

Modeling Hypoxia/Reoxygenation Injury in Proximal Tubular Epithelial Cells

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Abstract

Kidney transplantation accounts for approximately 60%-65% of all transplanted solid organs. Most donor kidneys are obtained from deceased individuals, requiring extended cold preservation, which is a known contributor to poor transplant outcomes. Despite current preservation strategies, ischemia-reperfusion injury (IRI) remains an unavoidable consequence of transient blood flow interruption, leading to oxygen and nutrient deprivation. Within the nephron, proximal tubular epithelial cells (PTECs) of the S3 segment are particularly susceptible to IRI due to their high metabolic demand and dependence on mitochondrial oxidative phosphorylation. At the molecular level, IRI disrupts mitochondrial metabolism and reduces ATP production, compromising the energy requirements of proximal tubular epithelial cells (PTECs) and promoting apoptosis and necrosis. To investigate these mechanisms and evaluate potential therapeutic strategies, robust and reproducible *in vitro* models of renal IRI that accurately recapitulate the metabolic vulnerability of PTECs are essential. Here, we describe a protocol for the induction and assessment of hypoxia/reoxygenation (H/R) injury in murine immortalized PTECs (IM-PTECs). The protocol includes detailed information on the medium composition and culture conditions required to maintain these cells, followed by the induction of H/R injury through controlled hypoxia and reoxygenation phases that closely mimic the ischemia and reperfusion events in transplanted kidneys. This model provides a valuable platform for evaluating the effects of different interventions on renal epithelial cells exposed to H/R injury. The impact of these treatments can be assessed through the analysis of the expression of markers associated with PT damage, as well as through the assessment of the mitochondrial respiratory function. Together, these readouts offer mechanistic insights into compound efficacy and cellular recovery processes, supporting the development of targeted therapies for renal IRI.

Introduction

According to the Global Observatory on Donation and Transplantation, kidney transplantation accounts for approximately 60%-65% of all solid organ transplants worldwide, with the number of procedures performed in 2023 being nearly three times higher than that of liver transplants, the latter being the second most common type. In 61% of kidney transplants performed that year, the organ originated from deceased donors¹, which involves a longer preservation period compared to transplants from living donors, where the waiting time is significantly shorter². During this preservation period, kidneys are usually preserved using static cold storage in order to slow down cellular metabolism, which reduces oxygen and energy demand and therefore limits ischemic injury³. However, static cold storage provides only partial protection, as it cannot fully sustain cellular metabolism or oxygen delivery, leading to progressive metabolic dysfunction during preservation^{4,5}. Alternatively, *ex vivo* normothermic preservation has emerged as a viable strategy to maintain the organ at near-physiological temperature, thereby supporting cellular metabolism and oxygenation, and consequently reducing ischemia-reperfusion injury (IRI)⁶.

However, during kidney transplantation, acute kidney injury (AKI) due to IRI is an unavoidable consequence. IRI results from the interruption of blood flow through the organ, leading to an imbalance between the supply and demand of oxygen and nutrients⁷. This process has a direct impact on the function of the transplanted kidney, and increases the risk of acute rejection and reduced long-term graft function^{8,9}.

PTECs-especially abundant in the renal cortex-are particularly vulnerable to IRI. These cells constitute approximately 60%-70% of the tubular epithelium and are

responsible for the reabsorption of most of the glomerular filtrate, including water, ions, glucose, and amino acids¹⁰. Their high metabolic activity is supported by an extensive mitochondrial network to meet ATP-dependent transport demands¹¹. Consequently, they are extremely sensitive to metabolic disturbances, such as oxygen deprivation and mitochondrial dysfunction, both of which are central features of IRI¹².

