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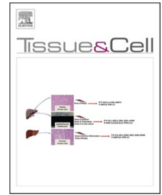

Assessment of the anti-inflammatory and biological properties of Bioroot Flow: A novel bioceramic sealer.

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A R T I C L E I N F O

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A B S T R A C T

Introduction: BioRoot Flow (BRF) is a novel premixed bioceramic sealer indicated for endodontic treatments, but the biological and immunomodulatory effects of this endodontic sealer on human periodontal ligament stem cells (hPDLSCs) have not been elucidated.

Methods: To ascertain the biological impact of BRF, TotalFill BC Sealer (TFbc), and AH Plus (AHP) on human Periodontal Ligament Stem Cells (hPDLSCs), assessments were conducted to evaluate the cytocompatibility, cellular proliferation, migratory capacity, osteo/cementogenic differentiation potential, the ability to form mineralized nodules, and the immunomodulatory characteristics of hPDLSCs following treatment with these endodontic sealers.

Results: Biological assays showed adequate cell metabolic activity and cell migration in BRF, while SEM assay evidenced that TFbc and BRF groups demonstrated a superior cell adhesion process, including substrate adhesion, cytoskeleton development, and spreading on the niche-like structures of the cement as compared to the AHP group. TFbc and BRF-treated groups exhibited a significantly lower IL6 and IL8 production than AHP (* $p < .05$). The bioceramic sealers stimulated heightened expression of BSP, CEMP-1, and CAP genes within a 7–14 day period. Notably, BRF and TFbc demonstrated a significant enhancement in the mineralization of hPDLSCs when compared to the negative control. Among these, cells treated with BRF showed a more substantial accumulation of calcium (*** $p < .001$).

Conclusions: Taken together, these findings indicate that BRF can potentially enhance cell differentiation by promoting the expression of essential genes related to bone and cement formation. In addition, BRF and TFbc displayed anti-inflammatory effects.

1. Introduction

Endodontic sealers play a fundamental role in biological interactions with the surrounding tissues, as they promote bonding between gutta-percha the dentin walls, in addition to allowing the sealing of secondary, lateral canals, apical deltas, and isthmuses, that induce healing when in contact with periapical tissues (Souza et al., 2023; Zamparini et al., 2023).

Among endodontic sealers, the hydrophobic epoxy-resin AH Plus sealer (Dentsply DeTrey, Konstanz, Germany) is considered the gold standard due to its long-term sealing integrity, low dimensional change, high radiopacity, allowing tag formation, and interfacial adaptation (Saghiri et al., 2023). However, some disadvantages, such as potential cytotoxicity, non-immunomodulatory properties, and difficulty of removal during retreatment, have favored the emergence of new materials (Kim et al., 2021).

Calcium silicate-based sealers or bioceramics have been successfully used in clinical endodontics (Santos et al., 2021). Various forms of

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bioceramics have been introduced as powder-liquid and premixed products (Kwak et al., 2023a). These materials with high concentrations of di- and trisilicates of calcium, hydroxyapatite, alumina, glass ceramics, zirconia, bioactive glass, and calcium phosphates were developed. These not only form calcium- and phosphate-based precipitates and promote remineralization of hard tissues, but also have immunomodulatory properties (Castro-Jara et al., 2023). Thus, it has been described that the therapeutic effects of bioceramic sealers are attributed to their anti-inflammatory and modulatory properties. These properties play a crucial role in maintaining bone homeostasis and facilitating the repair of damaged tissue. (Guo et al., 2023).

