]. In addition, hesperidin has the ability to inhibit the growth of harmful bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Prevotella* spp., *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, among others [3]. In particular, hesperidin can increase the abundance of *Faecalibacterium prausnitzii*, which inhibits NF- κ B activation and consequently attenuates the inflammatory response [65]. The inhibitory capacity of hesperidin in some bacteria may modify the composition of the intestinal microbiota acting as an immunomodulator and anti-inflammatory (\downarrow IL-1 β , TNF α , and IL6), with a direct relationship between the two effects [3]. In contrast, the effects of quercetin (flavonoid) intake (1g/day) for 3 weeks in trained cyclists were evaluated by Nieman et al. [62]. In this study, no significant changes in MCP1 plasma levels were observed after a 3-week supplementation and a 3-day period in which subjects cycled for 3 h/day at ~57% maximal work rate. Muscle biopsies showed a within-group significant post-exercise increase in muscle cytokine mRNA expression for IL6 and TNF α , but without differences between the quercetin and placebo groups [62]. No anti-inflammatory effect was observed after the

intake of quercetin. On the other hand, the placebo group showed a decrease in MCP1 at baseline and Post- P_{MAX} at post-intervention, possibly related to the decrease in TNF- α , as a positive correlation between MCP-1 and TNF- α concentrations after short-term exercise training has been previously demonstrated [66], indicating a relationship between these two cytokines.

Although there is no clear pattern of improvement in inflammatory markers in 2S-hesperidin, there is an enhancement in MCP1 (baseline and Post-REC) compared to the placebo in the second rectangular test at all points and at key times during recovery. However, further studies are needed to bring clarity to this question.

These 2S-hesperidin properties such as antioxidant and anti-inflammatory properties may be related: a decrease in oxidative stress during the exercise maximum intensity could modulate the inflammatory state in the acute phase of recovery. As has been shown in the studies presented in this publication, there is a close relationship between antioxidant and inflammatory status and their signaling pathways. Redox balance can be altered during periods of high intensity physical exercise and low rest periods, leading to a chronic oxidative stress state [15]. Moreover, high oxidative stress levels can inhibit exercise physiological adaptations, reducing performance and leading to overtraining [15]. Therefore, an optimal redox homeostasis is essential for a proper muscle physiological function (i.e., antioxidant status, biochemistry, signaling, bioenergetics and muscle contraction).

The effects of antioxidant supplementation on performance are a controversial topic, which still needs additional research. On one hand, it has been pointed out that the use of antioxidant substances may help to maintain optimal ROS levels in the muscle, avoiding possible decreases in performance [15]. On the other hand, it has been hypothesized that chronic antioxidant intake can hinder training adaptations, negatively affecting performance [17]. Different studies show that antioxidant intake does not prevent the exercise-induced activation of redox-sensitive signaling pathways [67]. A recent publication summarized the performance measurements that were carried out in this same intervention trial, along with the antioxidant and inflammatory marker results reported in this paper [7]. In this trial, amateur cyclists' supplementation (8 weeks) with 2S-hesperidin (500 mg/day) led to an increase in power production at estimated functional threshold power (2.3% = 6.40 W; $p = 0.049$) and maximum power (1.9% = 7.40 W; $p = 0.049$) during an incremental test after the intervention [7]. Thus, 2S-hesperidin does not appear to interfere with training-induced adaptations, improving performance while avoiding oxidative stress and inflammation.

The study described in this paper has some limitations. One limitation was the short recovery time after the rectangular test (20 min after exercise), in which changes in antioxidant and inflammatory markers were evaluated. Measurements 24 and 48 h after exercise would have provided valuable additional information; however, funding constraints made it impossible. Additionally, a larger sample would have given more statistical power to the reported results due to the high individual variability in some markers. Given the few studies carried out in this field, future research could shed light on the effectiveness of 2S-hesperidin as an ergogenic aid with antioxidant and inflammatory effects.

Differences with current results may be related to the different stage of the season in which studies were done in the sample used, and the different aerobic and anaerobic demand profiles of the used tests. In the same way, one of the factors influencing the variability of 2S-hesperidin effects in different studies may be its pharmacokinetics, and the resulting exposure of the body to hesperidin metabolites. It has been described that the concentration of 2S-hesperidin metabolites in plasma reaches its maximum peak 5–7 h after intake, being almost completely eliminated after 24 h. In the urine, the maximum peak of metabolites is usually found at 24 h of 2S-hesperidin intake, and its total excretion occurs after 48 h [68].

5. Conclusions

Supplementation with 2S-hesperidin (500mg/d) for 8 weeks improves the post-rectangular test antioxidant (\uparrow SOD and \downarrow AUC-GSSG) and inflammatory status during the acute phase of post-exercise recovery (\downarrow MCP1). This modulation in antioxidant and inflammatory markers can help cyclists to improve their recovery after intense efforts or long exercise sessions that, due to their characteristics, led to an increase in inflammation and oxidative stress. Unlike other polyphenols, 2S-hesperidin supplementation does not appear to interrupt adaptations produced by training in amateur cyclists, enhancing their performance [7].

Supplementary Materials: The following are available online at <https://www.mdpi.com/2076-3921/10/3/432/s1>, Supplementary File 1. Methodology oxidative, antioxidant and inflammatory markers.

Author Contributions: Conceptualization, F.J.M.-N., C.M.-P. and P.E.A.; methodology, F.J.M.-N., C.M.-P. and P.E.A.; formal analysis, F.J.M.-N., C.M.-P. and J.C.-V.; investigation, F.J.M.-N., C.M.-P. and J.C.-V.; resources, F.J.M.-N., C.M.-P. and J.C.-V.; data curation, F.J.M.-N., C.M.-P. and J.C.-V.; writing—original draft preparation, F.J.M.-N.; writing—review and editing, F.J.M.-N., C.M.-P. and J.C.-V.; visualization, C.M.-P.; supervision, C.M.-P. and P.E.A.; project administration, C.M.-P. and P.E.A.; funding acquisition, P.E.A. All authors have read and agreed to the published version of the manuscript.

Funding: The authors declare that this study has been financed by HTBA (Murcia, Spain), who kindly provided the product Cardiose®, but they did not participate in the experimental design, data collection, data analysis, interpretation of the data, writing of the manuscript, or in the decision to publish the results.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Catholic University of Murcia (CE091802).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data is contained within the article.

Acknowledgments: This study was supported by the Research Center for High Performance Sport of the Catholic University of Murcia and HTBA (Murcia, Spain). We would like to acknowledge Linda H. Chung for her help in this project. We also thank Iris Samarra, Antoni del Pino and Nuria Canela, from the Metabolomics facility of the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat, for their contribution to the urine analysis. The results of the current study do not constitute endorsement of the product by the authors or the journal.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary file 1. Methodology oxidative, antioxidant and inflammatory markers.**TBARS (Lipoperoxidation biomarker)**

Thiobarbituric acid reactive substances (TBARS) are by-products of the oxidative degradation of lipids by reactive oxygen species (lipid peroxidation), which is commonly used as an oxidative stress marker. The methodology consists of isolating the plasma lipid fraction by lipid precipitation with phosphotungstic acid, followed by a reaction with thiobarbituric acid (TBA) that forms an adduct, allowing for detection using UV-VIS spectrophotometer at a wavelength of 532 nm. The assay involves the reaction of malondialdehyde (MDA), a product of lipid peroxidation, with thiobarbituric acid (TBA) under high temperature and acidic conditions to form an MDA-TBA complex that can be measured colourimetrically. The coefficient of variation between replicas had to be less than or equal to 4.6 %.

Catalase (CAT)

CAT activity was determined using a UV-VIS spectrophotometer. The absorbance of H₂O₂ decreases at 240 nm proportional to its decomposition, so that the concentration of H₂O₂ is critical in this determination. The decrease in absorbance per unit time is the measure of catalase activity. This is expressed in per sec per gram of haemoglobin. The coefficient of variation between replicas must be less than or equal to 4.9 %.

Superoxide dismutase (SOD)

SOD activity was measured using an SD125 Ransod kit (Randox Ltd. Crumlin, United Kingdom). This method consists of the use of xanthine and xanthine oxidase to produce superoxide anion (O₂⁻), which responds with the 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) reactive and forms a red complex detectable at 420 nm. The SOD activity is then quantified by measuring the degree of inhibition of this reaction. The coefficient of variation between replicas must be less than or equal to 5.1 %.

Glutathione reduced (GSH)

The GSH was determined from whole blood, which was treated with perchloric acid to a final concentration of 6%, obtaining the supernatant after vortexing and centrifuging at 10,000 rpm for 10 minutes. After collecting the supernatants in vials, it was quantified using high-performance liquid chromatography (HPLC) with a

Waters ODS S5 NH2 Column (0.052, 25 cm) for separation purposes. The coefficient of variation for GSH between replicas must be less than or equal to 4.1 %.

Hemoxigenasa 1 (HO-1)

HO-1 ELISA kit applies the competitive enzyme immunoassay technique by utilizing an anti-HO-1 antibody and an HO-1-HRP conjugate. The assay sample and buffer are incubated together with HO-1-HRP conjugate in a pre-coated plate for one hour. After the incubation period, the wells are decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Finally, a stop solution yellow is formed. The intensity of the color is measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color is inversely proportional to the HO-1 concentration, since HO-1 from samples and HO-1-HRP conjugate compete for the anti-HO-1 antibody binding site. As there are a set number of sites and as more sites are occupied by HO-1 from the sample, there are fewer sites left to bind to the HO-1-HRP conjugate. A standard curve is plotted relating the intensity of the color (O.D.) to the concentration of standards. The HO-1 concentration in each sample is interpolated from this standard curve. The coefficient of variation between replicas must be less than or equal to 4.9 %.

Interleukin 6 (IL-6)

The DRG IL-6-ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of IL-6. Calibrators and samples react with the capture monoclonal antibody (MAB 1) coated on a microtiter well and with a monoclonal antibody (MAB 2) labelled with horseradish peroxidase (HRP). After an incubation period that allows for the formation of a sandwich (coated MAB 1 – human IL-6 – MAB 2 – HRP), the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB) is added and incubated. The reaction is stopped with the addition of a Stop Solution, and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is proportional to the IL-6 concentration. A calibration curve is plotted and IL-6 concentration in samples is determined by interpolation from the calibration curve. The use of the EASIA reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in a high sensitivity in the low range and in an extended calibration range. The coefficient of variation between replicas must be less than or equal to 4.4 %.

Tumoral necrosis factor α (TNF α)

The DRG TNF- α -ELISA is a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiterplate. The assay uses monoclonal antibodies (MAbs) directed against distinct epitopes of TNF- α . Standards and samples react with the capture monoclonal antibody (MAB 1), coated on microtiter well and with a monoclonal antibody (MAB 2) labelled with horseradish peroxidase (HRP). After an incubation period that allows for the formation of a sandwich (coated MAB 1 – human TNF- α – MAB 2 – HRP), the microtiterplate is washed to remove unbound enzyme labelled antibody. Bound enzyme-labelled antibody is measured through a chromogenic reaction. Chromogenic solution (TMB) is added and incubated. The reaction is stopped with the addition of a Stop Solution, and the microtiterplate is then read at the appropriate wavelength. The amount of substrate turnover is determined colourimetrically by measuring the absorbance, which is proportional to the TNF- α concentration.

A calibration curve is plotted, and TNF- α concentration in samples is determined by interpolation from the calibration curve. The use of the EASIA reader (linearity up to 3 OD units) and a sophisticated data reduction method (polychromatic data reduction) result in a high sensitivity in the low range and in an extended calibration range. For guidance, the results of 30 serum samples from apparently healthy persons with low CRP levels, ranged between 4.6 and 12.4 pg/ml, were used. The coefficient of variation between replicas must be less than or equal to 6.4 %.

Monocyte chemoattractant protein 1 (MCP-1)

An anti-human MCP-1 coating antibody is absorbed onto microwells. Human MCP-1 present in a sample or standard binds to antibodies absorbed to the microwells, and the horseradish peroxidase (HRP)-conjugated anti-human MCP-1 antibody is added and binds to human MCP-1 captured by the first antibody. Following incubation, unbound HRP-conjugated anti-human is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of human MCP-1 present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human MCP-1 standard dilutions and human MCP-1 concentration determined. The limit of detection of human MCP-1, defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations), was determined to be 2.3 pg/mL (mean of 6 independent assays). The coefficient of variation between replicas must be less than or equal to 4.7 %.



Cite this: DOI: 10.1039/d0fo03456h

8 weeks of 2S-Hesperidin supplementation improves muscle mass and reduces fat in amateur competitive cyclists: randomized controlled trial

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2S-Hesperidin is the main flavonoid of orange (*Citrus sinensis*). Previous researches have pointed its effects in muscle development and fat accumulation reduction, although most of these results have not been assessed in humans. The objective of this study is to evaluate the effect of chronic (8-weeks) intake of 2S-hesperidin on amateur cyclists' body composition. A double-blind, parallel and randomized trial, was carried out with 40 amateur cyclists that were divided in two groups, one taking 2S-hesperidin (500 mg d⁻¹, n = 20) and another taking placebo (500 mg d⁻¹ microcellulose, n = 20) for 8 weeks. Dual-energy X-ray absorptiometry (DXA) and anthropometric measurements were used to assess the effect of both treatments on body composition. In addition, the resting metabolic rate was measured. In comparison to placebo, DXA analysis showed a decrease in percentage body fat (%BF) (-10.4%; p = 0.035) and lower limb fat mass (-10.5%; p = 0.029) in favour of 2S-hesperidin. After evaluation of anthropometric data, a decrease in %BF (-3.7%; p = 0.006), total body fat (-3.0%; p = 0.047), Σ of 8 skinfolds (-6.1%; p = 0.008) was observed in 2S-hesperidin group, but not in placebo. Additionally, there was an increase in muscle mass percentage (1.0%; p = <0.001) and total muscle mass (1.7%; p = 0.011) after ingestion of 2S-hesperidin, with no changes in placebo. Chronic intake of 2S-hesperidin decreased fat mass in amateur cyclists, evaluated through different body composition measurement methodologies (DXA and anthropometry). In addition, 2S-hesperidin supplementation showed a promoting effect on muscle development.

Received 29th December 2020.
Accepted 28th March 2021

DOI: 10.1039/d0fo03456h
rsc.li/food-function

Published on 29 March 2021. Downloaded by Universidad de Murcia on 4/7/2021 9:09:12 AM.

1. Introduction

Cycling is a sport that presents a high aerobic component, but in which the anaerobic component is decisive for success in competitions.¹ The key physiological factors that could predict a cyclist's performance are: oxygen consumption (VO_{2MAX}),² maximum power output (MPO),^{3,4} ventilatory threshold 2 (VT2) at ~90%VO_{2MAX},⁵ power output (PO) and cycling efficiency.⁶ In addition, body composition control is essential for high-level athletes in order to monitor changes in different body components (fat, muscle, bone, residual, etc.) at different time points during the year, as it affects sports performance.⁷

It is known that having low BF is ideal for achieving high levels of sports performance, since high BF levels require higher energy expenditure to maintain the same running speed, and

may lead to a decrease in performance.⁸ Besides, it has been shown that a reduction in body weight can improve the climbing performance of cyclists.⁹ Indeed, dietary adjustments, in combination with physical exercise, are the best strategies to reduce BF, which in turn provides benefits (performance enhancement) to the athlete.¹⁰ Interestingly, in the last years, the use of phytochemical dietary supplements (including flavonoids) alone or in combination with exercise and/or diet has also shown beneficial modifications in body composition and sports performance in animals and humans.¹¹⁻¹³

One of the most studied flavonoids due to its antioxidant and anti-inflammatory properties is hesperidin, a flavonoid mainly found in citrus fruits.¹⁴ Hesperidin is a molecule that can be found in two isomeric forms, such as 2S and 2R, being the 2S-hesperidin the predominant form in nature.¹⁵ The biological behaviour of the two isomers is different, for example a 5.2-fold improvement in glucuronidation efficiency was observed for 2S-hesperetin compared to 2R-hesperetin *in vitro*, without any significant change in sulfonation kinetics.¹⁶ It should be noted that, despite a fresh squeeze orange juice mostly contains 2S-hesperidin, most commercialised hesperidin supplements contain a mixture of 2R and 2S forms.

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In vitro studies have shown that hesperidin increases adenine monophosphate activated protein kinase (AMPK), that controls mitochondrial biogenesis, glycemia, and fat oxidation; and reduces triglycerides (TG) accumulation in adipocytes.^{17,18} Hesperetin, the main hesperidin metabolite, has shown to increase intracellular ATP production and mitochondrial spare capacity in human myotubes.¹⁹ In animals with high-fat diets, it has been observed that the intake of citrus flavonoids (hesperidin and naringin) increases fatty acid β -oxidation, reverses AMPK activity and decreases weight and abdominal fat.^{18,20} Besides, the administration of hesperetin to aged mice completely reverted the age-related decrease of muscle fibre size and improved the running performance, due to a boost in mitochondrial energy supply and antioxidant effect.¹⁹

In humans, intake of glucosyl hesperidin (100–500 mg d⁻¹ for 6 weeks) has shown to reduce TG and low-density lipoprotein (LDL) levels.²¹ On the other hand, it has been observed that supplementation for 12 weeks with red-orange juice extract (400 mg d⁻¹), which is a source of 2S-hesperidin, decreased body weight, BMI, waist and hip circumference in healthy overweight human volunteers.²²

The effect of single 2S-hesperidin supplementation on body composition (fat and muscle mass) has not yet been studied. Nevertheless, 2S-hesperidin has shown performance improvements in amateur cyclists, both after acute intake (500 mg per single dose; improving anaerobic capacity¹²) and chronic intake (500 mg d⁻¹ for 8 weeks; improving aerobic capacity¹³ and antioxidant and inflammatory status²³). Overall, we hypothesised that (1) 2S-hesperidin treatment would help to decrease BF, and (2) 2S-hesperidin would improve the oxidation of fatty acids during resting metabolic rate test. Therefore, the purpose of this study was to determine if the intake of 2S-hesperidin during eight weeks improved body composition by densitometry and anthropometry in amateur cyclists. A secondary objective was to evaluate the effects of 2S-hesperidin supplementation on different variables of resting metabolic rate using indirect calorimetry.

2. Methodology

2.1 Participants

Forty healthy, male amateur competitive cyclists (AMA) completed the study (Table 1). Inclusion criteria were the following: 18–55 years, 19.0–25.5 kg m⁻² BMI, at least 3-years cycling of experience, and 6–12 h per week training. Participants were excluded if they: (a) were smokers (in the last 6 months) or regular alcohol drinkers (three to four days per week), (b) had metabolic, cardiorespiratory or digestive pathologies or anomalies, (c) had an injury in the last 6 months, (d) were consuming any type of supplementation or medication in the last 2 weeks and (e) had abnormal values in some parameter (haemogram, general biochemistry, transaminases, inflammation and virus) in the preliminary blood analysis. All participants were informed about the procedures and provided signed

Table 1 Baseline characteristics of male amateur competitive cyclists (N = 40)

	Cardiose®	Placebo	p-Value
Main characteristics			
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Weight (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI (kg m ⁻²)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
Training variables			
Km total (km)	1121.12 (534.99)	1082.43 (810.46)	0.868
HRavg (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
Wavg (W)	174.86 (15.79)	163.47 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are presented as mean (SD). BMI = body mass index; BF = body fat; Km total = total distance of all the training sessions carried out during the study period; HRavg = average heart rate of all the training sessions carried out during the study period; Wavg = average power output of all training sessions during the study period.

informed consent. The study was conducted according to the guidelines of the Helsinki Declaration for Human Research²⁴ and the protocol was approved by the Ethics Committee/ Institutional Review Catholic University of Murcia (code: CE091802), registered with ClinicalTrials.gov (identifier: NCT04597983).

2.2 Study design

A double-blinded, parallel and randomised experimental design was performed. Randomization was performed using computer software (Randomizer) to assign codes to the groups established in this study.²⁵ Participants were divided into two groups: 2S-hesperidin ($n = 20$) and placebo ($n = 20$). Depending on the group, participants took 500 mg of 2S-hesperidin (Cardiose® HTBA (HealthTech BioActives), Murcia, Spain) or 500 mg of placebo (microcellulose) per day for 8 weeks in the breakfast. Cardiose® is a natural orange extract that due to its unique manufacturing process, maintains most of the natural hesperidin isomeric form (NLT 85% 2S-hesperidin). The cyclists were instructed to continue their regular training schedule, and no significant differences between groups in the main variables of training load were observed (Table 1). Subjects in both groups were instructed not to consume foods high in citrus flavonoids (grapefruit, lemons or oranges) for 5 days prior to and during the study, which was verified by diet recalls records and urine analysis.

2.3 Procedures

Participants visited the laboratory three times. The first visit (V1) consisted of a medical examination and a blood analysis to evaluate health status, while second (V2) and third visits (V3) included resting metabolic rate (RMR) test, densitometry, anthropometry, urine sample and a 3-day recall diet questionnaire (Table 2). The supplementation of both treatments started at V2 after the completion of all tests and under the supervision of an investigator.

Table 2 Between-group comparisons in dietary intake of male amateur cyclists ($N = 40$)

	Pre-intervention			Post-intervention		
	2S-Hesperidin	Placebo	<i>p</i> -Value	2S-Hesperidin	Placebo	<i>p</i> -Value
Kilocalories	2163.60 (519.02)	2100.18 (515.77)	0.708	1974.09 (377.97)	2133.51 (437.98)	0.237
Carbohydrates (g)	245.72 (73.46)	221.93 (69.68)	0.312	216.58 (63.47)	248.26 (58.15)	0.117
Protein (g)	113.50 (25.21)	115.20 (25.37)	0.837	108.97 (23.05)	101.52 (23.67)	0.332
Lipids (g)	80.75 (27.24)	83.52 (23.65)	0.739	71.48 (17.61)	71.59 (18.89)	0.985

Values are presented as mean (SD). The mean values correspond to the average of the data collected in the 3-day recall diet questionnaire on pre-intervention (visit 2) and post-intervention (visit 3) days. * Significant differences for $p \leq 0.05$.