During IRI, the deprivation of oxygen disrupts mitochondrial oxidative phosphorylation, leading to ATP depletion and impaired reabsorption function^{13,14}. This initiates a metabolic switch from fatty acid oxidation (FAO) to anaerobic glycolysis and activation of the pentose phosphate pathway, which, while temporarily protective, is insufficient to fully meet the energetic and redox demands of these cells¹⁵. If the suppression of FAO persists during the repair phase, lipid accumulation¹⁶ and lipotoxicity ensue¹⁷, further damaging the cells and impairing their regenerative capacity¹⁸. This maladaptive metabolic state can ultimately drive tubular atrophy, interstitial fibrosis, and progression to chronic kidney disease¹⁹.

This metabolic impairment, driven by defective FAO, hinders the ability to meet the high energetic demands of the renal tubule^{17,19}. The resulting ATP depletion and lipid accumulation promote mitochondrial dysfunction and generation of reactive oxygen species, which activate pro-inflammatory signaling and leukocyte infiltration^{20,21,22}. Persistent inflammation and oxidative injury destabilize mitochondrial integrity, amplifying energy failure and eventually triggering apoptotic and necrotic cell death^{23,24}. During this acute-injury phase, specific biomarkers such as

Kidney Injury Molecule 1 (KIM-1) and Neutrophil Gelatinase-Associated Lipocalin (NGAL) are upregulated, serving as sensitive indicators of proximal tubular damage and early AKI²⁵.

Following acute injury, maladaptive repair mechanisms promote excessive extracellular matrix deposition, a hallmark of renal fibrosis²⁶. Among the extracellular matrix components, collagen, particularly type I collagen (encoded by the *Col1a1* gene), accumulates in the interstitial space, contributing to tubular atrophy and loss of functional parenchyma²⁷. This fibrotic remodeling is largely driven by tubular senescence and partial epithelial-to-mesenchymal transition (pEMT) in tubular epithelial cells, ultimately leading to kidney functional decline^{28,29,30}. The onset of renal pEMT and fibrosis assists the progression from AKI to chronic kidney disease (CKD), culminating in an irreversible loss of kidney function and compromised graft survival^{31,32}.

In this protocol, we describe an *in vitro* model of H/R injury using mouse SV40-immortalized proximal tubular epithelial cells (IM-PTECs), subjected to controlled H/R conditions. Most preclinical studies of renal IRI have traditionally relied on animal models, which reproduce the complex vascular and immune responses of the kidney but entail higher costs, ethical concerns, and considerably higher biological variability^{33,34}. These limitations have slowed the development of complementary cell-based systems valid for detailed mechanistic and high-throughput studies. This experimental setup allows for the reproducible simulation of oxygen deprivation and restoration, mimicking key features of IRI observed in transplanted kidneys. The model serves

as a valuable tool for studying the cellular and molecular mechanisms underlying tubular injury, biomarker expression, and fibrotic responses in a controlled environment. Moreover, it provides a relevant platform for the screening and evaluation of potential therapeutic strategies aimed at preventing or mitigating ischemic damage and improving post-transplant outcomes.

Protocol

The cells used in this protocol were not isolated within the context of this study. They were previously isolated and made available by other researchers³⁵. Therefore, no new animal experimentation was performed, and ethical approval was not required for the present work.

1. IM-PTEC culture

1. Prepare L3 medium as described in **Table 1**.
2. Thaw a vial of IM-PTECs. Culture approximately 1×10^6 cells at 33 °C in a humidified incubator under standard conditions (5% CO₂, atmospheric O₂) until 80% confluence (usually reached after 3 days).
NOTE: IM-PTECs are isolated from Immorto mice. For a detailed protocol on IM-PTECs isolation, please refer to³⁵.
3. Subculture cells 2x a week at an approximate 1:10 split ratio. For passaging, detach the adherent monolayer using 0.05% trypsin-EDTA for 2-3 min at 33 °C, neutralize with complete growth medium, and reseed in fresh culture flasks.