Among the bioceramic sealers available, TotalFill BC sealer (FKG Dentaire, La Chaux-de-Fonds, Switzerland) has shown chemical reactions that occur through interaction with the periapical tissues. These provide calcium release and alkalinization of the medium, chemical stability with the environment (Tanomaru-Filho et al., 2017; Zordan-Bronzel et al., 2019), induce biological and bioactive effects, exhibit a long-lasting antibacterial action, and promote interfacial adaptation and penetration into the root canal system (Lopez-Garcia et al., 2019). Another sealer with bioactive properties has recently been developed, i.e., BioRoot Flow (Septodont, Saint Maur Des Fosses, France), with components that include tricalcium silicate, calcium carbonate, zirconium oxide, propylene glycol, aerosil (silica), acrylamide/sodium acryloyldimethyltaurate copolymer, povidone, isohexadecane, and polysorbate (Bhandari et al., 2023). However, no information exists on its biological/bioactivity and immunomodulatory properties.

Among the many desirable properties of sealers is biocompatibility (Janini et al., 2023). This property refers to the ability of the material to interact with vital tissues without inducing adverse tissue reactions, such as systemic or local toxicity, mutagenicity, genotoxicity, or carcinogenicity. Therefore, these materials must demonstrate a high level of biocompatibility to promote tissue healing and improve the overall success of endodontic treatment (Sanz et al., 2021a). Bioactivity refers to the inherent ability of biomaterials to induce hydroxyapatite formation by stimulating the migration, proliferation, and osteogenic differentiation of precursor cells (Vallittu et al., 2018). Regarding endodontic biomaterials, the target precursor cells are mesenchymal stem cells from the oral cavity. These cells are multipotent and easily accessible for testing (Diomede et al. 2019). Specifically, dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) are often used for in vitro experimentation. For the assessment of the biological properties of endodontic sealers, the latter appear as relevant due to their contact in the event of sealer extrusion (Sanz et al., 2021).

Bioceramic materials are bioactive materials capable of facilitating hard tissue formation when in contact with pulpal or periodontal tissues (Sequeira et al., 2021; Sequeira et al., 2018). The mineralization potential is closely related to local inflammation and vascularization events, and the interaction between periapical cells plays a crucial role in achieving positive apical healing outcomes (Lopez-Garcia et al., 2020). Osseous tissue and cementum formation is crucial for periapical regeneration, aiming to create a "biological seal" to form an ideal environment for the repair of periapical lesions (Holland & Souza, 1985). Accordingly, it is paramount to investigate the impact of bioceramic sealers on the expression and release of cementogenic and osteogenic factors by precursor cells (Rodriguez-Lozano et al., 2019). The evaluation of bioactive properties involves the quantitative analysis of odontogenic, osteogenic, and cementogenic markers or genes, followed by the assessment of alizarin red staining (Shokrzadeh et al., 2023).

Consequently, the objective of this research was to assess the biological and immunomodulatory characteristics of BioRoot Flow and TotalFill BC Sealer, in comparison to the epoxy resin-based sealer AH Plus. The null hypothesis tested was that there would be no significant difference in the properties tested between the endodontic sealers.

2. Material and methods

2.1. Cell culture and characterizations of hPDLSCs

The protocol for isolating human periodontal ligament stem cells (hPDLSCs) was approved by the University of Murcia Ethics Committee (IRB number: 3686/2021). Before inclusion in this study, written informed consent was obtained from all subjects. hPDLSCs were obtained from the periodontal tissues from impacted third molars of patients (n=10; age, 16–22 years) and cultured in DMEM (Sigma-Aldrich Corporation, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich Corporation) and 1 % penicillin/streptomycin (Gibco-BRL, Gaithersburg, MD, USA) as previously reported (Lopez-Garcia et al., 2023). Subsequently, 1×10^5 cells were resuspended in 100 mL of PBS containing 1 % FBS, and the cocktail of fluorescence-conjugated specific monoclonal antibodies cocktail (CD14, CD20, CD34, CD45, CD73, CD90, and CD105; Miltenyi Biotec, Bergish Gladbach, Germany), to verify the mesenchymal phenotype of hPDLSCs by flow cytometry.