2.4 Testing

2.4.1 Medical exam. The medical exam included a medical history, resting electrocardiogram, and medical examination (auscultation, blood pressure, etc.) to confirm that the participants were healthy before they were enrolled in the study.

2.4.2 Dual-energy X-ray absorptiometry (DXA). Before (V2) and after (V3) intervention period, body weight and body composition (percentage of body fat (BF%), body fat (BF), lower limb fat mass (LLFM) and lean mass (LM)) were assessed in the morning with cyclists in a fasted state and wearing light clothes. Body composition was evaluated using a whole body DXA-scan (XR-46; Norland Corp., Fort Atkinson, WI). Discrimination of BF and lean body mass (LBM) was done with a computerised analysis of DXA-scan (Software Illuminatus DXA 4.4.0, Visual MED, Inc., Charlotte, NC and Norland CooperSurgical Company, Minneapolis, MN).

2.4.3 Anthropometry. The same researcher (ISAK Level-1 certified) performed the anthropometric measurements for both pre- and post-test assessments. Height and body weight were measured using a digital scale with a stadiometer for clinical use (SECA 780; Vogel & Halke GmbH & Co. Hamburg, Germany). Skinfold thickness was measured using Holtain Skinfold Calipers (Holtain, Ltd Crymych Pembrokeshire, UK), in accordance with the International Society for the Advancement of Kinanthropometry guidelines. Percentage of body fat was determined using the Faulkner equation,²⁶ while percentage of muscle mass was calculated using the modified Matiegka equation.²⁷ The sum of the eight skinfolds (triceps, subscapular, bicep, crestailiac, supraspinal, abdominal, thigh and calf) was also calculated.

2.4.4 Resting metabolic rate (RMR). Participants visited the laboratory in a fasted state for V2 and V3. Subjects were requested to abstain from caffeine or alcohol consumption for 24 h prior to the measurement. Diet was controlled 24 h before the visits with using the 24-hour diet recall conducted prior to the resting metabolic rate measurement at pre-intervention. Participants were asked to consume the same diet at post-intervention.

Resting metabolic rate (RMR) was measured by indirect calorimetry with metabolic cart (Metalyzer 3B; Cortex-medical, Leipzig, Germany). Tests were performed between 9 am and 11 am, and always at the same time in both pre- and post-intervention sessions. The room was dimly lit and quiet, and the

ambient temperature was at ~ 25 °C. Participants laid in a supine position wearing light clothing for 15 min. Data were then collected for 20 min, and only the middle 10 min were used to calculate the substrate-based utilisation of energy (carbohydrates and lipids).²⁸ The system was calibrated before each measurement following manufacturer recommendations.

2.4.5 Blood samples. Blood was collected into one 3.0 mL ethylenediaminetetraacetic acid (EDTA) tube for haemogram and another 3.5 mL polyethene terephthalate (PET) tube for overall health analysis. Red blood cell count was carried out in an automated Cell-Dyn 3700 analyser (Abbott Diagnostics, Chicago, IL, USA) using internal (Cell-Dyn 22) and external (Program of Excellence for Medical Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, haematocrit and haematimetric indexes were estimated. The blood extraction was performed by a nurse expert in the antecubital vein.

2.4.6 Hesperidin metabolites in urine. Urine samples, corresponding to the collection of urine 24 h before (V2) and after (V3) the supplementation in both groups for each participant, were frozen in liquid nitrogen after collection and thawed for its analysis. Fifty μ L of collected urine was mixed with 100 μ L of water with 1% formic acid containing the internal standard (rac-Hesperetin-d3). Then, the mixture was injected into LC-MS/MS (UHPLC 1290 Infinity II Series coupled to a QqQ/MS 6490 SeriesAgilent Technologies, Sta. Clara, CA, USA). The method was validated using a pool of samples by determining the limit of detection (MDL) and quantification (MQL), repeatability (expressed as relative standard deviation RSD), and accuracy (%). Metabolites were quantified by external standard calibration using rac-Hesperetin-d³ as the internal standard.

2.5 Statistical analyses

Statistical analysis was carried out using IBM Social Sciences software (SPSS, v.21.0, Chicago, IL, USA). Data are presented as mean and SD. Levene and Shapiro-Wilks tests were performed in order to check the homogeneity and normality of the data, respectively. Depending on the normality and homogeneity outcomes obtained, a Paired sample *T*-test or Wilcoxon signed-rank test were applied for analysing within-group pre-post differences. Likewise, a between-group comparison was calculated using an ANCOVA test with pre-test values as covariates (to eliminate the possible bias caused by the initial level of

each group in the different dependent variables) for all body composition and RMR variables. The significance level was determined at $p \leq 0.05$. Cohen's d effect sizes (ES) (95% confidence interval) were calculated for all comparisons. Threshold values for ES statistics were as follows: >0.2 small, >0.5 moderate, >0.8 large.²⁹ Relationships between levels of excreted hesperidin metabolites in urine and other evaluated parameters were analysed using Spearman correlation analysis (r). Significant differences were considered for $p \leq 0.05$.

3. Results

3.1 DXA

The results of body composition assessed by DXA are presented in Fig. 1. Within-group analysis showed a significant pre-post decrease in BF (-17.9% ; $p < 0.001$; ES = 0.50), BF% (-15.3% ; $p < 0.001$; ES = 0.49) and LLFM (-15.5% ; $p < 0.001$; ES = 0.56) in the 2S-hesperidin group, while there were no differences found in placebo ($p = 0.340$; ES = 0.14, $p = 0.441$; ES = 0.11 and $p = 0.469$; ES = 0.10, respectively). Furthermore, between-group comparisons revealed a significant decrease in %BF (-12.3% ; $p = 0.035$; ES = 0.67) and LLFM (-12.6% ; $p = 0.029$; ES = 0.66) in favour of 2S-hesperidin. Also, there was a trend with a moderate effect for a decrease in BF (-13.3% ; $p = 0.055$; ES = 0.61) in 2S-hesperidin compared to placebo.

Regarding LM, there were no pre-post significance in both placebo group (-1.1% ; $p = 0.082$; ES = 0.13) and in 2S-hesperidin group (0.4% ; $p = 0.461$; ES = 0.04). In addition, no significant differences with a moderate effect were observed between groups (1.5% ; $p = 0.115$; ES = 0.60). However, in the LM% a significant decrease (-1.8% ; $p = 0.009$; ES = 0.34) was found in placebo, without significant change (-0.3% ; $p = 0.649$; ES = 0.06) in 2S-hesperidin. No significant change with small effect was found when comparing groups (-1.5% ; $p = 0.116$; ES = 0.49).

3.2 Anthropometry

In anthropometry (Fig. 2), similar results to DXA were found. A significant pre-post decrease in the total body fat (TBF) (-3.1% ; $p = 0.047$; ES = 0.12), %BF (-3.9% ; $p = 0.006$; ES = 0.19) and \sum of 8 skinfolds (-6.5% ; $p = 0.008$; ES = 0.18) was observed in 2S-hesperidin, but no significant change in placebo ($p = 0.995$; ES = 0.01, $p = 0.775$; ES = 0.03 and $p = 0.721$; ES = 0.03, respectively). There were no significant differences with a small effect in TBF, %BF and \sum of 8 skinfolds between groups ($p = 0.221$, ES = 0.40, $p = 0.129$, ES = 0.48 and $p = 0.169$, ES = 0.43, respectively) in favour of 2S-hesperidin.

Regarding muscle mass (Fig. 2), a significant pre-post increase in total muscle mass (TMM) (1.8% ; $p = 0.011$; ES = 0.19) and percentage of muscle mass (%MM) (0.9% ; $p < 0.001$; ES = 0.26) was reported after ingestion of 2S-hesperidin, without any significant change in placebo ($p = 0.296$; ES = 0.07 and $p = 0.470$; ES = 0.07, respectively). Although there were no significant differences between groups, a trend with a

moderate effect ($p = 0.070$ and ES = 0.60) favouring an increase in %MM was observed in the 2S-hesperidin group.

3.3 Resting metabolic rate

Significant pre-post increase in CHO (Table 3) (32.4% ; $p = 0.001$; ES = 0.77 vs. 26.0% ; $p = 0.004$; ES = 0.71, respectively) and decrease in FAT (-29.5% ; $p = 0.005$; ES = 0.68 vs. -28.0% ; $p = 0.018$; ES = 0.73, respectively) were observed in 2S-hesperidin and placebo. Furthermore, a significant increase in RER (4.7% ; $p = 0.012$; ES = 0.72) after supplementation was found only in placebo group. There were no significant differences between groups.

3.4 Hesperidin metabolites in urine

Different 2S-hesperidin metabolites, mainly hesperetin glucuronides and sulfates, were analysed in the urine of the participants after 2S-hesperidin intake. The main metabolite detected was hesperetin-3-glucuronide, representing $78.9 \pm 5.0\%$ ($n = 20$) of the total, while hesperetin-7-glucuronide and hesperetin-7-sulfate made up $6.9 \pm 2.9\%$ ($n = 20$) and $14.7 \pm 4.1\%$ ($n = 20$) of the excreted metabolites. Despite the similarities in the excreted metabolites profile, a large interindividual variability was observed in the amount of excreted hesperidin metabolites, ranging from 2.3 to $37.5 \mu\text{mol}$. These differences between subjects indicate differences in the absorption and excretion of hesperidin, which have been previously reported.³⁰

4. Discussion

This randomised, placebo-controlled trial investigated the effect of eight weeks of 2S-hesperidin supplementation on body composition (DXA and anthropometry) in forty AMA. Main results are that the chronic intake of 2S-hesperidin caused a significant decrease in BF and a significant increase in muscle mass compared to placebo. However, similar significant changes were observed in fats and carbohydrates oxidation during RMR in both groups.

The effects of 2S-hesperidin may be heavily influenced by its bioavailability. In plasma, a maximum concentration is reached 5 to 7 hours after ingestion, with a total elimination at 24 hours.³¹ In contrast, metabolites in urine reach their maximum peak at 24 hours after ingestion, being almost completely eliminated after 48 hours.³¹ Additionally, high doses of hesperidin (1 L of orange juice; $9.28 \mu\text{mol h L}^{-1}$) led to higher area under the curve than low doses (0.5 L of orange juice; $4.19 \mu\text{mol h L}^{-1}$), and therefore, greater exposure of the organism to its metabolites.³¹

4.1 Body composition

Regarding DXA outcomes, this study showed improvements in body composition after 2S-hesperidin intake. Specifically, a significant decrease in the percentage of BF (-15.3%), total BF (-17.9%) and LLFM (-15.5%) were reported for the 2S-hesperidin group, while there were no changes in placebo. The power

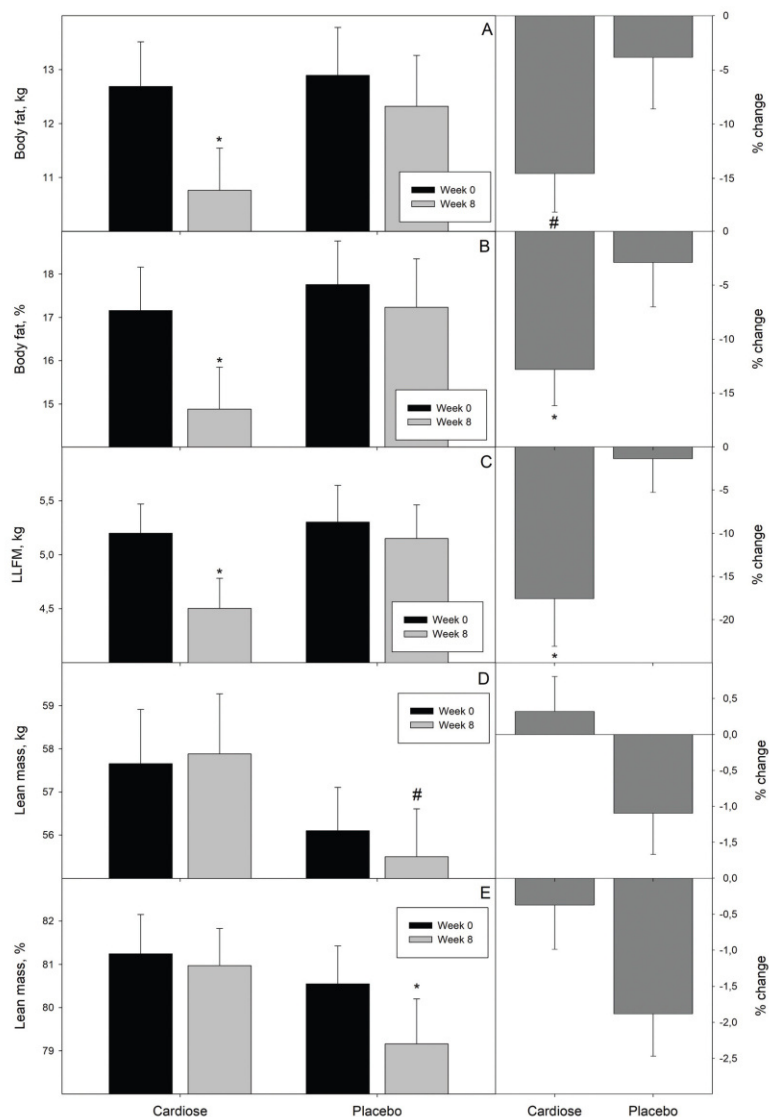


Fig. 1 Changes in body composition (A – body fat mass, B – body fat mass %, C – lower limbs fat mass, D – lean mass and E – lean mass%) after the intervention, evaluated by DXA, of amateur competitive cyclists (cardiose, $N = 20$; Placebo, $N = 20$). * means significant difference ($p < 0.05$). # trend ($p = 0.05-0.10$).

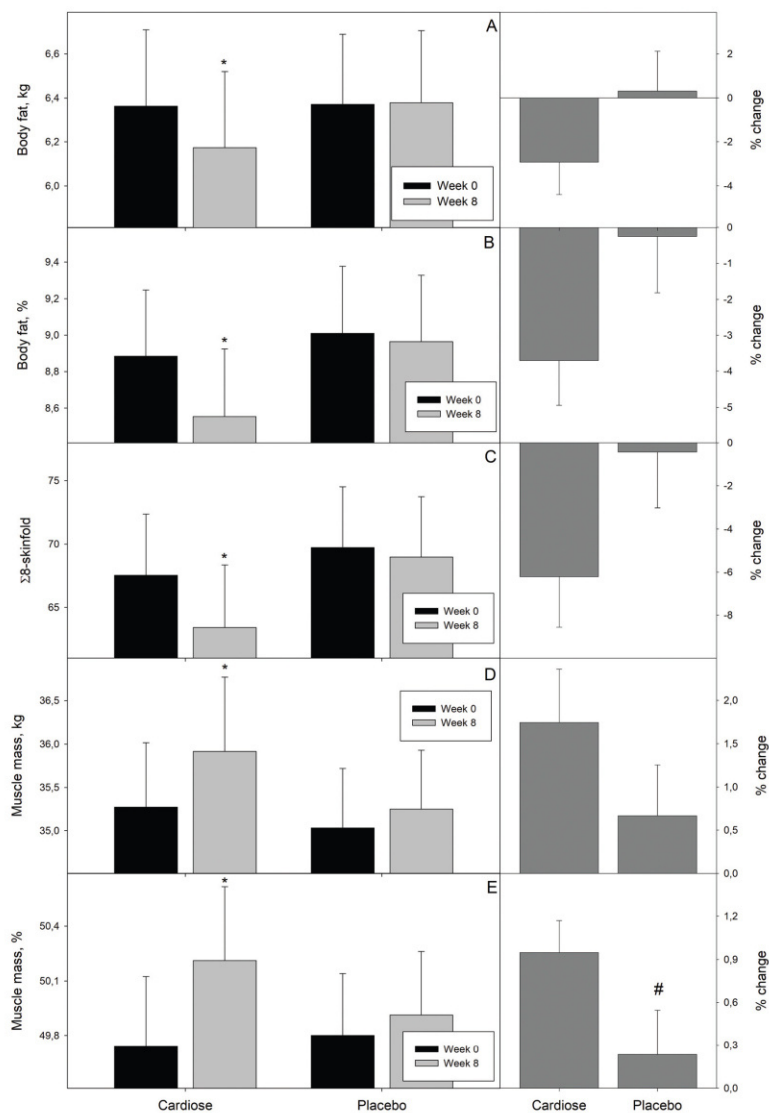


Table 3 Changes metabolic and substrate variables in resting metabolic rate (RMR) of male competitive cyclists ($N = 40$)

	2S-Hesperidin			Placebo		
	Pre-intervention	Post-intervention	<i>p</i> -Value	Pre-intervention	Post-intervention	<i>p</i> -Value
VO ₂ (mL min ⁻¹)	0.27 (0.02)	0.27 (0.03)	0.912	0.27 (0.03)	0.27 (0.04)	0.813
CO ₂ (L min ⁻¹)	0.23 (0.02)	0.24 (0.03)	0.471	0.23 (0.03)	0.24 (0.04)	0.169
VO ₂ R (ml kg ⁻¹ min ⁻¹)	3.79 (0.35)	3.78 (0.66)	0.904	3.88 (0.46)	3.82 (0.52)	0.659
RER	0.85 (0.07)	0.88 (0.05)	0.110	0.85 (0.05)	0.89 (0.04)	0.012
MET	1.08 (0.11)	1.08 (0.19)	0.966	1.11 (0.13)	1.09 (0.15)	0.600
CHO (g min ⁻¹)	8.48 (3.42)	11.23 (3.43)	0.001	9.12 (3.18)	11.49 (3.10)	0.004
FAT (g min ⁻¹)	3.71 (1.54)	2.62 (1.15)	0.005	3.64 (1.33)	2.62 (1.14)	0.018
Kcal day ⁻¹	1655.20 (181.78)	1654.37 (208.25)	0.988	1697.31 (155.27)	1678.98 (227.60)	0.757

Values are presented as mean (SD). VO₂ = volume of oxygen uptake; VCO₂ = volume of dioxide of carbon uptake; VO₂R = body mass oxygen consumption; RER = resting metabolic rate; MET = metabolic equivalent; CHO = carbohydrate oxidation; and FAT = fat oxidation.

of the data obtained with the DXA has a high reliability, as it is considered a precise and accurate method for measuring body composition.³²

To the best of our knowledge, there are no previous studies that have reported the effect of hesperidin intake on fat mass. In line with described results, Dallas *et al.*³³ showed improvements in body fat mass percentage (experimental -9.7% vs. placebo -3.2% ; significant group differences $p < 0.001$) after a 12-week intake of polyphenolic rich fruit extract (at least 20% of total flavanones) in combination with 30 min per week of physical activity in a healthy overweight population.

Similar results to those obtained in the DXA were found in anthropometric measurements, where a significant decrease in %BF (-3.9%), TFB (-3.1%) and Σ of 8 skinfolds (-6.5%) after ingestion of 2S-hesperidin was observed. In addition, in this group, inverse positive correlations were found between the total excretion of 2S-hesperidin metabolites in urine and fat mass percentage ($r = -0.592$; $p = 0.006$) and Σ of 8 skinfolds ($r = -0.550$; $p = 0.012$).

Both DXA and anthropometry fat component findings were similar to each other. The decrease in fat mass after the intake of 2S-hesperidin could be explained by an increase in the activation of SIRT1 and PGC-1 that would lead to over-expression of genes related to mitochondrial respiration and fatty acid oxidation at the muscular level.³⁴ The decrease in fat mass in the experimental group may also be due to higher fat oxidation in FatMax and VT1 after intake of 2S-hesperidin in AMA, whereas the placebo group significantly decreased its fatty acid oxidation.¹³ These findings suggest 2S-hesperidin as a useful tool for reducing fat mass in cyclists. Taking into account described results as well as that no dietary control was included in this study, the potential of 2S-hesperidin combined with a proper dietary control may be even greater.

Legaz *et al.*³⁵ investigated the relationship between changes in body composition and sports performance, observing that changes in the Σ of 6 skinfolds ($r = -0.660$, $p < 0.001$) and the ratio of extremity to trunk skinfolds (triceps, front thigh, medial cal/subscapular, iliac crest, abdominal) ($r = -0.600$, $p = 0.020$), were related to changes in running performance in top-class runners after three years follow-up. This suggests that a

decrease in fat mass is related to an enhancement in performance.

A recent publication by Martínez-Noguera *et al.*¹³ corresponding to the performance evaluation of this intervention study, reported a positive effect on performance after the ingestion of 2S-hesperidin (8 weeks) in amateur cyclists. This group increased the power output (PO) ($2.3\% = 6.40$ W; $p = 0.049$) at estimated functional threshold power (eFTP) and maximum power output (MPO) ($1.9\% = 7.40$ W; $p = 0.049$) in an incremental test. Participants also showed a decrease in FAT oxidation in FatMax (-65.6%) and VT1 (-65.7%) group during the rectangular test in the placebo group but no significant changes in the 2S-hesperidin.¹³ These results indicate a decrease in the capacity of fatty acid oxidation in placebo, which leads to lower utilization of fats at low to moderate intensities. Since it is known that a high percentage of the training volume of cyclists is performed in low and moderate-intensity, a decrease in the capacity of fat oxidation could lead to changes in body fat mass long term (weeks or months). Therefore, the changes found by Martínez-Noguera *et al.*¹³ would justify the reduction of fat mass after 2S-hesperidin supplementation described in this study.