Component	Quantity/Final Media Concentration
DMEM/F12	500 mL
Fetal calf serum (FCS)	10%

Penicillin	10,000 U/mL
Streptomycin	10,000 µg/mL
L-glutamine	0.02 µM
Insulin	5 µg/mL
Transferrin	5 µg/mL
Sodium Selenite	5 µg/mL
Triiodothyronine	40 pg/mL
Hydrocortisone	36 ng/mL
Epidermal Growth Factor	20 ng/mL
Interferon γ (IFN-γ)	10 ng/mL

Table 1: L3 medium composition.

2. Restrictive culture of IM-PTECs

1. Subculture as described in step 1.3 and transfer the entire cell suspension from one T-25 flask of IM-PTECs into a T-75 flask containing approximately 10 mL of L3 medium without interferon-γ (IFN-γ). Culture at 37 °C under standard conditions until 80% confluence (usually reached after 3 days).
2. Subculture as described in step 1.3 and transfer the entire cell suspension from one T-75 flask into two T-175 flasks containing approximately 20 mL of L3 medium without IFN-γ. Culture at 37 °C under standard conditions until 80% confluence (usually reached after 3 days).
3. Plate IM-PTECs for experiments by detaching cells from confluent cultures using 0.05% trypsin-EDTA, resuspending them in complete medium, and seeding at the desired density (approximately 1×10^5). Use 6-well plates for molecular marker expression analysis, or

96-well cell culture microplates suitable for mitochondrial respiration studies.

NOTE: For other experiments, use a suitable culture format.

1. When plating cells in a 96-well cell culture microplate for mitochondrial respiration studies, leave the four wells at the corners empty to use them as background correction. Leave the microplate for 1 h at room temperature before placing it in the incubator to allow cells to settle.

3. Hypoxia/reoxygenation procedure (Figure 1)

1. Refresh IM-PTECs by removing culture media and adding fresh L3 medium without IFN-γ (0.1 mL/well for 96-well plates or 2 mL/well for 6-well plates). Place cells into a hypoxia incubator at 1% O₂, 5% CO₂, at 37 °C for 48 h.

2. Remove cells from the hypoxia incubator and refresh them with L3 medium without IFN- γ . Place cells into a cell culture incubator at standard O₂ conditions and 5% CO₂ at 37 °C for 24 h.

NOTE: Hypoxic conditions can be achieved using either a hypoxia incubator or a modular hypoxia chamber connected to a controlled gas supply.