2.2. Material extracts and preparation

BioRoot Flow (BRF; Septodont, Saint Maur Des Fosses, France), TotalFill BC Sealer (TFbc; Innovative BioCeramix Inc., BC, Canada) and AH Plus sealer (AHP; Dentsply DeTrey, Konstanz, Germany) were tested in this study (Table 1). 30 cylindrical rubber molds were prepared, each measuring 5 mm in diameter and 2 mm in height. The molds were then disinfected by exposure to UV light for 30 min. Sealers were prepared according to the manufacturer's recommendations and allowed to set. Each disc was placed in a separate well of a 24-well plate and then immersed in fresh growth medium at 37 °C for 24 h. The original extracts (1:1) were prepared according to ISO 10993–5. Different dilutions (25 %, 50 %, and 100 % v/v) of these extraction media were then prepared as previously reported (Lopez-Garcia et al., 2019).

2.3. Cytocompatibility test

The assessment of metabolic activity of hPDLSCs in the presence of bioceramic sealers was conducted using the MTT assay as previously described (Lozano-Guillen et al., 2022). Briefly, hPDLSCs were seeded in 96-well culture plates (5×10^3 cells per well) and exposed to different concentrations (25 %, 50 %, and 100 %) of each sealer extract for 24, 48,

Table 1
Tested materials.

Materials	Manufacturer	Composition	Lot Number
BioRoot Flow	Septodont 58, Rue du Pont de Créteil 94107 Saint-Maur-des-Fossés Cedex-France	Tricalcium silicate, propylene glycol, povidone, calcium carbonate, AEROSIL, zirconium oxide, acrylamide / sodium acryloyldimethyltaurate copolymer, isohexadecane, polysorbate	**B29728A**
TotalFill BC Sealer	Innovative BioCeramix Inc. 101–8218 North Fraser Way Burnaby, BC V3N 0E9 Canada	Zirconium oxide, Tricalcium silicate, Dicalcium silicate, Calcium hydroxide.	**22003SP**
AH Plus	Dentsply, Konstanz, Germany	Epoxy paste: diepoxy, calciumtungstate, zirconium oxide, aerosol, and dyeAmine paste: 1-adamantane amine, N'dibenzyl-5 oxanonandiamine-1,9, TCD-diamine, calciumtungstate, zirconium oxide, aerosol, and siliconeoil	**2211000712**

and 72 h. Cells cultured in a complete medium and not exposed to any sealer were used as a negative control. At each selected timepoint, 5 mg/mL MTT reagent was added to each well, and the plates were incubated at 37 °C under 5 % CO_2 for 4 h. Finally, the mitochondrial activity was measured at 570 nm using a microplate reader (ELx800, Bio-Tek Instruments, Winooski, VT, United States).

2.4. Cell migration

The migratory capacity of hPDLSCs cultured with the bioceramic sealers was determined using *in vitro* wound healing assays. The hPDLSCs were seeded in 12-well plates (2×10^4 hPDLCs per well), and an artificial scratch was made using a 100 μL sterile pipette tip. The wounded areas of the treated and control groups were imaged at 0, 24, 48, and 72 h, and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, United States). Each material was tested three times in triplicate experimental conditions.

2.5. F-actin cytoskeleton staining

A phalloidin-based assay was conducted to detect any cytoskeletal variations in hPDLSCs consequent to their exposure to undiluted bioceramics (Lozano-Guillen et al., 2022). The cells were subjected to the treatment for a span of 72 hours and subsequently fixed with 4 % paraformaldehyde (PFA) (Merck Millipore, Darmstadt, Germany) for a duration of 10 minutes. Post-fixation, the cells were blocked for 30 minutes using 5 % bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, United States) and then incubated with TRITC-conjugated phalloidin (Invitrogen, Carlsbad, CA, United States) or phosphate-buffered saline for the controls, extending over an hour. Nuclear staining was carried out using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (ThermoFisher, Waltham, MA, USA), followed by the acquisition of fluorescent images using microscopy equipment (Leica, Wetzlar, Germany).