Concerning the muscle component, in DXA, the placebo group experienced a significant decrease in LM% (-1.4% ; -0.597 kg), while no changes were found in 2S-hesperidin (0.3% ; 0.224 kg). No-significant changes were found when comparing between groups, but a moderate effect in LM (-1.5% ; -0.822 kg) in favor of placebo was observed. In anthropometry, a significant increase in %MM (0.9%) and TMM (1.8%) was reported in the 2S-hesperidin group after the intervention period. A positive relationship was also found between urinary excretion of 2S-hesperidin metabolites and % MM ($r = 0.487$; $p = 0.029$), supporting the positive effect of 2S-hesperidin on muscle mass. Bieseman *et al.* found that hesperetin (main 2S-hesperidin metabolite) supplementation (50 mg kg⁻¹ d⁻¹), in addition to reducing oxidative stress in myotubes, completely reversed the age-related decline in muscle mass in mice and, thus, may have played a role in improving running performance.¹⁹ Regarding the relationship between hesperidin intake and muscle mass, Jeon *et al.*³⁶ observed pro-myogenic

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acid oxidation during exercise (low and moderate intensity) in cyclists with 2S-hesperidin supplementation of 8 weeks.¹³ Few studies have evaluated the effect of flavonoids, in our case of 2S-hesperidin, on the RMR of athletes or healthy people. Therefore, further research is needed to establish whether 2S-hesperidin may affect RER or RMR.

4.3 Limitations

The current study was conducted between September and December (in off-season). In this phase of the season, cyclists work at a lower training intensity and volume relative to other phases of the season. This is an important fact to be considered when comparing described results with other studies carried out during training seasons with different volumes and intensities. The selection of study dates was made based on the fact that it is usually more complicated to modify the habits of the cyclists and to control their evolution during the season, due to their own and individual periodization.

Another limitation is the sample size enrolled in this study. Despite the relatively low number of participants ($n = 40$), they are representative of the AMA population.

Future lines of research should describe precisely the molecular mechanisms and changes in gene expression (nutrigenomic effect) involved in 2S-hesperidin action for the promotion of muscle tissue and energy metabolism. It would be also interesting to investigate how modulation of intestinal microbiome induced by 2S-hesperidin may be related to the described significant effects on body composition.

5. Conclusions

The chronic intake of 500 mg of 2S-hesperidin improves body composition, decreases fat mass and increases muscle mass in AMA without modifying training and diet. These changes in body composition may provide an advantage to endurance athletes in maintaining or improving their physical condition during pre-season and start the season with less fat and more muscle. Besides, 2S-hesperidin appears to prevent an increase in the RER in rest metabolic rate, post intervention. Therefore, 2S-hesperidin is postulated to be a new ergogenic aid oriented to control fat and muscle mass in endurance athletes.

Author contributions

Conceptualization, F.J.M.N., C.M.-P. and P.E.A.; methodology, F.J.M.N., C.M.P. and P.E.A.; formal analysis, F.J.M.N., C.M.P. and J.C.V.; investigation, F.J.M.N., C.M.P., L.C., E.M.C. and J.C.V.; resources, F.J.M.N., C.M.P. and J.C.V.; data curation, F.J.M.N., C.M.P., J.C.V., L.C. and E.M.C.; writing—original draft preparation, F.J.M.N.; writing—review and editing, F.J.M.N., C.M.P., L.C. and J.C.V.; visualization, C.M.P.; supervision, C.M.P. and P.E.A.; project administration, C.M.P. and P.E.A.; funding acquisition, P.E.A. All authors read and approved the final manuscript.

Funding

The authors declare that this study has been financed by HTBA (Murcia, Spain), who kindly provided the product Cardiose®. However, they did not participate in the experimental design, data collection, data analysis, interpretation of the data, writing of the manuscript, or in the decision to publish the results.

Conflicts of interest

No conflict of interest.

Acknowledgements

This study was supported by the Research Center in High-Performance Sport of the Catholic University of Murcia and HTBA (Murcia, Spain).

We also thank Iris Samarra, Antoni del Pino and Núria Canela from the Metabolomics facility of the Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat for their contribution to the urine analysis.

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The American Journal of Clinical Nutrition
8-Week Supplementation of 2S-Hesperidin Improves Acid-base Status and Decreases Lactate at Low-moderate and Submaximal Intensities, Enhancing Recovery After an Incremental Test in Amateur Cyclists.
 --Manuscript Draft--

Manuscript Number:	AJCN-D-21-01338
Full Title:	8-Week Supplementation of 2S-Hesperidin Improves Acid-base Status and Decreases Lactate at Low-moderate and Submaximal Intensities, Enhancing Recovery After an Incremental Test in Amateur Cyclists.
Short Title:	Effects of 2S-Hesperidin on acid-base
Article Type:	Original Research Communications
Section/Category:	Dietary supplements
Keywords:	Flavonoids; endurance; exercise; performance; polyphenols and sport nutrition
Manuscript Classifications:	1.10: Dietary supplements; 1.30: Nutritional support; 1.36: Osteoporosis; 2.03: Body composition; 2.04: Bone health; 2.05: Carbohydrate metabolism; 2.08: Energy metabolism; 2.09: Exercise; 2.11: Gene/nutrient interaction; 2.12: Genetics: polymorphisms; 2.21: Nutritional assessment; 2.22: Phytochemicals; 2.23: Protein metabolism/amino acids; 4.07: Randomized controlled trials
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Abstract:	<p>Background</p> <p>Chronic supplementation with 2S-hesperidin has been shown to improve performance. However, to date, the mechanisms that have made this effect possible have not been explored.</p> <p>Objective</p> <p>Therefore, this study's aim was to assess whether changes in gasometry or acid-base status may be associated with improved performance after the intake of 2S-hesperidin.</p> <p>Methods</p> <p>40 amateur cyclists underwent a rectangular test in which capillary blood samples were taken at baseline, FatMax1, VT1, VT2, P MAX , FatMax2 and EPOC to measure gasometry and acid-base parameters.</p>

	<p>Results</p> <p>Increased CO₂ metabolism was found in FatMax1, VT1, FatMax2 and EPOC, and decreased Lac in FatMax1, VT1, VT2, FatMax2 and EPOC after 8 weeks of 2S-hesperidin ingestion. In addition, acid-base status was improved in the rectangular test and prevented a decrease in pO₂ in VT2, after 2S-hesperidin supplementation in amateur cyclists.</p> <p>Conclusions</p> <p>Chronic supplementation with 2S-hesperidin improved acid-base status and Lac at both low-moderate and submaximal intensities, improving recovery after exercise to exhaustion in amateur cyclists.</p>
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Opposed Reviewers:	
Additional Information:	
Question	Response
Number of words:	8700
Has this manuscript been posted to a preprint server?	No
REGISTRATION OF CLINICAL TRIALS	<p>Trial registration number:NCT04597983</p> <p>URL of registrationhttps://clinicaltrials.gov/ct2/show/NCT04597983</p>
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Cover Letter

Catholic University of Murcia

Campus de los Jerónimos, Guadalupe (Murcia)
30107 Spain
September/2021

Dear Editor-In-Chief,

We report the following document whose title is **“8-Week Supplementation of 2S-Hesperidin Improves Acid-base Status and Decreases Lactate at Low-moderate and Submaximal Intensities, Enhancing Recovery After an Incremental Test in Amateur Cyclists”** to be published as manuscript in *“The American Journal of Clinical Nutrition”*.

This research aimed to evaluate the 8-weeks 2S-hesperidin intake on gasometry and acid-base status at pre, during and post rectangular test in amateur cyclists. This is the first human study to demonstrate the capacity of 2S-hesperidin to lose fat and maintain muscle mass in amateur cyclists.

The novelty of this study is that it is the first human clinical trial to demonstrate that chronic intake of 2S-hesperidin improves acid-base status and decreases lactate at both low-moderate and submaximal intensities, enhancing recovery after maximal exercise.

The authors would like to express that:

1. This manuscript contains original material.
2. The paper is not under consideration elsewhere.
3. None of the paper's contents has been previously published.
4. All authors have read and approved the manuscript.
5. The results of the current study do not constitute endorsement of the product by the authors or the journal
6. The study and all its procedures were approved by the Human Subjects Ethics Committee of the Catholic University of Murcia, Spain, in accordance with the Helsinki Declaration.
7. Subjects were fully informed about all testing procedures and training protocols and written informed consent was obtained from each one.

8. English language was reviewed by a native person.

Best regards,

Francisco Javier Martínez-Noguera

1 **8-Week Supplementation of 2S-Hesperidin Improves Acid-**
2 **base Status and Decreases Lactate at Low-moderate and**
3 **Submaximal Intensities, Enhancing Recovery After an**
4 **Incremental Test in Amateur Cyclists.**

5

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12

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18 The author reported no funding received for this study

19

20 The authors declare no conflict of interest.

21

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24

25 The data described in the manuscript, codebook and analytical code will be made
26 publicly available upon request to the author for correspondence [e.g., application and
27 approval, payment, etc.] for review of the manuscript by the reviewers.

28

29 **Running title:** Effects of 2S-Hesperidin on acid-base

30

31 **Abbreviations:**

32 AaDpO₂ - Alveolar–arterial gradient

33 ABE - Actual base excess

34 AUC - Area under the curve

35 CO₂ - Carbon dioxide production

36 COHb - Carboxyhemoglobin

37 EPOC - Excess post-exercise oxygen consumption

38 FatMax - Intensity at which maximum fat oxidation

39 Glu - Glucose

- 40 Hb - Hemoglobin
- 41 HCO_3^- - Bicarbonate anion
- 42 Hct - Hematocrit
- 43 Lac - Lactate
- 44 MetHb - Methemoglobin
- 45 O_2 - Oxygen uptake
- 46 ODC - Oxygen dissociation curve
- 47 O_2Hb - Oxyhemoglobin
- 48 p_{50} - Oxygen partial pressure at 50% oxygen saturation
- 49 $p\text{CO}_2$ - Carbon dioxide partial pressure
- 50 P_{MAX} - Maximum power output
- 51 $p\text{O}_2$ - Oxygen partial pressure
- 52 RHb - deoxyhemoglobin
- 53 SBC - Standard bicarbonate
- 54 SBE - Standard base excess
- 55 Shunt - Relative physiological Shunt
- 56 $s\text{O}_2$ - Oxygen saturation
- 57 $t\text{CO}_2$ - Total blood carbon dioxide concentration
- 58 $t\text{O}_2$ - Total blood oxygen concentration
- 59 $\text{VO}_{2\text{MAX}}$ - Maximum O_2 uptake
- 60 VT1 - Ventilatory threshold 1
- 61 VT2 - Ventilatory threshold 2
- 62
- 63

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65

66 **Abstract**

67 Background: Chronic supplementation with 2S-hesperidin has been shown to improve
68 performance. However, to date, the mechanisms that have made this effect possible have
69 not been explored.

70 Objective: Therefore, this study's aim was to assess whether changes in gasometry or
71 acid-base status may be associated with improved performance after the intake of 2S-
72 hesperidin.

73 Methods: 40 amateur cyclists underwent a rectangular test in which capillary blood
74 samples were taken at baseline, FatMax1, VT1, VT2, P_{MAX}, FatMax2 and EPOC to
75 measure gasometry and acid-base parameters.

76 Results: Increased CO₂ metabolism was found in FatMax1, VT1, FatMax2 and EPOC,
77 and decreased Lac in FatMax1, VT1, VT2, FatMax2 and EPOC after 8 weeks of 2S-
78 hesperidin ingestion. In addition, acid-base status was improved in the rectangular test
79 and prevented a decrease in pO₂ in VT2, after 2S-hesperidin supplementation in amateur
80 cyclists.

81 Conclusions: Chronic supplementation with 2S-hesperidin improved acid-base status and
82 Lac at both low-moderate and submaximal intensities, improving recovery after exercise
83 to exhaustion in amateur cyclists.

84

85 **Registered in ClinicalTrials.gov (Identifier: NCT04597983)**

86

87 **Keywords:** Flavonoids, endurance, exercise, performance, polyphenols and sport
88 nutrition.

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119 **1. INTRODUCTION**

120

121 There are several factors, mainly training, nutrition and ergogenic aids, that affect
122 endurance performance (1). Endurance training produces a number of adaptations at the
123 cellular and systemic level with the aim of reducing the breakdown of the whole-body
124 homeostasis caused by exercise (2, 3). The type of training, training status, as well as the
125 diet composition affect the exercise response and adaptations in a positive or negative
126 way (4, 5). In addition, the intake of ergogenic aids has shown to modulate the adaptations
127 generated via resistance training, like reactive oxygen species (ROS) signaling, acid-base
128 balance, the redox state, training load, etc (4). These physiological mechanisms allow the
129 body to adapt to training and improve the athlete's performance.

130

131 The major goal for endurance athletes is to increase the ability to maintain the highest
132 speed or average power output for a given distance or time (speed/power output). This
133 depends on the speed and efficiency with which chemical energy can be converted into
134 mechanical energy within the skeletal muscle (6). Specifically, high-level endurance
135 athletes want to obtain the highest rate of aerobic metabolism that is sustainable over the
136 duration of an event, the upper limit of which is set by an athlete's maximum O₂ uptake
137 (VO_{2MAX}) (7). However, despite having the ability to maintain high values of VO_{2MAX} for
138 10-15 minutes during all-out efforts, the circulatory system is unable to sustain a linear
139 increase in the delivery of O₂ to the locomotor muscles, where the production of aerobic
140 energy becomes restricted and, consequently, accelerates anaerobic metabolism (8).

141

142 VO_{2MAX} is limited by the ability of the cardiorespiratory system to transport O₂ to the
143 muscles. The physiological factors that could limit VO_{2MAX} are: 1) the pulmonary
144 diffusing capacity, 2) maximal cardiac output, 3) oxygen carrying capacity of the blood,
145 and 4) skeletal muscle characteristics. The first three factors are classified as central
146 factors and the fourth is categorized as a peripheral factor (9). The limitation at the lung
147 level in highly trained athletes can be overcome with O₂-enriched air, as Powers et al.
148 (10) observed when they compared incremental tests (VO_{2MAX}) in normal air and O₂-

149 enriched air (26%) room conditions in highly-trained and normal subjects. They observed
150 that there was an increase in $\text{VO}_{2\text{MAX}}$ from 70.1 to 74.7 $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ and an increase in
151 arterial O_2 saturation (SaO_2) from 90.6% to 95.9% during the maximum work in the O_2 -
152 enriched air condition in highly-trained athletes, without significant changes in the normal
153 subjects.

154

155 Another limiting factor regarding performance and $\text{VO}_{2\text{MAX}}$ of endurance athletes is the
156 maximal cardiac output, since longitudinal studies have observed that training induces an
157 increase in $\text{VO}_{2\text{MAX}}$ because of an increase in maximal cardiac output (11, 12). Magnetic
158 resonance cross-sectional images showed an enhanced left ventricle (LV) mass (200 vs.
159 148 g) and volume (167 vs. 125 ml) in the endurance-trained group compared to matched
160 non-athletic controls (13).

161

162 The oxygen-carrying capacity is another factor that can limit the endurance athletes'
163 performance and $\text{VO}_{2\text{MAX}}$ (9). It's been observed that high concentration and above
164 normal total mass of hemoglobin (Hb), an oxygen transport molecule that supports
165 aerobic cellular metabolism, improves performance capacity in humans (14). Several
166 authors have observed how reinfusion of 900-1,350 mL of blood increases oxygen
167 transport capacity in the blood, demonstrating that this procedure can increase $\text{VO}_{2\text{MAX}}$
168 by 4-9% (15, 16). In line with previous research, an augmenting red blood cell volume
169 by means of blood transfusions or erythropoietin injections improves exercise
170 performance in healthy humans (17-19). In addition, several studies have found a strong
171 relationship between the number of capillaries by fiber in the vastus lateralis and $\text{VO}_{2\text{MAX}}$
172 ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) measured during cycle ergometry (20). The main significance of the
173 training-induced increase in capillary density in skeletal muscle is to maintain or lengthen
174 the average transit time (21). This improves the oxygen supply by maintaining the oxygen
175 extraction (a-v O_2 difference), even at high muscle blood flow rates (9).

176

177 Furthermore, the position of the O_2 dissociation curve (ODC), conveniently described by
178 P_{50} (PO_2 at which 50% Hb is saturated), has an important role in O_2 transport. Of course,
179 variations in P_{50} have ramifications not only in the periphery but also during O_2 loading
180 in the lungs. A high P_{50} opposes the association of O_2 in the lungs but favors its release to
181 the tissues and vice versa (22).

182

183 Physiologists have done extensive work to examine whether enzyme levels in the
184 mitochondria are a limiting factor for VO_{2MAX} within the muscle fibers, where the
185 mitochondria are the sites where O_2 is consumed in the last step of the electron transport
186 chain (9). In theory, doubling the number of mitochondria should double the number of
187 sites for O_2 uptake in the muscle. However, human studies show there's only a modest
188 increase VO_{2MAX} , despite a 2.2 times increase in mitochondria enzymes (20). Increasing
189 mitochondrial enzymes improves endurance performance rather than increase VO_{2MAX} ,
190 and low-intensity training may elicit small changes in mitochondrial enzymes without
191 any change in VO_{2MAX} , and *vice versa* (23-25). In addition, the increase in muscle
192 mitochondria may allow a slightly greater extraction of O_2 from the blood by the working
193 muscles, thus providing a small contribution in increasing VO_{2MAX} (26).

194 Additionally, skeletal muscle oxidative capacity, once thought to be indicated by
195 percentage of type 1 (oxidative) muscle fibers (27), correlates strongly with exercise
196 efficiency (28), which also accounts for a significant variation in exercise performance
197 among highly trained athletes (29, 30). Two metabolic effects of an increase in
198 mitochondrial enzymes are: 1) muscles adapted to endurance exercise will oxidize fat at
199 a higher rate (thus sparing muscle glycogen and blood glucose) and 2) there is decreased
200 lactate production during exercise (9). All these changes in the body are stimulus-
201 dependent (exercise time and intensity, nutritional status, temperature, etc.), but the intake
202 of ergogenic aids can also modify training adaptations (31).

203 In this sense, endurance athletes are increasing the use of ergogenic aids in the search of
204 improving performance, which include: dietary nitrates (32), β -alanine (33),
205 antioxidants(34), sodium bicarbonate (35), creatine (36) and polyphenols (37). Recently,
206 there is a worldwide research interest in the pleiotropic effect of polyphenols on the
207 immune system, chronic diseases and aging (38-42). The large family of polyphenols is
208 divided into 4 groups: flavonoids (e.g., hesperidin, hesperitin, etc.), phenolic acids,
209 stilbenes and lignans (38).

210

211 Specifically, hesperidin is a flavonoid of the flavanone family and is largely located in
212 high concentrations in citrus fruits (43), such as sweet orange (*Citrus sinensis*) (44). S and
213 R isomers are both found in hesperidin. 2S-hesperidin is predominantly the natural form
214 in citrus (45), whereas processed foods with high hesperidin content undergo a
215 transformation from S to R isomer (46). A 6-week consumption of the metabolite form

216 of hesperidin, hesperitin ($50 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) has shown to improve antioxidant status (GSH,
217 GSSG and GSH/GSSG) and running performance (exercise time) in aged mice (47).
218 Similar results were found with the 5-week intake of 2S-hesperidin (200 mg/kg), where
219 improvements in the test performance (running) until exhaustion (58%) and in the
220 antioxidant system (superoxide dismutase (SOD), glutathione peroxidase (GPx)) in the
221 liver and lymphoid tissue were observed in rats (48). In addition, Martínez-Noguera et al.
222 (2019) showed improvements in average power (2.27%), maximum speed (3.23%) and
223 total energy (Σ 4 sprint test) (2.64%) during a repeated sprint test (4 sprints of 30 sec)
224 following an acute intake of 2S-hesperidin (500 mg) in amateur cyclists (49). Recently,
225 the same authors demonstrated significant performance improvements in estimated
226 functional threshold power (eFTP) (2.33% = 6.40 W) and maximum power (1.93% = 7.40
227 W) during an incremental test after 8-weeks of 2S-hesperidin (500 mg/d) consumption in
228 amateur cyclists (50). They also observed an increase in power at maximum speed (1.08%
229 = 8.05 W) and a decrease in time at peak power (-11.17% = -376.5 ms) in the Wingate
230 test (one sprint of 30 sec) (50).

231

232 No previous articles that have studied the effect of 2S-hesperidin intake on oxygen
233 metabolism and acid-base state, and taking into account the results obtained on the
234 performance after intake with 500 mg of 2S-hesperidin during 8 weeks in amateur cyclists
235 (50), carried out in our laboratory and of which the data presented in this study are part.
236 We hypothesized that the enhancement of nitric oxide (NO) production following
237 prolonged ingestion of 2S-hesperidin may improve peripheral blood flow to muscles, as
238 has been demonstrated in several studies investigating the use of hesperidin and other
239 flavonoids (51-55). This would provide a better supply of oxygen and nutrients at high
240 intensities, which can be decisive in maintaining certain levels of work.

241 The main objective of this intervention study was to perform an exhaustive report of the
242 effects with 500 mg/d of 2S-hesperidin in amateur cyclists on markers related to O_2
243 metabolism and acid-base balance, from capillary blood sample during a rectangular test.
244 Secondly, was to be able to give an explanation to the findings found in our recently
245 published article (50), where was found an improvement in performance, in submaximal
246 and maximal level exercises after 2S-hesperidin intake in amateur cyclists for 8 weeks.

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252 **2. METHODOLOGY**

253

254 **2.1 Participants**

255 Forty healthy male amateur cyclists completed the study (**Table 1**). The inclusion criteria
256 were: 18-55 years, 19-25.5 kg·m⁻² BMI, at least 3-years cycling experience and 6-12 h·wk⁻¹
257 ¹ of training. Exclusion criteria were: a) smokers or regular alcohol drinkers, b) metabolic,
258 cardiorespiratory or digestive pathologies or anomalies, c) injury in the last 6 months, d)
259 intake any type of supplementation or drug in the last 2 weeks and) no normal values in
260 some parameter of the previous blood analysis. Prior to starting the study, participants
261 were informed about the procedures and signed the informed consent. The study was
262 conducted according to the guidelines of the Helsinki Declaration for Human Research
263 (56) and was approved by the Ethics Committee of the Catholic University of Murcia
264 (CE091802), registered in ClinicalTrials.gov (Identifier: NCT04597983). All participants
265 completed the study.