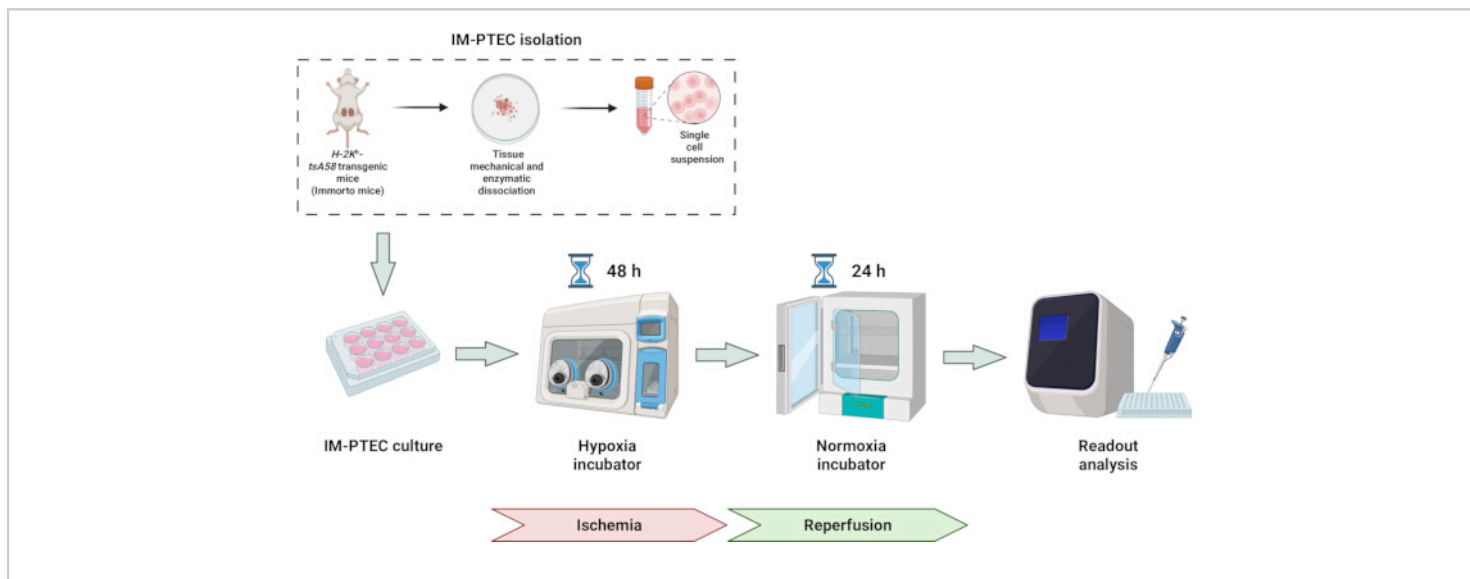


Figure 1: Schematic representation of the in vitro H/R model in immortalized proximal tubular epithelial cells (IM-PTECs). IM-PTECs derived from H-2Kb-tsA58 transgenic (Immorto) mice were cultured under normoxic conditions, exposed to hypoxia (1% O₂, 48 h), and subsequently reoxygenated (standard O₂ conditions, 24 h), for readout analysis. [Please click here to view a larger version of this figure.](#)

4. Analysis of the expression of molecular markers

1. RNA isolation
 1. Add 800 μ L of RNA isolation reagent to each well of a 6-well plate. Incubate for 5 min at room temperature. Transfer the samples to RNase-free 1.5 mL microcentrifuge tubes.
 2. Add 200 μ L of chloroform and mix well for 15 s by pipetting. Incubate at room temperature for 2-3 min.
 3. Centrifuge at 12,000 x g at 4 °C for 12 min. Transfer the aqueous top phase to a RNase-free 1.5 mL microcentrifuge tube.
 4. Add 500 μ L of isopropanol. Incubate at room temperature for 10 min. Centrifuge at 12,000 x g at 4 °C for 10 min.
 5. Discard the supernatant and wash the pellet with 1 mL of 70% ethanol. Centrifuge at 5,000 x g at 4 °C for 5 min. Discard the supernatant.
 6. Air-dry the pellet. Dissolve the pellet in 12 μ L of RNase-free water.

- Heat the sample at 56 °C for 15 min. Measure RNA concentration in a microvolume UV-Vis spectrophotometer.

NOTE: Always keep the samples on ice between incubation steps to avoid RNA degradation.

2. Reverse transcription

- On ice, add the following reaction components to a RNase-free 0.2 mL microcentrifuge tube: 10 µL of Master Mix (2x), 2 µL of Enzyme Mix, 1-5 µg RNA, and up to 20 µL of nuclease-free H₂O.
- Mix gently and incubate at 25 °C for 10 min followed by incubation at 50-55 °C for 10 min.
- Inactivate the reaction by heating at 85 °C for 5 min and chill on ice. Dilute cDNA at 1:5 or 1:10.

3. Real-Time Quantitative PCR (RT-qPCR)

- Prepare primer sets by mixing them at a final concentration of 10 µM (**Table 2**).
- For each gene to measure, prepare a Real-Time (RT) qPCR mix containing the following reagents per well: 5 µL of RT-qPCR Master Mix; 0.4 µL of 10 µM forward primer, 0.4 µL of 10 µM reverse primer, and 2.2 µL of nuclease-free H₂O.
- Add 8 µL of the RT-qPCR mix to each well of a 384-well PCR plate. Add 2 µL of cDNA sample to each well, leaving a drop on the edge of the well.
- After adding all the samples, tap the plate gently until all drops fall in the wells. Seal the plate firmly with its membrane. Spin the 384-well PCR plate.
- Measure in an RT qPCR System with the following program: 98 °C for 30 s, 40 cycles of 98 °C for 10 s followed by 60 °C for 30 s, and a melting curve at 95 °C.