2.6. Scanning electronic microscopy

hPDLSCs (5×10^3 cells/well) were cultured directly on the surface of the sealer discs (n=9) in 48-well plates and incubated for attachment in a 5 % CO_2 incubator at 37 °C for 72 h. The discs with the hPDLSCs were washed three times with PBS and then fixed in 2.5 % glutaraldehyde within 0.1 M Na-cacodylate buffer. The samples were then dehydrated with ethanol and culminated in hexamethyldisilane-assisted drying. The dry samples were coated with gold and palladium and scanned by (Jeol 6100 EDAX; Jeol Inc.) in three different regions at magnifications of 100×, 300× and 1500×.

2.7. Elisa assays

To analyze the ability of hPDLSCs to secrete anti-inflammatory molecules via ELISA, supernatants were obtained by culturing the cells in complete medium for 72 h at 37 ºC under the same conditions as above. The levels of the proinflammatory cytokines IL6 and IL8 were measured in the supernatants using specific human ELISA kits (Elabscience; Bethesda, MD, United States). Absorbance was measured at 450 nm using a microplate reader.

2.8. Gene expression analysis

Following 7 and 14 days of exposure to the experimental sealers (n=3), total cellular RNA was isolated from both the test and control groups using TRIzol reagent (Invitrogen Corporation) and subsequently converted into complementary DNA via a PrimeScript RT reagent kit (Takara Bio, Inc., Shiga, Japan), adhering to the guidelines provided by the manufacturer. The gene expression patterns of bone sialoprotein (BSP), cementum attachment protein (CAP), and cementum protein 1 (CEMP1) were evaluated using quantitative polymerase chain reaction (qPCR), as described in previous research(Sanz et al., 2020). The mRNA levels of these specific genes were standardized against GAPDH expression, which functioned as the reference gene, employing the 2-ΔΔCT approach. As a negative control, untreated hPDLSCs were used, while cells cultivated in StemMACS OsteoDiff Media (Miltenyi Biotec), a commercially available medium for osteogenic differentiation, served as the positive control. Replication of these experiments was performed in triplicate.

2.9. Alizarin Red S staining

The impact of bioceramic sealers on the capacity of hPDLSCs to produce calcified nodules was assessed using Alizarin Red S staining. The procedure for seeding cells and the composition of experimental groups were identical to those used in the gene expression tests, encompassing both negative (basal growth media) and positive controls (OsteoDiff media). After 21 days of cultivation, the cells were fixed with 4 % paraformaldehyde for 30 min and stained with alizarin red S (Sigma-Aldrich Corporation). Finally, the samples were photographed using a light microscope (Olympus CKX41; Olympus, Tokyo, Japan). Colorimetry was carried out by measuring the absorbance at 577 nm using a microplate reader. Each treatment and time point were replicated thrice to ensure the reliability of the results.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism v8.1.0 (GraphPad Software Inc., San Diego, CA, United States). Results are presented as the average of three independent experiments. A Q-Q plot was first used to assess the normality of the data distribution. Statistical significance was analyzed by one-way ANOVA followed by Tukey post-hoc test or Mann-Whitney test, depending on whether or not the data met the requirements for normality and homogeneity of variance requirements. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Characterization and cell viability

hPDLSCs exhibited a high expression of CD73, CD90, and CD105, while they did not express hematopoietic markers. This confirmed their mesenchymal profile (Fig. 1A). The results of the MTT assay, as shown in Fig. 1B, demonstrated higher toxicity of AHP than the other sealers at all time points ($p < .001$). No differences were observed between control and 1:2 and 1:4 of TFbc, and only a slight difference was observed with undiluted TFbc at 72 h. In general, bioceramic sealers showed increasing cell viability over time.

3.2. Cell migration and cell morphology

The wound healing assay results suggest that BRF did not significantly alter the behaviour of the cells at any time point compared to the untreated control group. On the other hand, the group of cells cultured with undiluted TFbc exhibited a reduced rate of wound closure compared to the control group at 24 h and 48 h ($p < .001$), while there was no significant difference between the two groups at 72 h. Remarkably, significant differences were observed in the cells treated with AHP compared to the untreated cells ($p < .001$); Fig. 2A,2B).