266

267 ****Insert table 1****268 **2.2 Study design**

269 This research was part of a larger, previously published study investigating the effect of
270 chronic hesperidin intake on the performance (50) and in body composition (57) in
271 amateur cyclists. To carry out this study, a double-blind (blinding carried out by the
272 company supplying the capsules), parallel and randomized experimental design was
273 conducted. Randomization was performed using computer software (Randomizer) to
274 assign codes to the groups established in this study (58). Participants were divided in two
275 groups: experimental (2S-hesperidin; n=20) and control (Placebo; n=20) groups. In
276 accordance to their group assignment, participants either took two capsules of 2S-
277 hesperidin (500 mg of 2S-hesperidin; HealthTech BioActives, Murcia, Spain) or placebo
278 (500 mg of microcellulose) for 8 weeks. The cyclists continued their normal training
279 schedule during the length of the study. Both groups had similar general and training
280 characteristics at the start of the study (**Table 1**).

281

282 **2.3 Procedures**

283 The experimental protocol required 7 visits to the laboratory Day 1 consisted of a medical
284 examination and health status blood analysis. Days 2 and 4 entailed a reminder 24-h diet
285 recall questionnaire and incremental test until exhaustion on a cycle ergometer. Days 3
286 and 5 involved of a 24-h diet recall questionnaire and rectangular test on a cycle ergometer
287 (**Figure 1** and **Table 2**). Participants ingested a standardized breakfast, composed of
288 95.16 gr carbohydrates (68%), 18.86 gr protein (14%) and 11.30 gr lipids (18%), which
289 was prescribed by a sports nutritionist, and was consumed 2.5 h before each testing
290 session (visits 2, 3, 4 and 5).

291

292 ****Insert figure 1****

293

294

295 ****Insert table 2****296 **2.3 Testing**

297

298 **2.3.1 Medical exam**

299 The medical exam included a medical history, resting electrocardiogram and medical
300 examination (auscultation, blood pressure, etc.), to confirm that the cyclist was healthy to
301 participate in the study.

302 **2.3.2 Maximal test**

303 An incremental step with final ramp test with a metabolic cart (Metalyzer 3B, Leipzig,
304 Germany) was performed to determine maximal zone of fat-burning (FatMax),
305 ventilatory thresholds 1 (VT1) and 2 (VT2), maximum power output (P_{MAX}) and VO_{2MAX} .
306 The test started at 35W and was increased by 35W every 2 min, followed by a final ramp
307 ($+35W \cdot min^{-1}$), which started when the RER was higher than 1.05, and continued until
308 exhaustion. To guarantee VO_{2MAX} value, the following criteria were checked: plateau in
309 the final VO_2 values (increase $\leq 2.0 ml \cdot kg^{-1} \cdot min^{-1}$ in the 2 last loads), maximal theoretical
310 HR $(220 - age) \cdot 0.95$ for a cycling test (59), $RER \geq 1.15$ and lactate $\geq 8.0 mmol \cdot l^{-1}$ (60,

311 61). Ventilatory thresholds were obtained using the ventilatory equivalents method
312 described by Wasserman (62).

313

314 **2.3.3 Rectangular test**

315 Prior to each exercise test, a resting oxygen measure was obtained by having participants
316 in the supine position for 20 min while breathing room air into a metabolic analysis
317 system, and only the middle 10 minutes (steady state) were used for the calculation of
318 oxygen consumption at rest (63). The rectangular test was performed on a cycle ergometer
319 using the power output values obtained from the maximal test (FatMax, VT1, VT2 and
320 P_{MAX}). The test consisted of 10-min at FatMax1 (intensity at which maximum fat
321 oxidation is given), 10-min at VT1 and 10-min at VT2, until exhaustion at P_{MAX} and 15-
322 min at FatMax2. As soon as the exercise was over, the subjects immediately laid down
323 on a bed so that excess post-exercise oxygen consumption (EPOC) could be measured for
324 30 min. Cardiorespiratory variables (VO₂, VO_{2R}, carbohydrate oxidation (CHO), fat
325 oxidation (FAT) and cycling economy) were determined for the different metabolic
326 zones. These metabolic data have been published separately (50).

327

328 **2.3.5 Blood samples**

329 Blood extraction was conducted by a certified nurse, where one 3-mL tube
330 ethylenediaminetetraacetic acid (EDTA) for hemogram and another 3.5-mL tube with
331 polyethene terephthalate (PET) for health analysis were obtained. Red blood cell count
332 was carried out in an automated Cell-Dyn 3700 analyzer (Abbott Diagnostics, Chicago,
333 IL, USA), using internal (Cell-Dyn 22) and external (Program of Excellence for Medical
334 Laboratories-PEML) controls. Values of erythrocytes, haemoglobin, hematocrit and
335 hematimetric indexes were estimated.

336

337 **2.3.6. ABL-90 (blood gas analyzer)**

338 The blood parameters of haematocrit, haemoglobin, oxyhaemoglobin, pH, lactate
339 (amperometry electrode using the enzyme, lactate oxidase) were determined arterialized
340 capillary blood from the fingertip at rest (pre), in 30 last seconds of FatMax1, in 30 last
341 seconds of VT1, in 30 last seconds of VT2, Post P_{MAX}, in 30 last seconds of FatMax2

12

342 and at the end of EPOC (at rest), were measured by ABL 90 FLEX blood gas analyzer
343 (Radiometer Medical ApS, Copenhagen, Denmark). The blood-gas analyzer was
344 calibrated at hourly intervals throughout the day, with internal reference standards. A
345 previous study indicated that ABL90 FLEX had good accuracy (64). The plastic capillary
346 tubes intended for blood samples were collected were preheparinized with electrolytically
347 balanced solid heparin. This significantly reduces the risk of clots and helps to ensure
348 reliable results without electrolyte bias. There are arterial and capillary (finger) blood gas
349 analyzers that can measure biochemical, electrolyte, liver and kidney function, metabolic
350 and hematological markers, which can detect physiological or pathological changes with
351 a small blood sample, in case of finger sample (65).

352

353 **2.4 Statistical analyses**

354 Data analysis was conducted using IBM Social Sciences software (SPSS, version 21.0,
355 Chicago, IL, USA). Descriptive statistics are presented as mean and standard deviation
356 (SD). Levene's and Shapiro-Wilk tests were applied to check the homogeneity and
357 normality of the data, respectively. A group \times time \times moment ANOVA was conducted to
358 analyze within-group and between-group differences in all dependent variables and for
359 every time-point of measurement (baseline (pre), FatMax1, VT1, VT2, P_{MAX}, FatMax2 y
360 EPOC) and in both moments (pre-test and post-test). In addition, the area under the curve
361 (AUC), resulting from the integration of the three time-points of measurement taken
362 during the rectangular test, was calculated for each variable. The AUC was used to
363 analyze pre-post differences both within groups and between groups. The within-group
364 differences in the AUC were analyzed by repeated-measures t-test, and between-group
365 comparisons in the AUC were conducted by applying an independent samples T-test.
366 Cohen's d effect size (ES) (95% confidence interval) was calculated for all comparisons.
367 Threshold values for ES statistics were as follows: > 0.2 small, > 0.5 moderate, > 0.8
368 large (66). Significant differences were considered when $p \leq 0.05$.

369

370 **3. RESULTS**

371 All subjects who participated in this study performed all the tests proposed in the study
372 and successfully completed the research. Recruitment was carried out 2 months before
373 the start of the study (end of September 2018).

374 **3.1 Rectangular test**

375 **3.1.1 Oxygen and carbon dioxide metabolism (capillary blood gases)**

376 Table 3 shows the intragroup changes in biomarkers of oxygen metabolism at baseline
377 (pre), FatMax1, VT1, VT2, P_{MAX}, FatMax2 and EPOC from the rectangular test. In the
378 2S-hesperidin group, there was a significant decrease at pre in O₂Hb (-1.67%; p=0.028;
379 ES=2.04), sO₂ (-1.43%; p=0.048; ES=1.83) and an increase in RHb (26.38%; p=0.048;
380 ES=1.81), after the supplementation period. On the other hand, placebo group showed a
381 significant increase at pre in pCO₂ (4.52%; p=0.029; ES=1.81) after the intervention. In
382 addition, significant differences in pO₂ at ΔBaseline (p=0.03; ES; 0.94) were also
383 observed between groups (Table 5).

384

385 When assessing intragroup changes at FatMax1 during the rectangular test (**Table 3**),
386 there were a significant increase in pCO₂ (3.07%; p=0.035; ES=1.75) and tCO₂ (9.92%;
387 p=0.037; ES=4.83), after the 2S-hesperidin intervention. However, the placebo group
388 showed a significant decline in Hct (-3.99%; p=0.043; ES=1.72) and Hb (-4.01%;
389 p=0.042; ES=1.97) at FatMax1. However, significant differences in pO₂ (p=0.01; ES;
390 0.33) and pCO₂ (p=0.05; ES; 0.50) in ΔFatMax1 were also observed between groups.
391 (Table 5).

392

393 In a subsequent stage of the rectangular test, specifically at VT1 (Table 3), the 2S-
394 hesperidin group experienced a significant increment in pCO₂ (3.62%; p=0.020; ES=1.80)
395 and tCO₂ (5.56%; p<0.001; ES=3.21) and a significant decrease in AaDpO₂ (-18.81%;
396 p=0.004; ES=2.87) and a downward trend with a large effect size in Shunt (-26.58%;
397 p=0.057; ES=2.20), after the supplementation period. When comparing intragroup pre-
398 post test at VT1 in placebo, we found no significant change. In addition, there was a
399 significant difference in Hb (p=0.04; ES; 1.24) at ΔVT1 and a trend with large effect size
400 in AaDpO₂ (p=0.09; ES; 1.42) were observed between groups (Table 5).

401

402 At VT2 (**Table 3**), the placebo group showed a significant increase in CO₂Hb (32.50%;
403 p=0.022; ES=1.94) and a significant decline in pO₂ (-7.94%; p=0.042; ES=1.76), after 8
404 weeks of intervention. In the 2S-hesperidin group, there was only a significant increase in

14

405 CO₂Hb (21.56%; p=0.020; ES=2.01) at VT2 (Table 3). In the Δ VT2 analysis, only a trend
406 with moderate effect size in O₂Hb (p=0.07; ES; 0.63) was found when comparing the
407 groups (Table 5).

408

409 At the maximum exercise phase of the rectangular test (P_{MAX}) the placebo group (Table
410 3) demonstrated a significant decrease in p50 (-11.14%; p=0.032; ES=2.25) after the
411 experimental period. In 2S-hesperidin group, no significant change in P_{MAX} was observed
412 post-intervention (Table 3).

413

414 After the maximum phase of the test, the cyclists performed another exercise phase in
415 FatMax (FatMax2) (Table 3). The 2S-hesperidin group showed a significant increase in
416 CO₂Hb (30.10%; p=0.012; ES=2.63), RHb (24.00%; p=0.015; ES=3.16), pCO₂(6.78%;
417 p=0.005; ES=2.34) and tCO₂ (10.58%; p=0.003; 2.20) and a significant decrease in O₂Hb
418 (-1.03%; p=0.006; ES=3.47), sO₂ (-0.93%; p=0.015; 3.18) and a downward trend in pO₂
419 (-9.71%; p=0.076; ES=2.24) post-intervention. In placebo, no significant pre-post
420 changes were observed at FaxMax2 (Table 3). There was a significant difference in Shunt
421 at Δ FatMax2 (p=0.05; ES; 1.69) between groups. (Table 5).

422

423 At resting EPOC (Table 3), 2S-hesperidin had a significant increase in pCO₂ (4.03%;
424 p=0.033; ES=2.51) and tCO₂ (6.94%; p=0.001; ES=2.61) following intervention. The
425 placebo showed a significant increase in COHb (3.32%; p=0.049; ES=1.35) at EPOC
426 post-intervention (Table 3). Moreover, significant differences in AaDpO₂ at Δ FatMax2
427 (p=0.05; ES; 1.06) were also observed between groups. (Table 5).

428

429 Finally, when comparing the intra-group areas under the curve (AUCs), in 2S-hesperidin,
430 there was a significant increase in RHb (9.91%; p=0.023; ES=0.61), pCO₂ (3.73%;
431 p=0.012; ES=0.54) and tCO₂ (6.17%; p=0.012; ES=0.73) with an upward trend in COHb
432 (17.16%; p=0.077; ES=0.49) post-intervention. In the placebo group, there was a
433 significant decrease in p50 AUC (-3.63%; p=0.040; ES=0.61). Moreover, we found an
434 upward trend in COHb AUC (11.23%; p=0.088; ES=1.63) and pCO₂ AUC (3.78%;

435 p=0.086; ES=0.50) post-intervention. When comparing the AUC between groups, there
436 was no significant difference between groups.

437 ***insert table 3***

438 ***insert figure 2***

439 ***insert figure 3***

440

441

442 3.1.2 Acid-base status (capillary blood gases)

443 Table 4 shows the intragroup changes in biomarkers of acid-base status at baseline (pre),
444 FatMax1, VT1, VT2, P_{MAX}, FatMax2 and EPOC achieved during the rectangular test. In
445 2S-hesperidin we no found significant changes post-intervention. Similarly, no
446 significant changes were found at pre (Table 4) in placebo.

447

448 When we measured acid-base status at FatMax1 (Table 4), the 2S-hesperidin group
449 showed a significant increase in HCO₃⁻ (10.30%; p=0.040; ES=4.85), Lac (-29.39%;
450 p=0.010; ES=2.26) and SBE (424%; p=0.046; ES=4.87) and an upward trend with a large
451 effect size SBC (10.36%; p=0.076; ES=5.26) and ABE (534%; p=0.059; ES=4.82) post-
452 intervention. In the placebo group, we found a significant decreased in Lac (-21.91%;
453 p=0.041; ES= 1.71) post-intervention. However, in ΔFatMax1, a trend with large effect
454 size in SBC (p=0.07; ES; 1.81) were observed between groups. (Table 5).

455

456 When we analyzed the changes in VT1 (Table 4), 2S-hesperidin had a significant increase
457 in HCO₃⁻ (5.55%; p=<0.001; ES=3.20), SBC (4.65%; p=0.001; ES=3.59), ABE (6500%;
458 p=0.001; ES=3.48) and SBE (1913%; p=0.001, ES=3.42) and a significant decrease in
459 Lac (-30.83%; p=0.003; ES=2.98), after the supplementation period. On the other hand,
460 no significant changes were found in the placebo group post-intervention. A significant
461 difference in SBE (p=0.01; ES; 4.59) in ΔVT1, a trend with large effect size in ABE
462 (p=0.08; ES; 5.05) were also observed between groups. In the ΔVT1 analysis, although
463 there were no significant differences in HCO₃⁻ (p=0.31; ES; 5.48), SBC (p=0.81; ES;

464 5.07) and Lac ($p=0.18$; ES; 3.39) between groups, these parameters had a large effect size
465 (Table 5).

466 In the submaximal exercise stage measurements (VT2) (Table 4), the 2S-hesperidin group
467 did not show any significant changes in acid-base state post-intervention. Similarly, there
468 were no significant changes in VT2 in placebo after the 8-week intervention.

469 At P_{MAX} (Table 4), the 2S-hesperidin group showed no significant pre-post change in
470 acid-base status. However, in the placebo group (Table 4), an upward trend was observed
471 at P_{MAX} in SBC (5.88%; $p=0.077$; ES=1.34) post-intervention.

472 During the rectangular test, after P_{MAX} decreased in intensity to FatMax2 (Table 4), 2S-
473 hesperidin showed a significant increase in pH (0.26%; $p=0.028$; ES=1.48), HCO_3^-
474 (10.81%; $p=0.003$; ES=2.19), SBC (7.68%; $p=0.006$; ES=2.01), and ABE (34.23%;
475 $p=0.034$; ES=1.62). Conversely, we found a significant decrease in Lac (-18.56; $p=0.018$;
476 ES=1.88) post-intervention. In placebo, no significant change was found at FatMax2 after
477 intervention.

478 Finally, at EPOC (Table 4), the 2S-hesperidin group showed a significant increase in
479 HCO_3^- (5.76%; $p=0.001$; ES=2.63), SBC (4.48%; $p=0.001$; ES=2.39), ABE (246.15%;
480 $p=0.001$; ES=2.42) and SBE (248.39%; $p<0.001$; ES=2.53) and a significant decrease
481 in Lac (-18.56%; $p=0.039$; ES=1.51), post-intervention. In placebo (Table 4), we found a
482 significant increase in HCO_3^- (3.40%; $p=0.045$; ES=1.36) at resting EPOC after
483 intervention.

484 When comparing the intra-group AUCs of the acid-base state, in 2S-hesperidin there was
485 a significant increase in pH (0.16%; $p=0.016$; ES=0.54), HCO_3^- (6.34%; $p=0.012$;
486 ES=0.74) SBC (5.07%; $p=0.017$; ES=0.79), with a downward trend in Lac (12.58%;
487 $p=0.057$; ES=0.51), post-intervention. After comparing AUCs in placebo, there was no
488 significant change after the intervention (Table 4). When comparing AUC between
489 groups, a significant change in pH (0.16%; $p=0.022$; ES=0.99) was found in favour of
490 2S-hesperidin, post-intervention. In the analysis of ΔAUC , significant differences were
491 observed in pH ($p=0.02$; ES; 1.03) (Table 5).

492 ***insert table 4***

493 ***insert table 5***

494 ***insert figure 4***

495

496 **4. DISCUSSION**

497 This study primarily aimed to determine if 8-weeks of 2S-hesperidin supplementation
498 (500 mg/d) has the ability to modify biomarkers of oxygen and carbon dioxide
499 metabolism and acid-base state and whether these possible changes can affect
500 performance in trained amateur cyclists. The second objective was to determine if any of
501 the changes in the previously mentioned parameters could explain improvement in
502 performance, as observed in our recently published article (50), of which this research is
503 a part. The most important findings of this study showed that the chronic intake of 2S-
504 hesperidin: i) increased R_{Hb} and decreased in oxygen parameters at baseline (0) ii)
505 increased dioxide carbon metabolism and state acid-base markers at FatMax1, iii)
506 increased dioxide carbon metabolism and state acid-base markers and decreased Shunt,
507 AaDpO₂ and Lac at VT1, iv) increased dioxide carbon metabolism and state acid-base
508 markers and decreased oxygen metabolism markers and Lac in FatMax2, v) increased
509 dioxide carbon metabolism and state acid-base markers and decreased Lac in EPOC post
510 intervention, and vi) increased dioxide carbon metabolism and state acid-base markers
511 and decreased Lac when comparing pre-post intervention AUCs. However, there were no
512 pre-post changes in the aforementioned parameters in placebo.

513

514 **4.1. Blood gas changes and acid-base at baseline (pre)**

515 At baseline, the 2S-hesperidin group showed a decrease in O₂Hb and sO₂ following the
516 intervention. However, there were pre-intervention significant differences in pO₂ (2S-
517 hesperidin -2.28 vs placebo -8.90, mmHg) at baseline between groups (Δ baseline; Table
518 5), indicating a greater decrease in resting pO₂. This difference may be due to the effect
519 of detraining because the study was conducted off-season to pre-season, where the
520 cyclists had decreased their training load. Detraining is defined as the partial or complete
521 loss of physiological, anatomical, and performance adaptations due to the reduction or
522 cessation of training (67). In young top-level road cyclists, after 5 weeks of training
523 cessation, a decrease in red blood cell count ($-6.6 \pm 4.8\%$) and Hb ($-5.4 \pm 4.3\%$) at rest
524 were observed (68). However, in our study, there were no significant changes in Hct and
525 Hb in both groups. The rapid decrease in blood volume after the first days of cessation of

526 training possibly has an important role in the processes that decrease maximal cardiac
527 output and, consequently, VO_{2MAX} (68).

528 Moreover, cessation of training results in a rapid decline in plasma volume primarily
529 mediated by a loss in intravascular protein content. Furthermore, cessation of training
530 results in a rapid decrease in plasma volume, mediated mainly by a loss of intravascular
531 protein content, which directly affects blood volume (69). However, to our knowledge,
532 there are studies that have observed the decrease in pO_2 after cessation or reduction of
533 training volume. Therefore, based on the findings of this study, chronic intake of 2S-
534 hesperidin could prevent the decline in resting pO_2 during a period when cyclists reduce
535 their training volume. Although, further studies are still needed in this regard.

536

537 **4.2. Blood gas changes and acid-base at FatMax1 and VT1**

538 Exercise performed in FatMax1 intensity that produces maximum fat oxidation rate,
539 translated as the % VO_{2MAX} , uses the oxidation of free fatty acids and intramuscular
540 triglycerides in skeletal muscle as its main source of energy (70). Both peak oxidative
541 maximum and FatMax are related to better-prolonged endurance performance, being an
542 important determinant of performance in elite endurance athletes (71). On the other hand,
543 being a zone close to the physiological-metabolic level of the FatMax, VT1 represents
544 the first increase in minute ventilation (VE) that is proportional to the increase in CO_2
545 output (VCO_2) generated by the HCO_3^- buffering of lactic acid. As a result, the ventilatory
546 equivalent for oxygen (VE/VO_2) increases with no change in the ventilatory equivalent
547 for carbon dioxide (V/VCO_2) (72). In these, two exercise zones (FatMax and VT1),
548 energy production is predominantly aerobic.