Gene	Primer sequences
Kim-1	F: 5' - TGGTTGCCTTCCGTGTCTCT
	R: 5' - TCAGCTCGGGAATGCACAA
Ngal	F: 5' - ATGTCACCTCCATCCTGGTCAG
	R: 5' - GCCACTTGACATTGTAGCTCTG
Acta2	F: 5' - GCCATCTTTCATTGGGATGGA
	R: 5' - CCCCTGACAGGACGTTGTTA
Col1a1	F: 5' - CCTCAGGGTATTGCTGGACAAC
	R: 5' - CAGAAGGACCTTGTTTGCCAGG

Table 2: Primer sequences used for RT-qPCR.

5. Mitochondrial respiratory function assessment

1. Day prior to assay

1. Turn on the oxygen consumption rate (OCR)/ extracellular acidification rate (ECAR) analyzer and allow it to warm up at least 5 h before the experiment.
2. Hydrate a sensor cartridge at 37 °C in a non-CO₂ incubator overnight.

2. Day of assay

1. Prepare assay medium for one 96-well cell culture microplate containing: 33.4 mL of DMEM medium pH 7.4; 0.875 mL of glucose 1 M (final concentration 25 mM); 0.35 mL of pyruvate 100 mM (final concentration 1 mM); and 0.35 mL of Glutamine 200 mM (final concentration 2 mM).
2. Prepare inhibitor solutions as per **Table 3**.

3. Remove the assembled Sensor Cartridge from the incubator. Load 25 µL of each inhibitor into its corresponding port using a multichannel pipette and a loading guide.
4. Remove cell culture growth medium from the cell culture microplate. Add 175 µL of pre-warmed (37 °C) assay medium to each well using a multichannel pipette.
5. Place the cell culture microplate into a 37 °C non-CO₂ incubator for 45-60 min prior to the assay.
6. Place the calibration plate with the loaded sensor cartridge in the OCR/ECAR analyzer for calibration.
7. Place the cell culture microplate in the instrument for measurement.
8. After the run, save the cell culture microplate for quantification of total protein for data normalization.

	[Cartridge] (µM)	[Assay medium] (µM)	Port A	Port B	Port C
Oligomycin	12	1.5	8.4 µL		
FCCP	9	1		6.3 µL	
Antimycin A	25	2.5			17.5 µL
Rotenone	12.5	1.25			8.75 µL
Assay medium			3.5 mL	3.5 mL	

Table 3: Inhibitor solutions for the assessment of mitochondrial respiration.

Representative Results

A time-course validation was first performed to determine the optimal hypoxia/reoxygenation (H/R) conditions to reproduce ischemic injury in IM-PTECs. Caspase-3 activation, a

hallmark of renal ischemia-reperfusion injury both in vivo and in vitro, was used as an indicator of model fidelity^{36,37}. We found that 48 h of hypoxia followed by 24 h of reoxygenation produced the greatest increase in caspase-3 activity. Cell viability, assessed by the MTT assay, remained comparable

to that of normoxic controls under these conditions, indicating that while apoptotic signaling was initiated, cells remained viable for subsequent pharmacological testing (**Supplementary Figure 1**). This validated protocol was then used to induce H/R injury through controlled hypoxia/reoxygenation of IM-PTECs.