Phalloidin staining revealed a significant decrease in F-actin density in hPDLSCs exposed to AHP, whereas a high confluence of cells with an increase in F-actin stress fibers and focal adhesion complexes were observed in the presence of control, TFbc, and BRF-treated groups (Fig. 2C).

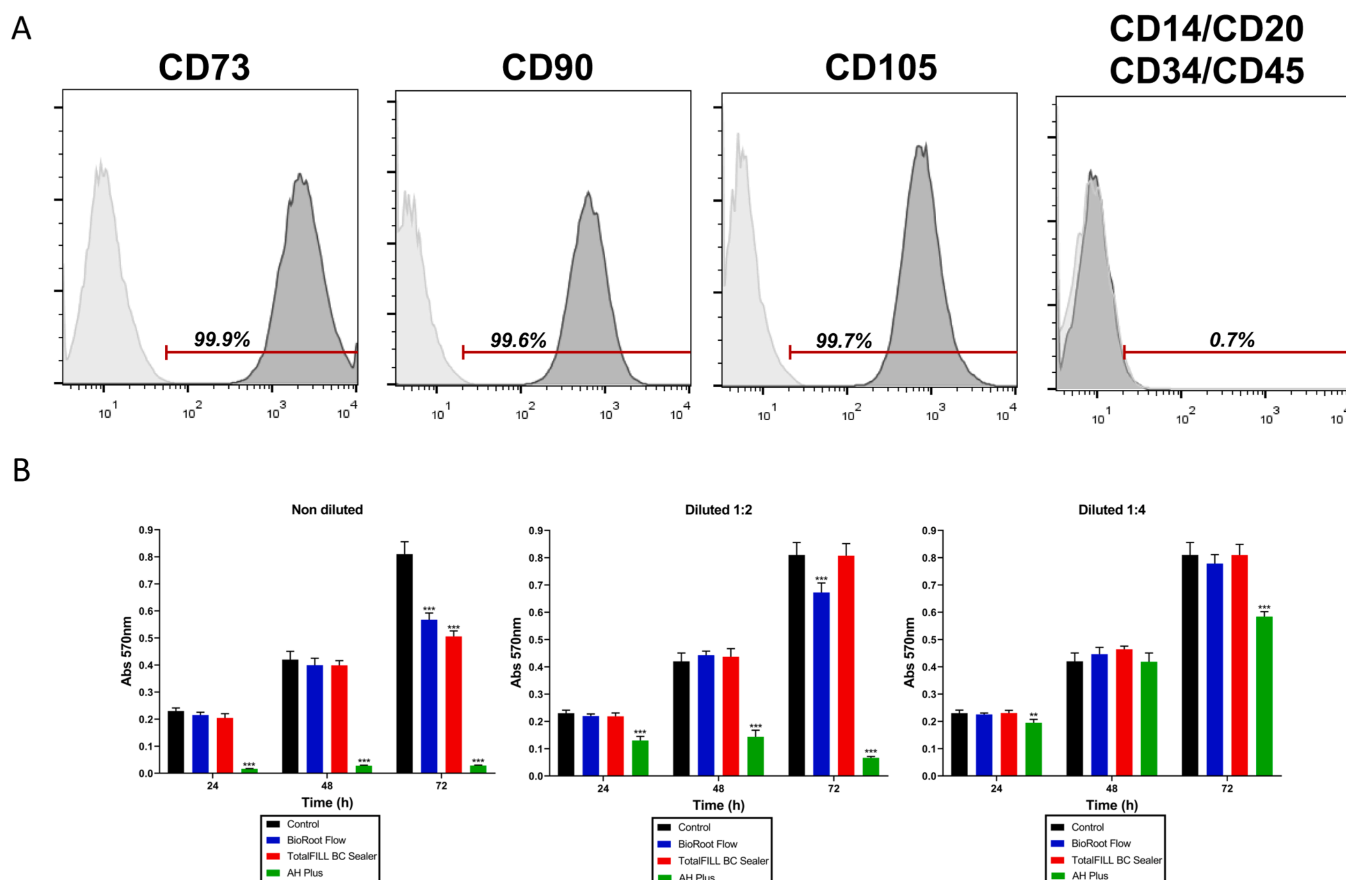

Fig. 1. A) Evaluation of hPDLSCs MSC immunophenotype through flow cytometry analysis. hPDLSCs were incubated in complete medium at 37º C for 96 hours. Subsequent assessment of MSC markers CD73, CD90, and CD105, along with hematopoietic markers CD14, CD20, CD34, and CD45 was conducted. Displayed are the mean fluorescence intensity values for each experimental condition. Histograms shown are representative of three separate experiments. B) Results of MTT assays at 24, 48, and 72 hours for hPDLSCs cultured with either the test or control groups. Asterisks denote statistically significant differences compared to the control group: * $p < .05$; ** $p < .01$; *** $p < .001$.

3.3. SEM and ELISA assays

SEM analysis at a magnification of 1500x revealed that the groups TFbc and BRF exhibited cellular filopodia attaching the cells to the surface of the calcium silicate cement. Additionally, these groups demonstrated enhanced cell adhesion, characterized by improved substrate attachment, spreading, and development of the cytoskeleton, on the niche-like formations of the cement. In contrast, the AHP group displayed a sparse cell population lacking fibroblastic morphology (round cells) (Fig. 3A). To verify whether the bioceramic sealers could attenuate the synthesis of proinflammatory cytokines, the levels of IL6 and IL8 were analyzed. For IL6 and IL8, production was significantly higher in AHP compared to control, TFbc, and BRF-treated groups ($p < .001$) (Fig. 3B). Interestingly, TFbc and BRF-treated groups had significantly lower IL6 production than the control ($p < .05$; $p < .01$, respectively) (Fig. 3B).

3.4. Gene expression and Alizarin Red assay