549 In our study, at FatMax1 and VT1, there were increases in pCO_2 and tCO_2 in 2S-
550 hesperidin. Moreover, this group also showed a decrease in $AaDpO_2$ and a downward
551 trend in Shunt after the in capillary blood intervention. No previous studies have
552 evaluated polyphenol intake on blood gas biomarkers. At intensities close to 50% of the
553 lactate threshold (LT) or VO_{2MAX} (FatMax and VT1), the values of arterial CO_2 pressure
554 ($PaCO_2$) and partial pressure of exhaled carbon dioxide ($PETCO_2$) remain stable (73). It
555 is well known that CO_2 is the obligatory product of aerobic catabolism in tissues and, as
556 it is produced at a higher rate, higher concentrations of H^+ will also be generated, thereby
557 increasing the production of HCO_3^- from carbon dioxide (74). But unlike H^+ , the effect

558 on total bicarbonate concentration is minimal (74). Although in the 2S-hesperidin group
559 the Δ pH values increased (alkalinisation), the placebo group showed a decrease in Δ pH
560 (acidification), without significant changes between groups, but there was a large effect
561 size in FatMax1 (ES=2.13) and VT1 (ES=2.50). Therefore, the increased pCO₂ and tCO₂
562 in the 2S-hesperidin group is not responsible for acidification (\downarrow pH) of the blood,
563 although at low-moderate intensities (FatMax1 and VT1) this situation does not have
564 much influence on performance. However, elevated pCO₂ post-intervention (2S-
565 hesperidin) could be a consequence of alterations in pulmonary ventilation, which was
566 observed in our recently published study (that is part of the same project as the present
567 research), where there was no worsening or improvement in VT1 performance (W) in
568 amateur cyclists following 8 weeks of 2S-hesperidin (500 mg/d) intake (50).

569

570 We detected an increase in HCO₃⁻ in FatMax1 and VT1 in the 2S-hesperidin group. In
571 addition, we observed an increase in SBC, ABE and SBE, with a decrease in Lac,
572 indicating an improvement in acid/base status and a lower contribution of glucose to
573 energy production at low intensities. These results could explain how the 8-week intake
574 of 2S-hesperidin was able to maintain fatty acid and carbohydrate oxidation compared to
575 placebo (2S-hesperidin: -12.9% and -0.45% vs placebo: -34.3% and 17.7%; respectively)
576 at VT1 intensities in amateur cyclists in a period where there was lower intensity and
577 volume of training) (50). This decrease in fatty acid oxidation and increase in
578 carbohydrate oxidation in placebo in VT1 found by Martínez-Noguera *et al.* (50) is linked
579 to the negative changes in aerobic metabolism (\downarrow Hct, \downarrow Hb, \downarrow tO₂ and \downarrow AaDpO₂) from this
580 study, although there were no significant changes in sO₂% and pO₂. In fact, changes in
581 Hb or by plasma volume expansion can slightly alter O₂ supply and thus O₂ kinetics in
582 humans (73).

583 The consequences of these changes would be a higher carbohydrate utilization at low-to-
584 moderate intensities in the placebo-supplemented cyclists. Therefore, chronic ingestion
585 of 2S-hesperidin could improve acid/base and lactate status, which may influence the
586 efficiency of the energy substrate used at low-to-moderate intensities (VT1), leading to
587 conservation of muscle glycogen for higher intensity phases in the final phase of a
588 competition in amateur cyclists.

589

590 4.3. Blood gas changes and acid-base at VT2

591 In some athletes, high-intensity endurance exercise induces a time-dependent decrease in
592 sO₂ of more than 5% compared to resting levels (~98%), where extreme declines in the
593 80% range have been reported (75). The desaturation of O₂Hb during exercise is based
594 on respiratory and non-respiratory factors. Non-respiratory factors, like metabolic
595 acidosis and hyperthermia, produces a rightward shift in the O₂Hb dissociation curve (76).
596 In high performance athletes (77), arterial desaturation of O₂Hb is due to a fall in PaO₂
597 (78) secondary to an abnormally widening of AaDpO₂ (75). In healthy untrained
598 individuals, AaDpO₂ is reported to decline to 20 to 30 mmHg during maximal exercise;
599 however, in some elite athletes, this difference can be as high as 35-50 mmHg (79).

600 In contrast, our study found no post-intervention changes in AaDpO₂ in both groups (2S-
601 hesperidin: -1.23 mmHg vs placebo: 0.29 mmHg) at VT2. However, only the placebo
602 group showed a decrease in pO₂ (-7.94%) with a non-significant decrease in O₂Hb, sO₂
603 and increase in COHb but with a large effect size. In addition, we found a trend with a
604 moderate effect size between groups in O₂Hb for Δ VT2. These results are in line with our
605 previously reported study that showed a decrease in VO₂ (L/min) (-8.3%; $p \leq 0.01$) and
606 oxygen consumption relative to weight (VO₂R) (mL/kg/min) (-8.9%; $p \leq 0.01$) at VT2
607 (10 min) when performing a rectangular test (cycloergometer) amateur cyclists who
608 ingested placebo (8-weeks) but not in the 2S-hesperidin group (50). In this same study,
609 we also performed an incremental test where we found a non-significant decrease in
610 power output at VT2 (WVT2) (-3.1%; -8.9W; $p=0.264$) in placebo and a non-significant
611 increase (1.0% = 2.9 W; $p=0.642$) in 2S-hesperidin post-intervention. It is important to
612 make clear that this study (50) and the data presented in this paper are part of the same
613 project and were carried out at the same time. There are currently no other studies that
614 have used other polyphenols as markers of oxygen metabolism and with which we can
615 compare our results. There is evidence that a decrease in SpO₂ (77.9%; O₂Hb saturation
616 estimated using a pulse oximeter) manipulated by a low FIO₂ (0.15) led to a decrease in
617 mean power output (MPO) (-23.3%) and increase in time (32 s) in trained male cyclists
618 performing a 5-K time trial (TT) compared to normoxic conditions (FIO₂, 0.21) (80). In
619 addition, they observed that, when generating a hyperoxia state by means of a FIO₂ of 1.0
620 reaching a SpO₂ of 100%, cyclists increased MPO (18.1%) and decreased time (-19.5 s)
621 in a 5K TT compared to normoxic conditions. This demonstrates that changes in arterial

622 oxygen can affect performance. It has been shown that for every 1% reduction in SaO₂
623 below 95%, there is a 1-2 decrease in VO_{2MAX} (81).

624 Arterial desaturation during exercise may be due to an insufficient hyperventilatory
625 response secondary to a low chemo-responsiveness as a consequence of a low response
626 to circulating chemical stimuli, such as catecholamines, adenosine, protons or potassium
627 (82) and O₂ and CO₂ (83) and/or mechanical constraints on the airway (75). Inadequate
628 ventilatory responses during exercise reduces the alveolar partial pressure of O₂ (PAO₂),
629 which negatively affects arterial blood gas status and SaO₂ (84). Previously, it has been
630 reported that EIAH (SaO₂ ≤ 91%), involves an increase in AaDpO₂, which combined with
631 a minimal alveolar hyperventilatory response, results in a reduction in pO₂ (85). Several
632 authors suggest that one consequence of EIAH is that even small amounts of EIAH have
633 a significant negative effect on limiting the transport and utilization of O₂ during maximal
634 exercise (81, 86). Dempsey and Wagner et al. (87) observed SaO₂ values <93%
635 (moderate and severe EIAH) in highly trained athletes, which was related to decreases in
636 ventilatory equivalents for CO₂, showing that a worsening in the hyperventilatory
637 response may be associated with the development of EIAH.

638 Several authors have reported that hesperidin may prevent the decline in pO₂ during
639 maximal exercise through different mechanisms. Liu et al. (52) showed that hesperetin
640 (hesperidin metabolite) increases NO release from endothelial cells in a dose-dependent
641 manner and up-regulates endothelial nitric oxide synthase (eNOS) expression. In
642 addition, hesperetin plus naringenin ameliorates airway structural remodeling (improved
643 basement membrane thickness and smooth muscle hypertrophy) with a significant
644 reduction of inflammatory cells and lowering of mucus plug formation in murine chronic
645 asthma mode (54).

646 Furthermore, flavonoids (a subgroup of polyphenols to which hesperidin belongs) can
647 facilitate an increase in mitochondrial Ca₂⁺ levels by acting on the mitochondrial Ca₂⁺
648 uniporte (53). This mechanism can up-regulate respiratory rate and ATP production and
649 stimulate eNOS, thus increasing NO synthesis (51, 55, 88). NO-induced vasodilation may
650 increase oxygen supply to active muscles, which could improve performance (55, 89).

651 Another factor that can affect blood oxygenation is the Shunt, which is defined as blood
652 that enters the arterial system without coming in contact with ventilated areas of the lung
653 (90, 91). In our study, no significant intra-group pre-post differences were found, but

654 placebo did show a non-significant increase with a large effect size (ES= 1.61) in VT2.
655 Another important limiting factor for arterial pO₂ is the diffusion problem caused by the
656 presence of pulmonary edema during high-intensity exercise, as fluid in the lungs
657 increases the effective thickness of the alveolar wall and decreases the area of gas
658 exchange (92, 93). The edema prevents air from reaching pulmonary capillaries, resulting
659 in perfusion without ventilation and a physiologic right-to-left shunt, thereby creating
660 greater hypoxemia (94). One possible mechanism of action of hesperidin in oxygen
661 metabolism is the inhibition of histamine and expression of mRNA and proteins histamine
662 receptor H1 in the hypothalamus and brainstem regions in rats that consumed hesperidin
663 (95). In addition, the anti-inflammatory effect of hesperidin has been demonstrated in a
664 rat model with lung damage, where there was a decreased presence of TGF- β 1, IL-1 β ,
665 IL-4, IL-10 and TNF- α in the lung tissues (96). The activation of the immune system may
666 influence the lung's response. For example, basophilic, granulocytes and mast cells are
667 degranulated, as indicated by the enhanced ratio of plasma histamine to total histamine in
668 the blood during intense exercise, and may contribute to the increased permeability of the
669 lung microvasculature (97). In addition, Martinez-Noguera *et al.* (98) observed a decrease
670 in monocyte chemoattractant protein 1 at basal levels after a period of recovery from
671 exertion to exhaustion and decline in the area under the curve of the rectangular test
672 performed by amateur cyclists.

673 Wei *et al.* (99) showed that the administration of hesperidin significantly decreased the
674 number of infiltrating inflammatory cells and Th2 cytokines in bronchoalveolar lavage,
675 goblet cell hyperplasia and mucus hypersecretion compared with the ovalbumin-induced
676 group of mice. On the other hand, hesperidin methylchalcone acts as an inhibitor of
677 increased bradykinin-induced microvascular permeability and prevents leukotriene B₄-
678 histamine, which induces vascular leakage, thus reducing the formation of edema in an
679 animal model of venous insufficiency (100). In particular, an elevated concentration of
680 IL-1 β and IL-8 in plasma during exercise may partly explain the increase in %H
681 associated with EIAH in highly trained athletes, showing how a reaction of the
682 inflammatory system can influence histamine concentrations and consequently EIAH
683 (101). Therefore, chronic intake of 2S-hesperidin could prevent a decrease in pO₂, thus
684 avoiding a decrease in oxygen consumption and performance in cyclists in a period of
685 decreased training intensity and volume (off-season to preseason).

686 We also observed in this clinical trial, a non-significant intra-group increase in both
687 treatments but with a large effect size in pH for the 2S-hesperidin group (2S-hesperidin:
688 0.22% vs placebo: 0.11%) and no significant changes but a moderate effect size (ES=
689 0.52) (between-group comparison) in Δ VT2. Together with these changes, we also found
690 a non-significant intra-group decrease in both groups but with a large effect size in 2S-
691 hesperidin (2S-hesperidin: -16.6% vs placebo: -3.8%) and no significant changes but a
692 large effect size in Δ VT2.

693 Changes in pO_2 in the placebo group in VT2 could be largely contributed by anaerobic
694 metabolism, but this is not evident in our study, as there were no significant changes in
695 Lac post-intervention. However, the lower lactate production in the 2S-hesperidin group
696 seems to indicate a lower anaerobic contribution of glycolysis or a better lactate clearance,
697 thereby leading to better pH levels. Due to the influence of acidosis on the development
698 of fatigue, an increase in pO_2 , a lower intracellular lactate accumulation and an increase
699 in intracellular pH can increase exercise capacity independently of VO_{2MAX} (102).

700 Administration of lemon peel flavonoids (LPF) for 4 weeks in mice decreased post-
701 intervention lactic acid levels following a swim to exhaustion test, improving time-to-
702 exhaustion and endogenous antioxidant status (103). As mentioned above, Martínez-
703 Noguera et al (50) found an improvement in VT2 (\uparrow W) performance after 8 weeks of 2S-
704 hesperidin intake in amateur cyclists using the same rectangular test as the current study,
705 which could be linked to lower levels of Lac in VT2 post-intervention.

706 Therefore, chronic intake of 2S-hesperidin could have the capacity to prevent a decrease
707 in pO_2 in VT2, in which anaerobic metabolism is mainly involved and can be described
708 as "hard" or "high intensity" exercise (104). This parameter plays an important role in the
709 performance of extreme endurance competitions, such as 3-week races, like Tour of
710 France(105).

711

712 **4.4. Blood gas changes and acid-base at P_{MAX}**

713 The P_{MAX} during an incremental test until exhaustion is a mechanical expression of
714 aerobic capacity (106). In addition, it is known that cycling at an output power equivalent
715 to VO_{2MAX} or P_{MAX} is not sustainable in endurance events, and that sustainable output
716 powers measured on the ergometer are around the lactate threshold, the onset of blood

717 lactate accumulation (OBLA) or the ventilation threshold (typically 75 to 90% of P_{MAX})
718 (107). Maintaining maximum cycling power production is an important quality for
719 performance in competitive cyclists, due of the strong relationship between power
720 production and endurance cycling performance (107-109).

721 When assessing changes in capillary blood samples at P_{MAX} , we found a decrease in p50
722 in the placebo group (-11.14%) coupled with a non-significant increase with a large effect
723 size in sO_2 (0.72%) and decrease in pO_2 (-4.53%). However, there was no significant
724 change in any parameter in 2S-hesperidin, but a non-significant decrease with large effect
725 size in pO_2 (-8.33%) and p50 (-7.34%). To our knowledge, this study is the first to
726 examine blood gas parameters following polyphenol supplementation. It is known that
727 the p50, indicating the affinity of Hb- O_2 , is expressed as the pO_2 value at 50% saturation
728 of Hb with O_2 (110). The standard p50 in humans is 26.9 mmHg at pH 7.4 and 37°C
729 (111), which was the methodology used in our study. There are factors that can modify
730 the p50 (Hb- O_2 affinity) due to an increase in hydrogen ions (acidosis), temperature, 2,3-
731 bisphosphoglycerate (2,3-BPG) and pCO_2 that decrease Hb- O_2 affinity with higher p50
732 values and a rightward shift of the ODC (112). During physical exercise and depending
733 on the intensity of the exercise, an increase in local temperature occurs at the muscle level
734 and produces more H^+ and CO_2 , which leads to a better release of oxygen (112). This is
735 mainly due to the conversion of CO_2 to bicarbonate and H^+ by carbonic anhydrase,
736 consequently decreasing pH and Hb- O_2 affinity (112). In addition, CO_2 has a specific
737 effect on Hb- O_2 affinity at constant pH (113). However, an increase in p50 in venous
738 blood (decrease in Hb- O_2 affinity) would benefit oxygen-deficient tissues (110). In our
739 study, although the p50 finger capillary blood (ΔP_{MAX} ; ES=0.84, between groups)
740 decreased, there were no changes in markers of acid-base status in placebo, which could
741 indicate a higher rate of fatigue that would anticipate physical exhaustion in P_{MAX} .
742 Nevertheless, in the study we recently published, the placebo group decreased
743 performance and the 2S-hesperidin group improved performance during the rectangular
744 test after eight weeks of supplementation (50).

745 During maximal exercise, the SaO_2 appears to be affected by the reduction in pH, and this
746 is of particular importance when PaO_2 is low (114). Maximal exercise produces an
747 extreme lactate spill-over to blood which decreases the pH to below 7.1 and, according
748 to the ODC, this is critical for SaO_2 (115). Indeed, to test the effect of pH on SaO_2 , it has
749 been found that the infusion of sodium bicarbonate maintains a stable blood buffer

750 capacity, thereby attenuating acidosis and increasing SaO₂ from 89% to 95%, and
751 enabling exercise capacity to increase (115).

752 Although both groups had a non-significant decrease in pO₂, a significant decrease in p50
753 was only observed in placebo, indicating a leftward shift of OCD, leading to an increase
754 in Hb-O₂ affinity, in the capillary blood sample. This effect is in line with the non-
755 significant increase but with a large effect size of sO₂. Therefore, it is plausible that 2S-
756 hesperidin intake could prevent a decrease in p50 in finger capillary blood and would
757 improve O₂ delivery at the tissue level at maximal exertion (P_{MAX} → rectangular test),
758 which could be associated with an increase in maximal power in 2S-hesperidin vs
759 placebo, as we observed in a recently published study but an incremental test (study which
760 is part of the same project as the current project) (50). But this is only a hypothesis, as we
761 did not measure p50 in venous blood, which would be the most appropriate to verify OCD
762 changes at the tissue level. More studies are needed in this regard.

763

764 **4.5. Blood gas changes and acid-base at FatMax2**

765 At FatMax2 were increase in variables of the CO₂ metabolism (pCO₂ and tCO₂),
766 hemoglobin (COHb and RHb) and Shunt (19%), but with inverse direction, there was a
767 decreased in oxygen metabolism markers (O₂Hb, sO₂, pO₂ and tO₂) in 2S-hesperidin.
768 When evaluating the changes in the placebo group at FatMax2, no significant changes
769 were found. In view of the results, we cannot clearly say that the decrease in markers of
770 oxygen metabolism is due to an increase in AaDpO₂ in 2S-hesperidin, but there were
771 significant differences in Shunt at ΔFatMax2 in the comparison between groups.

772 Previous studies have demonstrated that lung volume and function are temporarily
773 impaired after exercise, suggesting small-airway closure and possible subclinical edema,
774 all contributing to a decreased diffusion capacity (116, 117). In addition, hydrogen ion
775 accumulation and CO₂ decrease pH, which induces metabolic acidosis during and post-
776 exercise, which subsequently stimulates hyperventilation due to respiratory compensation
777 and decreases the arterial pCO₂ concentration (118). Based on the classic concept of Hill
778 and Lupton (119), elevated post-exercise oxygen uptake is indicative of oxygen debt,
779 which is the result of phosphocreatine resynthesis and lactate metabolism processes.
780 These mechanisms could be responsible for the increase in CO₂ markers and decrease in
781 O₂ markers in 2S-hesperidin in finger capillary blood, coupled with changes in ventilation

782 (hypoventilation) and an increased oxygen deficit generated from post P_{MAX} to FatMax2,
783 as no changes in p50 were observed post-intervention.

784 However, only the 2S-hesperidin group enhanced acid-base status markers (\uparrow pH, \uparrow HCO₃⁻
785 \cdot \uparrow SBC) and decreased Lac in FatMax2 post-intervention, suggesting that there was a
786 lower anaerobic contribution, as Lac is a sensitive biomarker of non-oxidative glycolysis
787 (120), as well as indicating a better washout of Lac in the transition from post P_{MAX} (very
788 high intensity exercise) to FatMax2 (low-moderate intensity exercise). In addition, a
789 marker of Lac and H⁺ clearance is VCO₂ production (nonmetabolic) relative to VO₂ (121,
790 122), and this is in line with our results, as CO₂ increased and O₂ decreased markers in
791 FatMax2 in the 2S-hesperidin group. The decrease in Lac could be explained by
792 improvements in the intracellular to extracellular transfer of lactate for oxidation or
793 convention to glucose and glycogen (123, 124).

794 Some phytochemicals with high flavonoid content have shown lower lactate
795 concentrations compared to placebo after exercise to exhaustion, for example after 6
796 weeks of drone pupae extract intake in mice (-27%) (125) and after 30 days of
797 pericarpium citri reticulatae extract supplementation in rats (126). In addition,
798 consumption of 500 mL/d of orange juice and 1 hour of aerobic training (3 times a week
799 for 3 months) in women decreased blood Lac concentration by 27% in the experimental
800 group compared to 17% in the control group after exercise to exhaustion (127). These
801 findings suggest that the experimental group showed less muscle fatigue and better
802 response to training. On the other hand, when purified Chestnut flower flavonoids were
803 administered to mice, a decrease in Lac and an increase in lactate dehydrogenase (LDH)
804 were observed after a swim to exhaustion test (128). LDH is expressed in several tissue
805 cells and is involved in the glycolytic pathway by facilitating the redox reaction between
806 pyruvic acid and lactic acid (reversible reaction) with concomitant actions of NADH and
807 NAD⁺, playing an important role in the quenching of Lac during high endurance exercise
808 (129). However, more research is needed to explain precisely the mechanisms by which
809 flavonoids can decrease Lac after high-intensity exertion.

810 Another mechanism that may have elicited post-intervention decrease in Lac in FatMax2
811 is the increased peripheral blood flow in leg muscles via increased production of NO, as
812 mentioned above, enhancing recovery, which is characterized by greater changes in Lac
813 allowing transport of lactate to other tissues for oxidation and allowing greater
814 phosphocreatine resynthesis and less accumulation of metabolites (130). Therefore, based

815 on our findings, chronic intake of 2S-hesperidin can decrease Lac levels and improve
816 markers of acid-base status, which offers cyclists greater recovery after maximal exertion.

817

818 **4.6. Blood gas changes and acid-base at EPOC**

819 In the post-exercise recovery period (EPOC), there was an increase in oxygen
820 consumption, which consists of a rapid and a prolonged component. A review in 2003
821 concluded that EPOC can last for several hours after exercise, but it can also be transient
822 and minimal (131). A relative shift from carbohydrate to fat as a substrate source is a
823 consistent finding after prolonged strenuous exercise (131). Since the energy equivalent
824 of oxygen is lower with fat as a substrate compared to carbohydrate (free fatty acids: ~4.7
825 mol ATP/mol oxygen; glucose: ~5.1 mol ATP/mol oxygen), some of the EPOC may be
826 explained by this substrate shift. It has been estimated that the substrate changes after
827 exhaustive submaximal exercise accounts for 10-15% of the observed EPOC (132).

828

829 In the last phase of the protocol that was performed at rest (EPOC), 2S-hesperidin
830 reported an increase in markers of CO₂ metabolism (pCO₂, tCO₂) and acid-base status
831 (pH, HCO₃⁻, SBC, ABE and SBE) and a decrease in Lac. However, in the placebo group,
832 only an increase in HCO₃⁻ and an increase in tCO₂ post-intervention were detected.
833 Furthermore, when evaluating changes between groups, significant differences in
834 AaDpO₂ at ΔEPOC were found.