After the ischemic insult, RT-qPCR was performed to determine the expression of several markers of kidney

damage (**Figure 2**). RT-qPCR showed that, in the context of IRI, the expression of *Kim-1*^{38,39} and *Ngal*^{40,41}, known markers of acute kidney injury, was significantly increased. Furthermore, the expression of *Acta2*^{42,43} and *Col1a1*^{44,45,46}, markers of renal pEMT/fibrosis, were also increased. In addition to being a kidney pEMT/fibrosis marker, *Col1a1* expression can be correlated with CKD severity⁴⁷. /p>

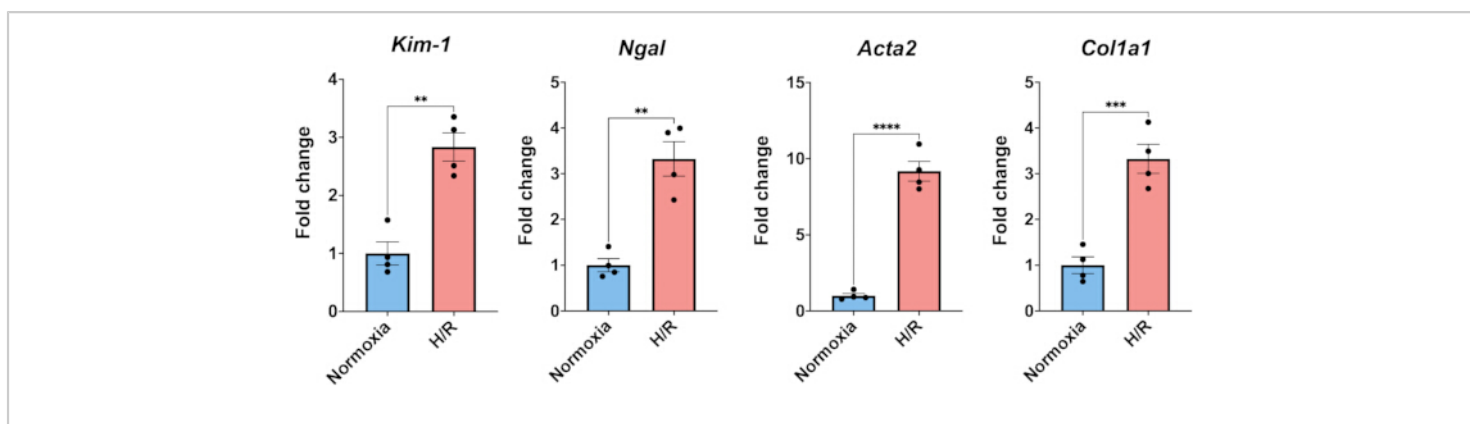


Figure 2: Molecular expression of kidney injury markers under normoxia or hypoxia/reoxygenation (H/R). After H/R, the expression of the kidney injury molecular markers *Kim-1*, *Ngal*, *Acta2*, and *Col1a1* is significantly increased in IM-PTECs. Data are presented as mean $\pm$ standard error of the mean (SEM) from four biological replicates per condition. Statistical significance was assessed using an unpaired two-tailed Student's t-test. Significance levels are indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). [Please click here to view a larger version of this figure.](#)

Mitochondrial respiration studies were also performed to determine the mitochondrial respiratory capacity after H/R (**Figure 3**). Through the measurement of the Oxygen Consumption Rate (OCR), we determined that, after the

ischemic challenge, all mitochondrial respiration parameters (basal respiration, ATP production, proton leak, maximal respiration, and spare capacity) were reduced, pointing towards hindered mitochondrial metabolism.

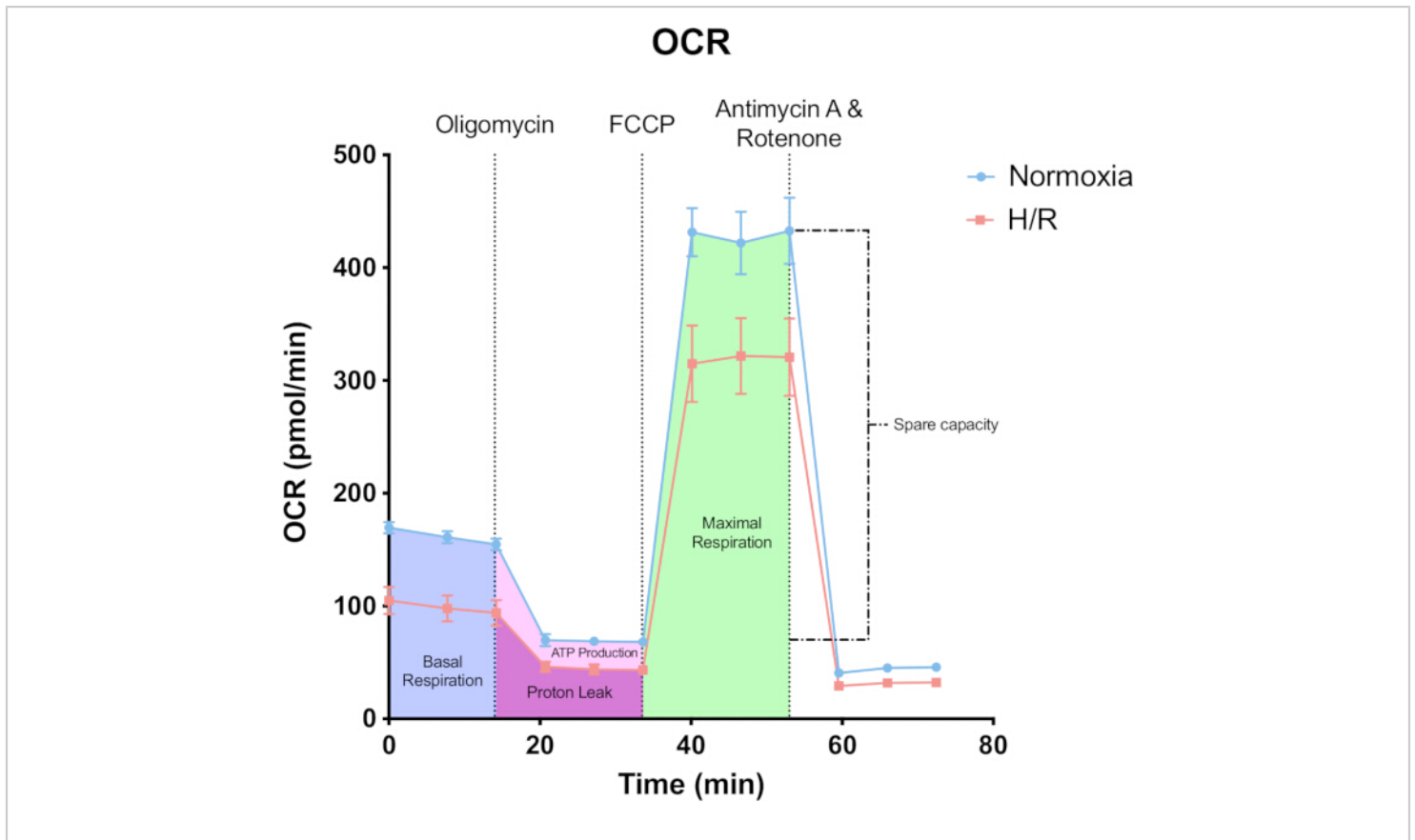


Figure 3: OCR (oxygen consumption rate) in IM-PTECs under normoxic or hypoxic conditions. Following hypoxia/reoxygenation (H/R), IM-PTECs show a reduced mitochondrial respiratory function. Mitochondrial respiration was assessed by sequentially adding electron transport chain inhibitors: oligomycin, FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazine), antimycin A, and rotenone. Data are presented as mean $\pm$ standard error of the mean (SEM) from four biological replicates per condition. [Please click here to view a larger version of this figure.](#)

These results confirm that the H/R protocol effectively induces molecular and metabolic alterations consistent with ischemic renal injury in IM-PTECs^{48,49}. This protocol therefore provides a useful framework to reproduce renal ischemia-reperfusion-like conditions *in vitro*.