835

836 In general, the changes observed in EPOC are in line with those found in FatMax2, where
837 the 2S-hesperidin group improved acid-base status and decreased post-intervention Lac,
838 giving 2S-hesperidin a recuperative effect after high-intensity exertion followed by low-
839 intensity exertion. In addition, reduced Lac levels in 2S-hesperidin indicate a lower
840 energy contribution from carbohydrates via the anaerobic pathway.

841

842 **4.6. Blood gas changes and acid-base in AUCs**

843 When assessing the AUCs, which reflect the overall changes, the 2S-hesperidin group
844 showed an increase in capillary blood markers in RHB, pCO₂, tCO₂, pH, SBC, and HCO₃⁻

845 , but a decrease in Lac. In contrast, the placebo only showed a decrease in p50 post-
846 intervention. In addition, significant changes in pH were found in Δ AUC ($p=0.02$;
847 $ES=1.03$) when comparing the groups.

848 These findings partially reaffirm the changes found in some phases of the rectangular
849 protocol, as the changes shown in pCO_2 , tCO_2 HCO_3^- and SBC in FatMax1, VT1,
850 FatMax2 and EPOC are related to significant differences in the AUC in 2S-hesperidin.
851 However, changes in Lac shown in FatMax1, VT1, FatMax2 and EPOC were related to
852 a downward trend with a moderate effect size in the AUC in 2S-hesperidin.

853 Overall, our data confirm improvements in acid-base status (low and moderate intensity
854 exercise) and decrease in Lac (low-moderate and high intensity exercise) after 8 weeks
855 2S-hesperidin (500 mg/d) supplementation in amateur cyclists.

856 **Limitations**

857 Ideally, blood gases should be obtained in arterial blood. However, indwelling arterial
858 catheters for sampling arterial blood are not always feasible and desirable. Thus, indirect
859 methods were used to assess blood gases in the present study. Therefore, the degree to
860 which the measurements provided an accurate proxy for arterial measures should be
861 considered. Arterial blood gases (pO_2 and pCO_2) during exercise could be estimated by
862 using capillary blood samples (pO_2 and pCO_2). Previous studies found that capillary
863 blood samples are in good agreement with arterial blood samples for partial pressure of
864 carbon dioxide, but not for partial pressure of oxygen. The main cause of underestimation
865 of pO_2 in capillary blood samples could be insufficient arterialization of blood due to
866 venous admixture. The capillary blood sample method requires adequate blood flow in
867 the arm to enable a sufficient volume of blood to be sampled without additional external
868 pressure during sampling.

869 The period of the season in which the study was conducted was in late September and
870 mid-December, also known as their pre-season. During the preseason, cyclists decrease
871 their training volume and intensity with respect to other times of the season. Thus, our
872 results should be taken with caution, considering the preseason. If the study had been
873 carried out in another period of the season where cyclists had more training volume and
874 intensity, the results could have been different, in a positive way. Our sample size could
875 have been larger, but due to budget constraints and planning to run the tests in 2 months,
876 it made it unfeasible to recruit more subjects.

877

878 **5. CONCLUSIONS**

879 Chronic ingestion of 2S-hesperidin improved acid-base status (\uparrow pH, HCO_3^- and SBC) at
880 low-moderate exercise intensities (FatMax1, VT1, FatMax2 and EPOC) and decreased
881 Lac at low-moderate and submaximal intensities (FatMax1, VT1, VT2, FatMax2 and
882 EPOC) in amateur cyclists. In addition, 2S-hesperidin intake prevented the decrease in
883 pO_2 at VT2, which is associated to the prevention of a decrease in VO_2 and performance
884 in periods of less training volume and load (50). These findings position 2S-hesperidin as
885 a new ergogenic aid, which may help cyclists to improve performance at high intensities
886 (VT2) and recovery after very high intensity exercise (P_{MAX}). Furthermore, the
887 improvements in acid-base status and decrease in Lac in FatMax1 and VT1 generated
888 after 2S-hesperidin ingestion are linked to the maintenance of fatty acid oxidation in
889 FatMax1 and VT1 (50), indicating a greater contribution of the aerobic pathway at low-
890 moderate intensities relative to placebo.

891

892 **Acknowledgments:** This study was supported by the Research Center for High
893 Performance Sport of the Catholic University of Murcia and HTBA (Murcia, Spain). We
894 would like to acknowledge Linda H. Chung for her help in this project. We also thank
895 Iris Samarra, Antoni del Pino and Nuria Canela, from the Metabolomics facility of the
896 Centre for Omic Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat,
897 for their contribution to the urine analysis. The results of the current study do not
898 constitute endorsement of the product by the authors or the journal.

899 **Authors' contributions:** Designed research, F.J.M.N., C.M.P. and P.E.A.; conducted
900 research, F.J.M.N., C.M.P. and J.C.V.; provided essential reagents, F.J.M.N., C.M.P. and
901 J.C.V.; analyzed data, F.J.M.N., C.M.P. and J.C.V.; wrote paper, F.J.M.N. All authors
902 read and approved the final manuscript.

903 **Funding:** The authors declare that this study has been financed by HTBA (Murcia,
904 Spain), who kindly provided the product Cardiose®, but they did not participate in the
905 experimental design, data collection, data analysis, interpretation of the data, writing of
906 the manuscript, or in the decision to publish the results.

907 **Conflicts of Interest:** The authors declare no conflict of interest.

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Table 1. Baseline general characteristics and training variables of the cyclists.

	2S-Hesperidin	Placebo	p-value
Age (years)	35.0 (9.20)	32.6 (8.90)	0.407
Body mass (kg)	71.0 (6.98)	70.4 (6.06)	0.773
Height (cm)	175.3 (6.20)	176.5 (6.10)	0.541
BMI (kg·m⁻²)	23.1 (1.53)	22.6 (1.43)	0.292
BF (%)	8.9 (1.63)	9.0 (1.64)	0.803
VO₂MAX (L·min⁻¹)	3.99 (0.36)	3.98 (0.63)	0.971
VO₂MAX (mL·kg⁻¹·min⁻¹)	57.5 (6.97)	57.9 (9.53)	0.880
HR_{MAX} (bpm)	184.9 (11.11)	183.2 (8.68)	0.593
VT1 (%)	50.9 (5.63)	50.0 (4.78)	0.610
VT2 (%)	84.9 (5.85)	84.1 (5.70)	0.644
Training variables			
Total distance (km)	1121.12 (534.99)	1082.43 (810.46)	0.868
HR_{AVG} (bpm)	144.76 (8.88)	137.48 (13.11)	0.067
W_{AVG} (W)	174.86 (15.79)	163.47 (32.49)	0.435
RPE	6.34 (0.82)	6.33 (1.16)	0.975

Values are expressed as mean (SD). BMI = body mass index; BF = body fat; VO_{2max} = maximum oxygen volume; VT1 = ventilatory threshold 1 (aerobic); VT2 = ventilatory threshold 2 (anaerobic); Total distance = of all the training sessions carried out during the study period; HR_{avg} = average heart rate of all the training sessions carried out during the study period; W_{avg} = average power output of all training sessions during the study period and RPE = rating of perceived exertion of all training sessions during the study

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Table 2. Between-group comparisons in dietary intake of cyclists.

	Pre-intervention			Post-intervention		
	2S-Hesperidin	Placebo	p-value	2S-Hesperidin	Placebo	p-value
Kcal	2163.6 (519.02)	2100.2 (515.77)	0.708	1974.1 (377.97)	2133.5 (437.98)	0.237
Kcal/BM	31.1 (9.34)	30.2 (8.71)	0.768	27.9 (6.53)	30.3 (6.46)	0.249
CHO (g)	245.7 (73.46)	222.0 (69.68)	0.312	216.6 (63.47)	248.3 (58.15)	0.117
CHO/BM	3.5 (1.31)	3.2 (1.14)	0.416	3.1 (1.08)	3.5 (0.94)	0.173
PRO (g)	113.5 (25.21)	115.2 (25.37)	0.837	109.0 (23.05)	101.5 (23.67)	0.332
PRO/BM	1.6 (0.41)	1.7 (0.48)	0.778	1.5 (0.35)	1.5 (0.42)	0.596
LP (g)	80.8 (27.24)	83.5 (23.65)	0.739	71.5 (17.61)	71.6 (18.89)	0.985
LP/BM	1.2 (0.45)	1.2 (0.37)	0.758	1.0 (0.27)	1.0 (0.29)	0.823

Values are expressed as mean (SD). Kcal = kilocalories; CHO = carbohydrates; PRO = protein; LP = lipids; BM = body mass. The mean values correspond to the average of all 24-hour diet recall data collected at pre-intervention (visits 2, 3 and 4) and post-intervention (visits 5, 6 and 7). * indicates significant differences ($p \leq 0.05$).

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Table 3. Changes in biomarkers of oxygen metabolism in capillary finger blood at baseline (0), FatMax1, ventilatory threshold 1 (VT1), ventilatory threshold 2 (VT2), power output maximum (P_{MAX}), FatMax2 and EPOC during the rectangular test. Values are mean (SE).

		2S-Hesperidin										Placebo														
		Pre	FatMax1	VT1	VT2	Pmax	FatMax2	EPOC	AUC	Pre	FatMax1	VT1	VT2	Pmax	FatMax2	EPOC	AUC	Pre	FatMax1	VT1	VT2	Pmax	FatMax2	EPOC	AUC	
Hematocrit (%) (Hct)	Pre	47.77 (0.88)	47.15 (0.79)	48.21 (0.70)	48.57 (0.86)	49.09 (0.93)	48.45 (0.74)	45.74 (0.69)	286.54 (13.08)	48.13 (1.00)	48.13 (0.91)	47.37 (0.79)	47.36 (0.98)	49.90 (1.06)	47.74 (0.84)	46.09 (0.79)	287.63 (13.49)	48.13 (1.00)	48.13 (0.91)	47.37 (0.79)	47.36 (0.98)	49.90 (1.06)	47.74 (0.84)	46.09 (0.79)	287.63 (13.49)	
	Post	48.37 (0.56)	47.08 (0.87)	46.98 (0.72)	49.01 (0.61)	50.05 (0.78)	48.17 (0.71)	46.15 (0.80)	287.68 (9.69)	47.34 (0.64)	46.21 (1.00)	47.04 (0.82)	48.40 (0.70)	49.39 (0.89)	47.89 (0.81)	45.48 (0.91)	285.37 (16.89)	47.34 (0.64)	46.21 (1.00)	47.04 (0.82)	48.40 (0.70)	49.39 (0.89)	47.89 (0.81)	45.48 (0.91)	285.37 (16.89)	
	P-value	0.568	0.930	0.102	0.536	0.293	0.654	0.618	0.743	0.510	0.043	0.690	0.205	0.621	0.831	0.514	0.381	0.510	0.043	0.690	0.205	0.621	0.831	0.514	0.381	
ES	0.64	0.08	1.65	0.48	0.96	0.35	0.55	0.08	0.72	1.72	0.38	0.97	0.44	0.16	0.71	0.11	0.08	0.72	1.72	0.38	0.97	0.44	0.16	0.71	0.11	
Hemoglobin (g/dL) (Hb)	Pre	15.58 (0.28)	15.39 (0.26)	15.73 (0.23)	15.85 (0.28)	16.01 (0.31)	15.80 (0.24)	14.92 (0.23)	94.03 (4.55)	15.70 (0.32)	15.70 (0.29)	15.44 (0.26)	15.45 (0.32)	16.27 (0.35)	15.59 (0.28)	15.03 (0.26)	93.81 (4.41)	15.70 (0.32)	15.70 (0.29)	15.44 (0.26)	15.45 (0.32)	16.27 (0.35)	15.59 (0.28)	15.03 (0.26)	93.81 (4.41)	
	Post	15.79 (0.19)	15.37 (0.29)	15.33 (0.24)	15.99 (0.20)	16.32 (0.26)	15.71 (0.23)	15.05 (0.26)	94.13 (3.23)	15.44 (0.21)	15.07 (0.33)	15.35 (0.27)	15.79 (0.23)	16.11 (0.29)	15.62 (0.26)	14.84 (0.30)	93.09 (5.53)	15.44 (0.21)	15.07 (0.33)	15.35 (0.27)	15.79 (0.23)	16.11 (0.29)	15.62 (0.26)	14.84 (0.30)	93.09 (5.53)	
	P-value	0.545	0.953	0.104	0.573	0.300	0.644	0.622	0.923	0.523	0.042	0.741	0.206	0.641	0.895	0.531	0.397	0.523	0.042	0.741	0.206	0.641	0.895	0.531	0.397	
ES	0.69	0.07	1.66	0.46	0.95	0.35	0.54	0.22	0.72	1.97	0.32	0.97	0.42	0.10	0.67	0.15	0.22	0.72	1.97	0.32	0.97	0.42	0.10	0.67	0.15	
Oxyhemoglobin (%) (O ₂ Hb)	Pre	93.69 (0.72)	93.43 (0.43)	92.79 (0.61)	92.25 (0.72)	93.34 (0.53)	95.19 (0.27)	92.31 (0.62)	560.02 (6.11)	93.55 (0.82)	93.86 (0.50)	92.43 (0.70)	93.46 (0.82)	92.73 (0.60)	94.82 (0.31)	92.32 (0.71)	560.26 (5.46)	93.55 (0.82)	93.86 (0.50)	92.43 (0.70)	93.46 (0.82)	92.73 (0.60)	94.82 (0.31)	92.32 (0.71)	560.26 (5.46)	
	Post	92.13 (0.52)	93.62 (0.47)	92.87 (0.42)	91.48 (0.51)	92.92 (0.54)	94.18 (0.34)	91.55 (0.75)	556.93 (6.76)	92.96 (0.60)	94.10 (0.54)	93.41 (0.48)	92.17 (0.58)	93.40 (0.62)	95.13 (0.38)	91.46 (0.86)	560.45 (5.94)	92.96 (0.60)	94.10 (0.54)	93.41 (0.48)	92.17 (0.58)	93.40 (0.62)	95.13 (0.38)	91.46 (0.86)	560.45 (5.94)	
	P-value	0.028	0.772	0.909	0.348	0.481	0.006	0.282	0.107	0.442	0.742	0.211	0.169	0.322	0.427	0.282	0.870	0.442	0.742	0.211	0.169	0.322	0.427	0.282	0.870	
ES	2.04	0.40	0.12	0.99	0.74	3.47	1.13	0.47	0.66	0.44	1.28	1.43	1.02	0.91	1.11	0.03	0.47	0.66	0.44	1.28	1.43	1.02	0.91	1.11	0.03	
Carboxy-hemoglobin (%) (COHb)	Pre	0.646 (0.07)	0.623 (0.06)	0.631 (0.07)	0.538 (0.05)	0.446 (0.04)	0.485 (0.05)	0.662 (0.05)	3.38 (1.10)	0.500 (0.08)	0.510 (0.07)	0.490 (0.08)	0.400 (0.06)	0.370 (0.04)	0.430 (0.06)	0.580 (0.06)	2.85 (8.18)	0.500 (0.08)	0.510 (0.07)	0.490 (0.08)	0.400 (0.06)	0.370 (0.04)	0.430 (0.06)	0.430 (0.06)	0.580 (0.06)	2.85 (8.18)
	Post	0.777 (0.10)	0.685 (0.10)	0.723 (0.10)	0.654 (0.06)	0.515 (0.06)	0.631 (0.08)	0.723 (0.09)	3.96 (2.05)	0.600 (0.12)	0.580 (0.11)	0.580 (0.11)	0.530 (0.07)	0.420 (0.07)	0.450 (0.09)	0.620 (0.10)	3.17 (1.05)	0.600 (0.12)	0.580 (0.11)	0.580 (0.11)	0.530 (0.07)	0.420 (0.07)	0.450 (0.09)	0.450 (0.09)	0.620 (0.10)	3.17 (1.05)
	P-value	0.067	0.288	0.090	0.020	0.089	0.012	0.194	0.077	0.209	0.290	0.144	0.022	0.277	0.744	0.453	0.088	0.077	0.209	0.290	0.144	0.022	0.277	0.744	0.453	0.088

ES	1.87	0.94	1.17	2.01	1.75	2.63	1.13	0.49	1.22	0.91	0.98	1.94	1.08	0.31	0.63	1.63
Pre	5.08 (0.69)	5.22 (0.43)	5.97 (0.59)	7.39 (0.77)	5.52 (0.54)	3.62 (0.26)	6.33 (0.59)	17.96 (2.71)	5.20 (0.78)	4.90 (0.49)	6.33 (0.67)	5.60 (0.88)	6.09 (0.61)	3.97 (0.29)	6.40 (0.67)	17.09 (4.24)
Post	6.42 (0.53)	5.08 (0.44)	5.74 (0.41)	7.15 (0.51)	5.85 (0.54)	4.49 (0.30)	7.04 (0.74)	19.74 (3.30)	5.71 (0.61)	4.60 (0.50)	5.29 (0.47)	6.53 (0.58)	5.41 (0.61)	3.77 (0.35)	7.17 (0.85)	18.15 (3.70)
P-value	0.048	0.821	0.729	0.759	0.559	0.015	0.313	0.023	0.489	0.667	0.180	0.316	0.307	0.598	0.335	0.264
ES	1.81	0.30	0.37	0.30	0.59	3.16	1.13	0.61	0.59	0.56	1.41	0.97	1.01	0.62	1.05	0.23
Pre	0.677 (0.03)	0.723 (0.03)	0.608 (0.05)	0.600 (0.17)	0.700 (0.03)	0.700 (0.04)	0.700 (0.03)	4.05 (0.81)	0.750 (0.04)	0.760 (0.04)	0.750 (0.06)	0.540 (0.20)	0.810 (0.03)	0.780 (0.04)	0.760 (0.03)	4.66 (0.46)
Post	0.685 (0.03)	0.631 (0.05)	0.685 (0.03)	0.715 (0.04)	0.708 (0.03)	0.700 (0.03)	0.692 (0.03)	4.13 (0.39)	0.750 (0.03)	0.740 (0.05)	0.730 (0.03)	0.770 (0.04)	0.770 (0.04)	0.700 (0.03)	0.770 (0.03)	4.47 (0.60)
P-value	0.832	0.120	0.149	0.549	0.786	1.000	0.812	0.714	1.000	0.761	0.736	0.299	0.223	0.101	0.786	0.235
ES	0.23	2.58	1.41	0.63	0.24	0.00	0.28	0.09	0.00	0.48	0.31	1.08	1.06	1.68	0.31	0.38
Pre	94.84 (0.69)	94.69 (0.44)	93.94 (0.60)	92.51 (0.78)	94.38 (0.54)	96.36 (0.27)	93.57 (0.59)	566.10 (6.59)	94.73 (0.79)	95.03 (0.50)	93.59 (0.68)	94.32 (0.89)	93.83 (0.62)	95.99 (0.30)	93.50 (0.68)	566.91 (5.67)
Post	93.48 (0.54)	94.84 (0.44)	94.16 (0.42)	92.75 (0.51)	94.05 (0.55)	95.46 (0.31)	92.87 (0.75)	564.48 (6.56)	94.20 (0.61)	95.33 (0.51)	94.64 (0.47)	93.38 (0.58)	94.51 (0.62)	96.20 (0.36)	92.72 (0.86)	567.55 (6.18)
P-value	0.048	0.813	0.741	0.762	0.573	0.015	0.321	0.473	0.480	0.671	0.181	0.315	0.314	0.594	0.332	0.549
ES	1.83	0.31	0.35	0.30	0.57	3.18	1.10	0.23	0.61	0.55	1.41	0.97	1.00	0.63	1.05	0.10
Pre	74.33 (4.27)	74.62 (2.21)	78.54 (4.97)	70.95 (3.29)	90.40 (4.00)	91.62 (3.67)	70.82 (2.49)	477.01 (43.84)	74.56 (4.27)	76.09 (2.21)	69.99 (4.97)	79.11 (3.29)	87.80 (4.00)	89.28 (3.67)	71.32 (2.49)	475.62 (32.00)
Post	72.05 (1.73)	74.13 (1.46)	76.63 (2.77)	69.64 (1.37)	82.84 (2.05)	82.72 (2.40)	69.55 (2.14)	454.45 (23.35)	68.57 (1.73)	76.46 (1.46)	73.48 (2.77)	72.83 (1.37)	83.82 (2.05)	87.67 (2.40)	69.32 (2.14)	465.93 (21.43)
P-value	0.572	0.852	0.754	0.653	0.109	0.076	0.578	0.136	0.145	0.888	0.568	0.042	0.387	0.739	0.385	0.130

Deoxyhemoglobin (%) (RHb)

Methemoglobin (%) (MetHb)

Oxygen saturation (%) (sO₂)

Oxygen partial pressure (mmHg) (pO₂)

ES	0.49	0.21	0.35	0.37	1.74	2.24	0.47	0.48	1.30	0.16	0.65	1.76	0.92	0.40	0.74	0.28
Pre	42.14 (0.79)	42.01 (0.69)	41.18 (0.77)	38.59 (1.60)	35.42 (1.17)	35.38 (0.96)	39.72 (0.59)	233.55 (15.20)	39.35 (0.90)	40.42 (0.79)	40.34 (0.88)	35.19 (1.83)	34.22 (1.34)	34.12 (1.10)	38.04 (0.68)	221.69 (15.13)
Post	42.99 (0.76)	43.30 (0.73)	42.67 (0.51)	39.95 (0.73)	36.38 (1.06)	37.78 (0.81)	41.32 (0.64)	242.25 (11.97)	41.13 (0.86)	41.41 (0.83)	40.62 (0.58)	38.13 (0.83)	34.99 (1.21)	34.76 (0.92)	39.45 (0.73)	230.07 (13.63)
P-value	0.213	0.035	0.020	0.421	0.433	0.005	0.033	0.012	0.029	0.145	0.681	0.133	0.581	0.468	0.091	0.086
ES	1.02	1.75	1.80	0.79	0.77	2.34	2.51	0.54	1.81	1.15	0.29	1.47	0.53	0.53	1.91	0.50
Pre	9.14 (0.17)	8.98 (0.15)	9.14 (0.16)	9.07 (0.18)	9.37 (0.16)	9.47 (0.15)	8.64 (0.16)	54.83 (2.63)	9.22 (0.18)	9.24 (0.16)	8.95 (0.18)	9.04 (0.20)	9.50 (0.18)	9.31 (0.16)	8.70 (0.18)	55.00 (2.47)
Post	9.11 (0.13)	8.98 (0.15)	8.92 (0.14)	9.14 (0.11)	9.49 (0.17)	9.28 (0.15)	8.64 (0.19)	54.81 (1.92)	9.01 (0.14)	8.89 (0.17)	8.99 (0.15)	9.13 (0.12)	9.45 (0.19)	9.34 (0.17)	8.53 (0.21)	54.57 (3.30)
P-value	0.847	1.000	0.128	0.627	0.506	0.156	1.000	0.973	0.275	0.026	0.799	0.594	0.807	0.835	0.423	0.385
ES	0.18	0.00	1.27	0.38	0.72	1.20	0.00	0.01	1.05	1.95	0.20	0.41	0.26	0.17	0.88	0.16
Pre	28.39 (0.73)	26.91 (0.52)	26.28 (0.42)	21.51 (1.11)	15.06 (0.65)	20.51 (0.92)	25.52 (0.52)	137.88 (10.80)	26.98 (0.80)	26.17 (0.56)	26.74 (0.46)	19.52 (1.21)	14.07 (0.71)	19.68 (1.00)	24.68 (0.56)	131.85 (12.65)
Post	28.16 (0.74)	29.58 (1.19)	27.74 (0.38)	22.87 (0.56)	15.82 (0.66)	22.68 (0.78)	26.95 (0.43)	146.39 (9.25)	27.19 (0.36)	26.42 (1.29)	26.55 (0.41)	21.22 (0.61)	15.04 (0.71)	20.44 (0.85)	25.50 (0.47)	135.91 (10.61)
P-value	0.754	0.037	< 0.001	0.206	0.203	0.003	0.001	0.012	0.794	0.853	0.630	0.149	0.141	0.303	0.049	0.082
ES	0.29	4.83	3.21	1.15	1.10	2.20	2.61	0.73	0.24	0.40	0.36	1.30	1.26	0.69	1.35	0.29
Pre	26.54 (1.30)	26.29 (0.56)	29.46 (1.97)	29.37 (1.28)	34.07 (1.41)	29.60 (1.91)	26.72 (0.76)	171.66 (18.34)	25.82 (1.42)	25.90 (0.61)	25.87 (2.16)	30.99 (1.40)	34.28 (1.55)	28.99 (2.09)	26.84 (0.84)	172.38 (9.32)
Post	27.04 (0.54)	26.50 (0.61)	28.09 (0.93)	28.25 (0.54)	31.57 (0.68)	27.95 (0.76)	26.95 (0.84)	167.72 (7.17)	25.08 (0.59)	26.13 (0.67)	26.42 (1.02)	28.87 (0.59)	30.46 (0.75)	28.06 (0.83)	27.25 (0.92)	166.13 (9.11)
P-value	0.753	0.791	0.473	0.395	0.114	0.429	0.843	0.483	0.666	0.788	0.790	0.150	0.032	0.683	0.744	0.040

ES	0.35	0.35	0.65	0.82	1.64	0.80	0.27	0.20	0.48	0.35	0.23	1.39	2.25	0.40	0.44	0.61
Pre	11.72 (2.64)	11.68 (1.58)	15.54 (1.72)	17.64 (2.67)	9.42 (2.21)	6.84 (0.97)	14.23 (1.66)	73.17 (19.54)	12.58 (2.64)	11.78 (1.58)	15.70 (1.72)	11.20 (2.67)	11.96 (2.21)	7.75 (0.97)	15.97 (1.66)	72.72 (19.06)
Post	15.94 (1.38)	11.26 (1.52)	11.41 (1.35)	18.27 (1.63)	11.77 (0.95)	8.62 (0.92)	16.01 (2.02)	75.27 (12.14)	14.62 (1.38)	9.97 (1.52)	12.62 (1.35)	15.92 (1.63)	11.86 (0.95)	7.37 (0.92)	17.01 (2.02)	73.56 (14.36)
P-value	0.135	0.856	0.057	0.818	0.349	0.180	0.284	0.747	0.459	0.437	0.146	0.097	0.968	0.769	0.527	0.785
ES	1.46	0.24	2.20	0.22	0.97	1.67	0.98	0.10	0.71	1.05	1.64	1.61	0.04	0.36	0.57	0.04
Pre	32.84 (2.70)	29.07 (1.75)	32.81 (1.87)	33.99 (4.67)	22.27 (2.49)	23.10 (1.74)	34.63 (1.99)	168.93 (19.52)	31.48 (2.38)	27.38 (1.54)	33.50 (1.65)	33.17 (4.12)	25.31 (2.20)	20.62 (1.53)	34.66 (1.76)	170.28 (26.25)
Post	28.87 (1.87)	26.63 (1.80)	26.64 (1.88)	32.76 (1.93)	23.39 (2.07)	21.76 (2.45)	33.96 (2.50)	161.62 (22.46)	33.86 (1.65)	25.36 (1.59)	29.74 (1.66)	33.46 (1.70)	25.61 (1.83)	23.96 (2.16)	36.30 (2.20)	167.42 (10.78)
P-value	0.256	0.343	0.004	0.794	0.756	0.619	0.778	0.316	0.435	0.373	0.035	0.945	0.924	0.175	0.438	0.671
ES	1.28	1.22	2.87	0.23	0.39	0.67	0.29	0.35	0.90	1.19	2.06	0.06	0.12	1.96	0.85	0.10

AaDpO₂ = difference between the alveolar concentration (A) of oxygen and the arterial (a) concentration of oxygen. In bold are p-values = ≤0.05 and trends between 0.05-0.08.

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Table 4. Changes in the acid-base state and metabolic substrate in capillary finger blood at baseline (0), FatMax1, ventilatory threshold 1 (VT1), ventilatory threshold 2 (VT2), power output maximum (P_{MAX}), FatMax2 and EPOC during the rectangular test. Values are mean (SE).

		2S-Hesperidin												Placebo											
		Pre	FatMax1	VT1	VT2	Pmax	FatMax2	EPOC	AUC	Pre	FatMax1	VT1	VT2	Pmax	FatMax2	EPOC	AUC	Pre	FatMax1	VT1	VT2	Pmax	FatMax2	EPOC	AUC
pH	Pre	7.417 (0.01)	7.395 (0.01)	7.392 (0.01)	7.326 (0.01)	7.202 (0.02)	7.344 (0.01)	7.395 (0.01)	44.07 (0.13)	7.422 (0.01)	7.398 (0.01)	7.405 (0.01)	7.320 (0.02)	7.188 (0.02)	7.341 (0.01)	7.399 (0.01)	44.07 (0.17)	7.405 (0.01)	7.388 (0.02)	7.399 (0.01)	7.328 (0.01)	7.202 (0.02)	7.352 (0.01)	7.394 (0.01)	44.07 (0.15)
	Post	7.404 (0.01)	7.419 (0.02)	7.402 (0.01)	7.342 (0.01)	7.213 (0.01)	7.363 (0.01)	7.403 (0.01)	44.14 (0.08)	7.407 (0.01)	7.388 (0.02)	7.399 (0.01)	7.328 (0.01)	7.202 (0.02)	7.352 (0.01)	7.394 (0.01)	44.07 (0.15)	7.407 (0.01)	7.388 (0.02)	7.399 (0.01)	7.328 (0.01)	7.202 (0.02)	7.352 (0.01)	7.394 (0.01)	44.07 (0.15)
	p-value	0.248	0.131	0.121	0.254	0.364	0.028	0.213	0.016	0.238	0.564	0.317	0.572	0.292	0.231	0.412	0.895	0.238	0.564	0.317	0.572	0.292	0.231	0.412	0.895
Bicarbonate anion (mmol/L) (HCO₃⁻)	ES	1.11	3.21	1.87	1.07	0.69	1.48	1.25	0.54	1.15	1.15	1.11	0.46	0.79	0.78	0.77	0.00	1.15	1.15	1.11	0.46	0.79	0.78	0.77	0.00
	Pre	27.09 (0.73)	25.63 (0.51)	25.03 (0.41)	20.32 (1.07)	13.98 (0.62)	19.43 (0.90)	24.29 (0.50)	130.72 (10.40)	25.76 (0.79)	24.92 (0.55)	25.49 (0.44)	18.43 (1.17)	13.01 (0.68)	18.64 (0.98)	23.50 (0.54)	125.00 (12.27)	25.76 (0.79)	24.92 (0.55)	25.49 (0.44)	18.43 (1.17)	13.01 (0.68)	18.64 (0.98)	23.50 (0.54)	125.00 (12.27)
	Post	26.85 (0.32)	28.27 (1.18)	26.42 (0.34)	21.65 (0.55)	14.71 (0.63)	21.53 (0.76)	25.69 (0.42)	139.01 (8.94)	25.94 (0.35)	25.15 (1.29)	25.06 (0.37)	20.04 (0.60)	13.94 (0.69)	19.36 (0.83)	24.30 (0.46)	128.54	25.94 (0.35)	25.15 (1.29)	25.06 (0.37)	20.04 (0.60)	13.94 (0.69)	19.36 (0.83)	24.30 (0.46)	128.54
p-value	0.744	0.040	<0.001	0.200	0.204	0.003	0.001	0.012	0.828	0.864	0.231	0.156	0.136	0.304	0.045	0.122	0.828	0.864	0.231	0.156	0.136	0.304	0.045	0.122	
Standard bicarbonate (mmol/L) (SBC)	ES	0.31	4.85	3.20	1.16	1.08	2.19	2.63	0.74	0.20	0.38	0.89	1.27	1.26	0.69	1.36	0.26	0.20	0.38	0.89	1.27	1.26	0.69	1.36	0.26
	Pre	26.29 (0.76)	24.79 (0.46)	24.31 (0.29)	20.19 (0.84)	14.67 (0.49)	20.04 (0.72)	23.88 (0.42)	129.73 (7.73)	25.41 (0.86)	24.36 (0.52)	24.97 (0.34)	18.85 (0.96)	13.94 (0.56)	19.59 (0.82)	23.56 (0.47)	126.23 (9.58)	25.41 (0.86)	24.36 (0.52)	24.97 (0.34)	18.85 (0.96)	13.94 (0.56)	19.59 (0.82)	23.56 (0.47)	126.23 (9.58)
	Post	25.78 (0.24)	27.36 (1.35)	25.44 (0.31)	21.16 (0.44)	15.22 (0.49)	21.58 (0.61)	24.95 (0.33)	136.31 (7.67)	25.12 (0.28)	24.28 (1.54)	24.61 (0.35)	19.74 (0.50)	14.76 (0.56)	20.31 (0.70)	24.03 (0.37)	128.30 (8.21)	25.12 (0.28)	24.28 (1.54)	24.61 (0.35)	19.74 (0.50)	14.76 (0.56)	20.31 (0.70)	24.03 (0.37)	128.30 (8.21)
p-value	0.516	0.076	0.001	0.222	0.165	0.006	0.001	0.017	0.744	0.960	0.267	0.322	0.072	0.227	0.164	0.111	0.744	0.960	0.267	0.322	0.072	0.227	0.164	0.111	
Lactate (mmol/L)	ES	0.63	5.26	3.59	1.08	1.04	2.01	2.39	0.79	0.31	0.14	0.98	0.85	1.34	0.80	0.90	0.20	0.31	0.14	0.98	0.85	1.34	0.80	0.90	0.20
	Pre	1.66 (0.14)	2.62 (0.32)	2.53 (0.24)	8.45 (0.88)	14.46 (0.86)	6.98 (0.74)	3.61 (0.42)	36.72 (8.50)	1.77 (0.15)	2.92 (0.34)	2.25 (0.27)	8.68 (0.96)	15.35 (0.93)	7.46 (0.81)	4.23 (0.45)	39.32 (6.76)	1.77 (0.15)	2.92 (0.34)	2.25 (0.27)	8.68 (0.96)	15.35 (0.93)	7.46 (0.81)	4.23 (0.45)	39.32 (6.76)
	Post	1.56 (0.14)	2.62 (0.32)	2.53 (0.24)	8.45 (0.88)	14.46 (0.86)	6.98 (0.74)	3.61 (0.42)	36.72 (8.50)	1.77 (0.15)	2.92 (0.34)	2.25 (0.27)	8.68 (0.96)	15.35 (0.93)	7.46 (0.81)	4.23 (0.45)	39.32 (6.76)	1.77 (0.15)	2.92 (0.34)	2.25 (0.27)	8.68 (0.96)	15.35 (0.93)	7.46 (0.81)	4.23 (0.45)	39.32 (6.76)

Table 4. Changes in the acid-base state and metabolic substrate in capillary finger blood at baseline (0), FatMax1, ventilatory threshold 1 (VT1), ventilatory threshold 2 (VT2), power output maximum (P_{MAX}), FatMax2 and EPOC during the rectangular test. Values are mean (SE).

2S-Hesperidin																	
Placebo																	
	Pre	FatMax1	VT1	VT2	Pmax	FatMax2	EPOC	AUC	Pre	FatMax1	VT1	VT2	Pmax	FatMax2	EPOC	AUC	
pH	Pre	7.417 (0.01)	7.395 (0.01)	7.392 (0.01)	7.326 (0.01)	7.202 (0.02)	7.344 (0.01)	7.395 (0.01)	44.07 (0.13)	7.422 (0.01)	7.398 (0.01)	7.405 (0.01)	7.320 (0.02)	7.188 (0.02)	7.341 (0.01)	7.399 (0.01)	44.07 (0.17)
	Post	7.404 (0.01)	7.419 (0.02)	7.402 (0.01)	7.342 (0.01)	7.213 (0.01)	7.363 (0.01)	7.403 (0.01)	44.14 (0.08)	7.407 (0.01)	7.388 (0.02)	7.399 (0.01)	7.328 (0.01)	7.202 (0.02)	7.352 (0.01)	7.394 (0.01)	44.07 (0.15)
	p-value	0.248	0.131	0.121	0.254	0.364	0.028	0.213	0.016	0.238	0.564	0.317	0.572	0.292	0.231	0.412	0.895
ES	1.11	3.21	1.87	1.07	0.69	1.48	1.25	0.54	1.15	1.15	1.11	0.46	0.79	0.78	0.77	0.00	
Bicarbonate anion (mmol/L) (HCO ₃ ⁻)	Pre	27.09 (0.73)	25.63 (0.51)	25.03 (0.41)	20.32 (1.07)	13.98 (0.62)	19.43 (0.90)	24.29 (0.50)	130.72 (10.40)	25.76 (0.79)	24.92 (0.55)	25.49 (0.44)	18.43 (1.17)	13.01 (0.68)	18.64 (0.98)	23.50 (0.54)	125.00 (12.27)
	Post	26.85 (0.32)	28.27 (1.18)	26.42 (0.34)	21.65 (0.55)	14.71 (0.63)	21.53 (0.76)	25.69 (0.42)	139.01 (8.94)	25.94 (0.35)	25.15 (1.29)	25.06 (0.37)	20.04 (0.60)	13.94 (0.69)	19.36 (0.83)	24.30 (0.46)	128.54
	p-value	0.744	0.040	< 0.001	0.200	0.204	0.003	0.001	0.012	0.828	0.864	0.231	0.156	0.136	0.304	0.045	0.122
ES	0.31	4.85	3.20	1.16	1.08	2.19	2.63	0.74	0.20	0.38	0.89	1.27	1.26	0.69	1.36	0.26	
Standard bicarbonate (mmol/L) (SBC)	Pre	26.29 (0.76)	24.79 (0.46)	24.31 (0.29)	20.19 (0.84)	14.67 (0.49)	20.04 (0.72)	23.88 (0.42)	129.73 (7.73)	25.41 (0.86)	24.36 (0.52)	24.97 (0.34)	18.85 (0.96)	13.94 (0.56)	19.59 (0.82)	23.56 (0.47)	126.23 (9.58)
	Post	25.78 (0.24)	27.36 (1.35)	25.44 (0.31)	21.16 (0.44)	15.22 (0.49)	21.58 (0.61)	24.95 (0.33)	136.31 (7.67)	25.12 (0.28)	24.28 (1.54)	24.61 (0.35)	19.74 (0.50)	14.76 (0.56)	20.31 (0.70)	24.03 (0.37)	128.30 (8.21)
	p-value	0.516	0.076	0.001	0.222	0.165	0.006	0.001	0.017	0.744	0.960	0.267	0.322	0.072	0.227	0.164	0.111
ES	0.63	5.26	3.59	1.08	1.04	2.01	2.39	0.79	0.31	0.14	0.98	0.85	1.34	0.80	0.90	0.20	
Lactate (mmol/L)	Pre	1.66 (0.14)	2.62 (0.32)	2.53 (0.24)	8.45 (0.88)	14.46 (0.86)	6.98 (0.74)	3.61 (0.42)	36.72 (8.50)	1.77 (0.15)	2.92 (0.34)	2.25 (0.27)	8.68 (0.96)	15.35 (0.93)	7.46 (0.81)	4.23 (0.45)	39.32 (6.76)

(Lac)	1.68 (0.13)	1.85 (0.24)	1.75 (0.23)	7.05 (0.52)	14.21 (0.79)	5.49 (0.67)	2.94 (0.35)	32.10 (6.58)	1.84 (0.15)	2.28 (0.26)	2.30 (0.25)	8.35 (0.56)	14.82 (0.86)	7.22 (0.73)	3.65 (0.38)	37.47 (8.54)
Post																
p-value	0.871	0.010	0.003	0.134	0.730	0.018	0.039	0.057	0.680	0.041	0.833	0.741	0.503	0.702	0.098	0.391
ES	0.15	2.26	2.98	1.49	0.28	1.88	1.51	0.51	0.38	1.71	0.19	0.32	0.53	0.28	1.17	0.25
Pre	100.00 (2.91)	86.25 (2.97)	93.58 (3.28)	96.83 (3.37)	129.08 (6.17)	114.25 (6.08)	101.00 (6.45)	616.46 (68.76)	101.82 (3.04)	87.00 (3.10)	87.82 (3.43)	94.27 (3.52)	123.73 (6.44)	112.73 (6.35)	93.00 (6.74)	611.50 (73.23)
Post	100.00 (2.57)	87.75 (3.24)	95.42 (2.79)	95.08 (3.58)	118.67 (5.92)	109.83 (5.38)	99.58 (3.62)	598.91 (69.45)	98.55 (2.69)	91.09 (3.38)	89.00 (2.91)	93.82 (3.74)	116.91 (6.19)	106.73 (5.62)	92.73 (3.78)	591.40 (64.12)
p-value	1.000	0.677	0.589	0.616	0.090	0.487	0.782	0.248	0.231	0.282	0.738	0.900	0.277	0.368	0.959	0.255
ES	0.00	0.47	0.52	0.48	1.57	0.68	0.20	0.24	0.99	1.22	0.32	0.12	0.98	0.87	0.04	0.25
Pre	2.14 (0.79)	0.50 (0.52)	-0.02 (0.35)	-5.21 (1.14)	-13.13 (0.78)	-5.20 (1.03)	-0.52 (0.50)	26.57 (8.72)	1.23 (0.90)	0.18 (0.59)	0.75 (0.40)	-7.02 (1.30)	-14.25 (0.89)	-6.09 (1.17)	-0.91 (0.57)	30.22 (10.04)
Post	1.72 (0.28)	3.17 (1.31)	1.28 (0.36)	-3.84 (0.57)	-12.28 (0.74)	-3.42 (0.79)	0.76 (0.38)	24.13 (8.72)	0.92 (0.32)	-0.12 (1.49)	0.30 (0.41)	-5.71 (0.65)	-13.00 (0.85)	-5.12 (0.90)	-0.35 (0.44)	26.76 (8.15)
p-value	0.604	0.059	0.001	0.200	0.169	0.034	0.001	0.472	0.739	0.846	0.238	0.279	0.082	0.290	0.154	0.103
ES	0.50	4.82	3.48	1.13	1.02	1.62	2.42	0.26	0.31	0.46	1.04	0.92	1.28	0.76	0.90	0.32
Pre	2.59 (0.85)	0.72 (0.59)	0.08 (0.42)	-5.67 (1.25)	-14.04 (0.81)	-5.93 (1.43)	-0.62 (0.57)	29.22 (9.82)	1.31 (0.92)	0.06 (0.64)	0.79 (0.45)	-7.67 (1.36)	-15.25 (0.88)	-5.59 (1.56)	-1.31 (0.62)	33.13 (10.89)
Post	2.14 (0.33)	3.77 (1.41)	1.61 (0.41)	-4.10 (0.65)	-10.71 (1.79)	-3.88 (0.90)	0.92 (0.46)	26.76 (9.37)	1.21 (0.36)	0.15 (1.54)	0.51 (0.45)	-5.94 (0.71)	-14.08 (1.95)	-6.24 (0.97)	-0.62 (0.50)	29.67 (8.62)
p-value	0.606	0.046	0.001	0.187	0.106	0.128	< 0.001	0.509	0.916	0.954	0.500	0.180	0.591	0.651	0.104	0.174
ES	0.50	4.87	3.42	1.17	3.87	1.34	2.53	0.23	0.10	0.13	0.57	1.18	1.23	0.38	1.03	0.29

In bold are p-values = ≤0.05 and trends between 0.05-0.08.

Table 5. Comparison of pre-post-intervention differences between each of the rectangular test points (Baseline, FatMax1, VT1, VT2, P_{MAX}, FatMax2, EPOC) and AUC between groups.