Supplementary Figure 1. Cell viability and caspase-3 activity under normoxia and hypoxia/reoxygenation (H/R) conditions. (A) Cell viability. Cell viability was assessed by MTT assay after exposure to normoxia or hypoxia/reoxygenation (H/R). Results are expressed as relative viability (%) normalized to normoxic controls. (B) Cleaved caspase-3. Cleaved caspase-3 activity (nmol/min/mg protein) was quantified in cells subjected to different durations of normoxia (N), hypoxia (H), and reperfusion (R). Data are presented as mean $\pm$ SEM, and each dot represents an

individual biological replicate. [Please click here to download this figure.](#)

Discussion

Most preclinical data on renal IRI-induced AKI is generated through *in vivo* models^{50,51,52,53}, due to the lack of established *in vitro* models. This, added to the limited availability of well-characterized murine immortalized proximal tubular epithelial cell lines, constrains *in vitro* research, making the development of this protocol a valuable asset. This method provides a more accessible and reproducible platform to study renal H/R injury mechanisms under controlled conditions, while also reducing the need for animal use.

Furthermore, this protocol predominantly utilizes standard laboratory equipment, facilitating its implementation without the need for specialized and expensive instruments. The only exception is the requirement for a hypoxia incubator, which is typically available in cancer research laboratories to recreate hypoxic tumor microenvironments^{54,55,56}. This accessibility supports broader adoption and promotes reproducibility across different laboratories.

A critical consideration in this protocol is the correct culture of IM-PTECs. Cells must be cultured at 33 °C and supplemented with IFN- γ to avoid downregulation of SV40 activity. Before experimental procedures, IM-PTECs are then shifted to restrictive conditions for 7 days prior to starting the experiments. Restrictive culture is achieved by increasing the temperature to 37 °C and removing interferon-gamma (IFN- γ) from the medium for 1 week, which decreases SV40 large T antigen expression—a viral protein encoded by Simian Virus 40, used to immortalize the cells^{35,57}—causing cells to lose their immortalized status and promoting a more differentiated and physiologically relevant phenotype⁵⁸.

Notably, IM-PTECs can develop prominent cytoplasmic vacuoles after several days in culture; if vacuolation occurs, hydroxycortisone should be omitted from the culture medium to prevent or reverse this change.

While the use of immortalized PTECs offers the advantage of stability and ease of maintenance, researchers may also consider using primary proximal tubular epithelial cells to increase physiological relevance. However, mouse primary PTECs present greater donor-to-donor variability^{59,60} and have limited proliferative capacity^{61,62}, making them less suitable for high-throughput or long-term experiments. Their use may be more appropriate for validation studies or when studying specific patient-derived responses.

This protocol provides a valuable platform to investigate the molecular mechanisms underlying H/R injury, identify and evaluate potential therapeutic approaches, and explore cellular responses such as inflammation, oxidative stress, and cell death pathways in a controlled environment^{63,64}.

Nevertheless, an important limitation of this model is that it does not reproduce certain features of ischemia observed in kidney transplantation, such as hypothermia and nutrient deprivation during cold storage⁹. These conditions markedly influence cellular metabolism and injury patterns, and their absence may restrict the translation of findings to the clinical setting^{65,66}.

In conclusion, this *in vitro* H/R model in IM-PTECs provides a reproducible, accessible, and ethically favorable approach to study renal ischemia-reperfusion injury under controlled conditions. While it does not fully recapitulate the complexity of *in vivo* ischemia, it represents a valuable complementary tool to elucidate cellular mechanisms, screen therapeutic

candidates, and reduce reliance on animal experimentation in kidney research.

Disclosures

The authors declare that they have no conflicts of interest with this work to disclose

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