	Between-Group Comparison							
	Δ Baseline	Δ FatMax1	Δ VT1	Δ VT2	Δ P _{MAX}	Δ FatMax2	Δ EPOC	Δ AUC
Hct (%)								
<i>Differences</i>	-0.252 (0.64)	0.334 (0.94)	0.990 (1.04)	0.040 (0.74)	1.485 (1.82)	0.189 (0.90)	0.343 (0.59)	3.402 (4.35)
<i>P-value</i>	0.70	0.72	0.35	0.96	0.39	0.83	0.56	0.44
<i>Effect size</i>	1.26	2.23	1.17	0.81	1.56	0.66	1.19	0.27
Hb (g/dL)								
<i>Differences</i>	-0.252 (0.33)	0.393 (0.27)	-0.568 (0.26)	0.234 (0.32)	0.334 (0.42)	-0.124 (0.25)	0.127 (0.26)	0.829 (1.40)
<i>P-value</i>	0.70	0.15	0.04	0.47	0.44	0.62	0.62	0.56
<i>Effect size</i>	1.28	1.77	1.24	0.86	1.51	0.58	1.15	-0.25
O₂Hb (%)								
<i>Differences</i>	-6.230 (5.48)	-5.609 (3.98)	3.321 (4.45)	-7.165 (3.89)	1.546 (5.55)	-0.931 (3.68)	-2.655 (2.97)	-3.282 (2.26)
<i>P-value</i>	0.26	0.17	0.46	0.07	0.78	0.80	0.38	0.16
<i>Effect size</i>	1.39	0.08	1.27	0.63	1.76	2.19	0.15	0.50
COHb (%)								
<i>Differences</i>	5.315 (5.19)	4.444 (4.13)	-3.763 (5.30)	5.395 (3.09)	-0.031 (5.93)	-3.753 (4.07)	-1.099 (2.70)	0.264 (0.37)
<i>P-value</i>	0.31	0.29	0.48	0.09	1.00	0.36	0.69	0.49
<i>Effect size</i>	0.43	0.13	0.04	0.31	0.46	2.25	0.45	0.23
RHb (%)								
<i>Differences</i>	-0.825 (1.88)	-0.732 (2.06)	-0.379 (2.53)	0.145 (1.67)	-1.981 (4.24)	2.405 (2.47)	2.220 (1.94)	0.718 (1.10)
<i>P-value</i>	0.66	0.73	0.88	0.93	0.65	0.34	0.26	0.52
<i>Effect size</i>	1.22	0.25	1.16	1.40	1.68	3.07	0.09	0.25
MetHb (%)								
<i>Differences</i>	-0.060 (0.72)	0.138 (0.61)	-0.963 (0.72)	0.785 (0.97)	-1.048 (0.85)	-0.381 (0.45)	-0.584 (0.78)	0.274 (0.28)
<i>P-value</i>	0.93	0.82	0.19	0.43	0.23	0.40	0.46	0.34
<i>Effect size</i>	0.03	1.19	1.78	0.57	1.61	1.83	0.53	0.49

	<i>Differences</i>	-0.855	-0.968	0.500	-1.574	-0.165	1.371	0.352	-2.263
sO₂ (%)	<i>P-value</i>	(0.75)	(0.84)	(0.84)	(1.60)	(1.81)	(0.83)	(0.86)	(2.67)
	<i>Effect size</i>	0.26	0.26	0.56	0.33	0.93	0.11	0.69	0.41
		1.20	0.24	1.17	1.39	1.65	3.08	0.11	0.26
	<i>Differences</i>	1.898	2.030	0.801	2.380	1.634	0.765	1.028	-12.868
pO₂ (mmHg)		(0.86)	(0.79)	(0.87)	(1.55)	(2.22)	(1.17)	(0.75)	(15.19)
	<i>P-value</i>	0.03	0.01	0.36	0.13	0.47	0.52	0.18	0.41
	<i>Effect size</i>	0.94	0.33	0.90	1.72	0.80	1.53	0.32	0.28
	<i>Differences</i>	0.001	0.031	0.012	-0.020	-0.001	0.008	0.005	0.330
pCO₂ (mmHg)		(0.01)	(0.02)	(0.01)	(0.02)	(0.02)	(0.01)	(0.01)	(5.01)
	<i>P-value</i>	0.96	0.05	0.11	0.23	0.96	0.43	0.46	0.95
	<i>Effect size</i>	1.31	0.50	1.92	0.91	0.15	2.18	0.25	0.14
	<i>Differences</i>	-0.005	-0.087	0.026	-0.023	0.010	0.024	-0.030	0.409
tO₂ (mmol/L)		(0.04)	(0.07)	(0.05)	(0.18)	(0.06)	(0.04)	(0.04)	(0.80)
	<i>P-value</i>	0.91	0.22	0.63	0.90	0.88	0.60	0.43	0.61
	<i>Effect size</i>	0.99	2.53	1.79	0.09	0.91	1.64	0.86	0.00
	<i>Differences</i>	0.142	4.332	-0.010	-0.125	2.992	0.561	0.146	4.457
tCO₂ (mmol/L)		(0.50)	(4.21)	(0.42)	(0.52)	(6.71)	(0.52)	(0.38)	(3.64)
	<i>P-value</i>	0.78	0.31	0.98	0.81	0.66	0.29	0.70	0.24
	<i>Effect size</i>	0.58	1.94	4.60	0.31	0.33	2.07	1.65	0.54
	<i>Differences</i>	0.085	-0.180	0.126	0.059	-0.646	-0.097	-0.068	2.308
p50 (mmHg)		(0.18)	(0.21)	(0.28)	(0.41)	(0.50)	(0.28)	(0.15)	(6.43)
	<i>P-value</i>	0.64	0.40	0.65	0.89	0.21	0.73	0.66	0.72
	<i>Effect size</i>	0.77	0.03	0.98	0.74	0.84	0.34	0.16	0.23
	<i>Differences</i>	0.011	0.037	-0.033	-0.009	-0.013	-0.041	0.012	1.268
Shunt (%)		(0.03)	(0.03)	(0.04)	(0.01)	(0.02)	(0.02)	(0.02)	(7.49)
	<i>P-value</i>	0.74	0.30	0.37	0.53	0.47	0.05	0.53	0.87
	<i>Effect size</i>	0.81	0.61	0.52	1.52	1.00	1.69	0.46	0.01
	<i>Differences</i>	-0.250	1.924	-3.316	-0.433	-2.060	-1.300	-1.176	-4.448
AaDpO₂ (mmHg)		(0.53)	(2.14)	(1.90)	(0.64)	(1.68)	(0.93)	(0.58)	(9.68)
	<i>P-value</i>	0.64	0.38	0.09	0.50	0.23	0.17	0.05	0.65
	<i>Effect size</i>	2.02	0.18	1.42	0.35	0.25	1.89	1.06	0.32

	<i>Differences</i>	-0.029	-0.072	0.198	-0.149	-0.136	0.055	0.066	0.077
	<i>P-value</i>	(0.23)	(0.32)	(0.28)	(0.27)	(0.36)	(0.24)	(0.28)	(0.03)
pH	<i>Effect size</i>	0.90	0.82	0.49	0.58	0.71	0.83	0.81	0.02
	<i>Effect size</i>	0.17	2.13	2.50	0.52	0.24	0.94	2.01	1.03
	<i>Differences</i>	-2.650	-4.495	-4.053	10.906	2.786	2.013	5.558	4.752
	<i>P-value</i>	(5.33)	(4.14)	(3.90)	(7.43)	(19.73)	(3.34)	(6.17)	(3.57)
HCO₃⁻ (mmol/L)	<i>Effect size</i>	0.62	0.29	0.31	0.18	0.89	0.55	0.37	0.20
	<i>Effect size</i>	0.55	1.92	5.48	0.27	0.36	2.08	1.66	0.59
	<i>Differences</i>	-0.100	0.348	-0.063	0.079	0.777	0.035	0.134	4.522
	<i>P-value</i>	(0.16)	(0.19)	(0.26)	(0.47)	(0.53)	(0.29)	(0.17)	(2.63)
SBC (mmol/L)	<i>Effect size</i>	0.55	0.07	0.81	0.87	0.15	0.91	0.44	0.11
	<i>Effect size</i>	0.27	1.81	5.07	0.10	0.68	1.53	1.95	0.67
	<i>Differences</i>	0.060	-0.118	0.974	-0.760	1.055	0.372	0.484	-2.772
	<i>P-value</i>	(0.70)	(0.60)	(0.71)	(0.96)	(0.84)	(0.43)	(0.80)	(3.03)
Lac (mmol/L)	<i>Effect size</i>	0.93	0.85	0.18	0.43	0.22	0.40	0.55	0.37
	<i>Effect size</i>	0.28	0.45	3.39	1.15	0.37	2.04	0.30	0.39
	<i>Differences</i>	0.635	0.435	-0.511	0.918	-0.872	-0.096	0.205	2.558
	<i>P-value</i>	(0.74)	(0.44)	(0.64)	(0.93)	(0.79)	(0.32)	(0.62)	(21.82)
Glu (mg/dL)	<i>Effect size</i>	0.40	0.33	0.43	0.33	0.28	0.77	0.74	0.91
	<i>Effect size</i>	1.26	0.71	0.19	0.37	0.60	0.25	0.22	0.18
	<i>Differences</i>	3.550	0.853	7.737	6.515	-2.786	2.524	0.211	1.020
	<i>P-value</i>	(3.23)	(4.13)	(4.30)	(3.91)	(7.51)	(6.50)	(5.42)	(4.00)
ABE (mmol/L)	<i>Effect size</i>	0.28	0.84	0.08	0.11	0.71	0.70	0.97	0.80
	<i>Effect size</i>	0.13	2.09	5.05	0.05	0.62	0.97	2.05	0.10
	<i>Differences</i>	0.763	-1.609	-8.400	-5.592	1.642	-6.097	-4.703	0.994
	<i>P-value</i>	(3.65)	(3.42)	(2.86)	(3.81)	(8.82)	(5.26)	(3.58)	(4.50)
SBE (mmol/L)	<i>Effect size</i>	0.84	0.64	0.01	0.15	0.85	0.25	0.20	0.83
	<i>Effect size</i>	0.39	1.98	4.59	0.14	1.05	2.00	2.17	0.07

Hct = hematocrit, **Hb** = hemoglobin, **O₂Hb** = oxyhemoglobin, **COHb** = carboxyhemoglobin, **RHb** = deoxyhemoglobin, **MetHb** = methemoglobin, **sO₂** = oxygen saturation, **pO₂** = oxygen partial pressure, **pCO₂** = carbon dioxide partial pressure, **tO₂** = total blood oxygen concentration, **tCO₂** = total blood carbon dioxide concentration, **p50** = oxygen partial pressure at 50% oxygen saturation, **Shunt** = relative physiological shunt, **AaDpO₂** = alveolar-arterial gradient, **HCO₃⁻** = bicarbonate anion, **SBC** = standard bicarbonate, **Lac** = lactate, **Glu** = glucose, **ABE** = actual base excess and **SBE** = standard base excess. In bold are p-values ≤ 0.05 and trends between 0.05-0.08.

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Figure

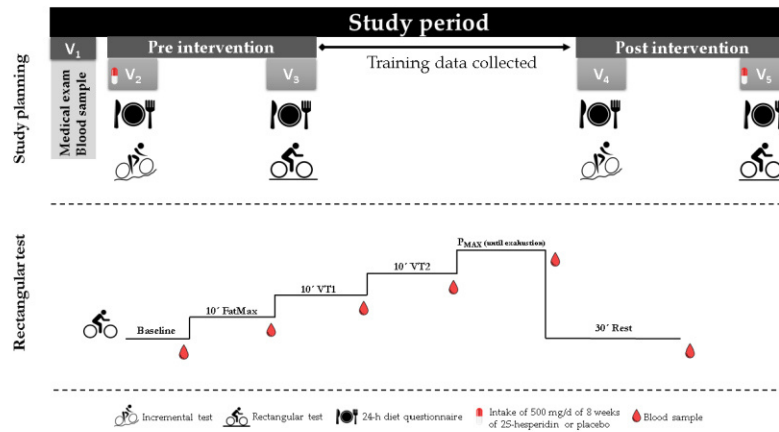
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Figure 1. Study planning.

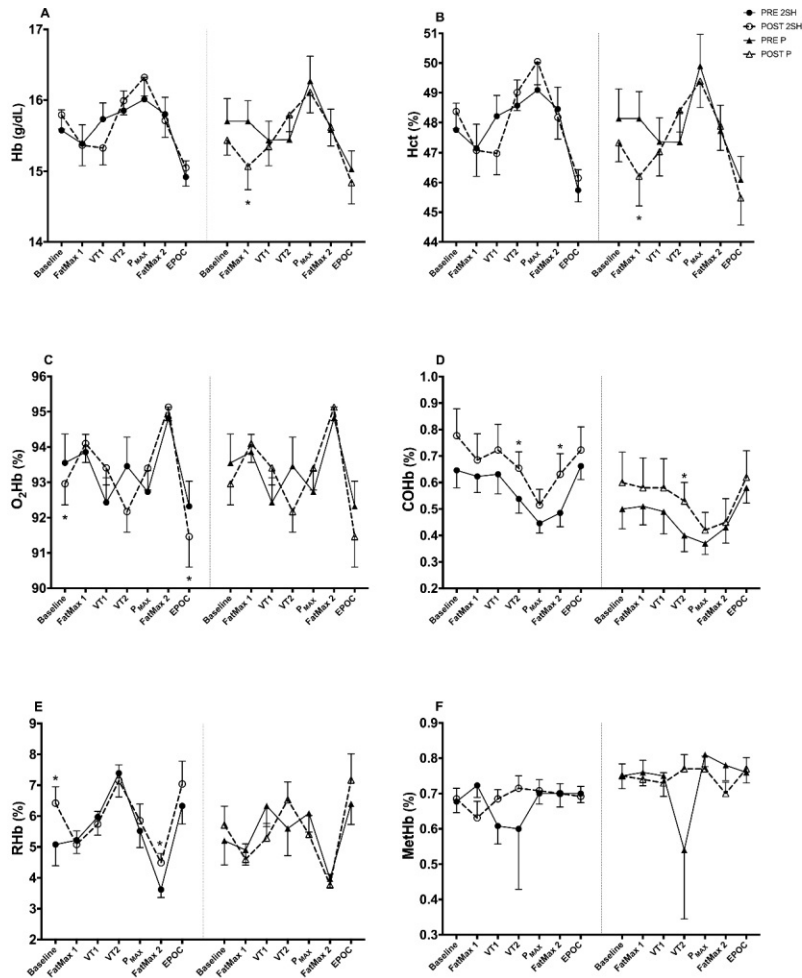


Figure 2. Differences between pre- and post-intervention within-group in finger capillary blood gas parameters at different points of the rectangular test (A-F). * $p < 0.05$.

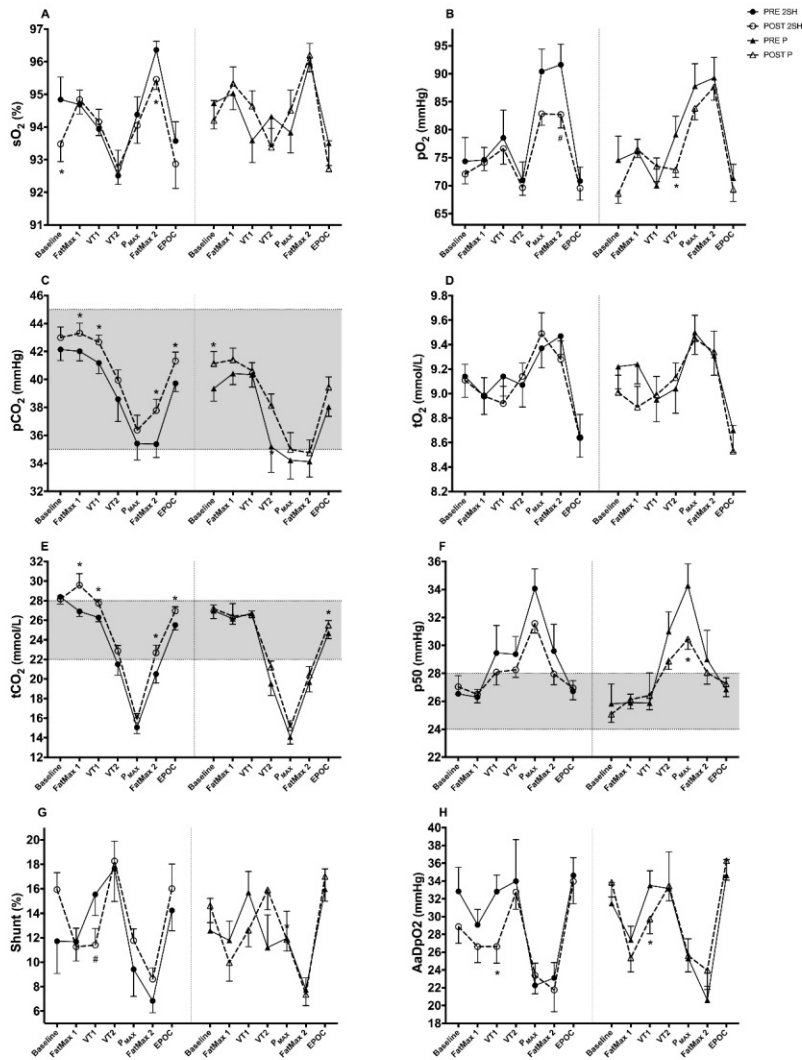


Figure 3. Differences between pre- and post-intervention within-group in finger capillary blood gas parameters at different points of the rectangular test (A-H). * p < 0.05. # p = 0.05-0.08.

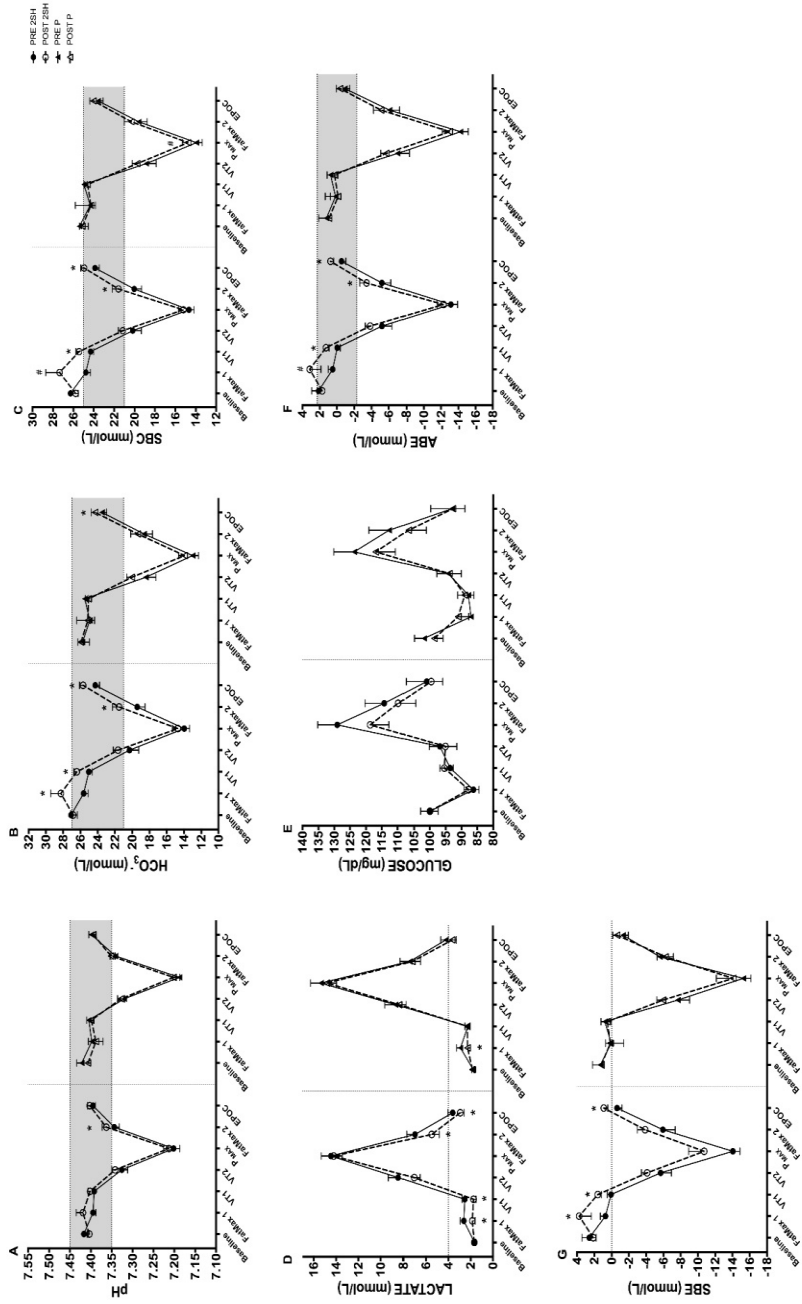
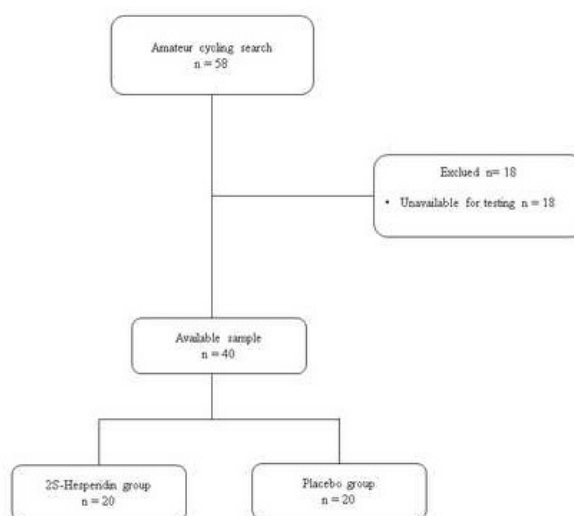


Figure 4. Differences between pre- and post-intervention intra-group differences in acid-base status parameters, lactate and glucose at different points of the rectangular test (A-G). * $p < 0.05$, † $p = 0.05-0.08$.

Flow chart of eligible participants

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