The effects of bioceramic sealers on the expression profiles of genes associated with osteo/cementogenic differentiation and mineralization were further investigated by qRT-PCR and Alizarin Red analysis. As shown in Fig. 4A, the gene expression levels of BSP, CEMP-1, and CAP were significantly higher in the TFbc group compared to the control group (and relatively lower in the Osteodiff group at day 7 ($p < .01$). In addition, the expression levels of BSP, CEMP-1, and CAP were increased in the BRF group as compared to the control group at day 14 ($p < 0.01$). Mineralized nodules are indicative of osteo/cementogenic differentiation. After incubation for 21 days, alizarin red staining indicated that mineral deposition was more significant in the BRF group than in the TFbc and AHP groups ($p < .001$) (Fig. 4B). Collectively, these results provide compelling evidence that TFbc and BRF promote osteo/cementogenic differentiation of hPDLSCs (Fig. 4B).

4. Discussion

In the present study, we aimed to compare the biological and immunomodulatory properties of BioRoot Flow and TotalFill BC Sealer, with those of the epoxy resin-based AH Plus sealer in vitro. Our findings showed variability in the materials' responses tested under most experimental conditions. Based on these results, we reject the null hypothesis that there are significant differences between the three tested materials in terms of their biological and immunomodulatory effects.

Recently, numerous bioceramic or calcium silicate-based materials have been introduced to the market (Camilleri et al., 2022; Castro-Jara et al., 2023). Several authors have focused mainly on studying three materials: BioRoot RCS, Endosequence BC Sealer, and AH Plus, with the latter being the most studied material (Kebudi Benezra et al., 2018). However, no information is available on the anti-inflammatory and biological properties of BioRoot Flow. Therefore, we included three materials in this study: TotalFill BC Sealer, AH Plus, and BioRoot Flow. All premixed sealers tested in this study were incubated as fixed eluates. This is consistent with most in vitro studies on the biological interaction between calcium silicate-based sealers and dental stem cells (Ratnayake

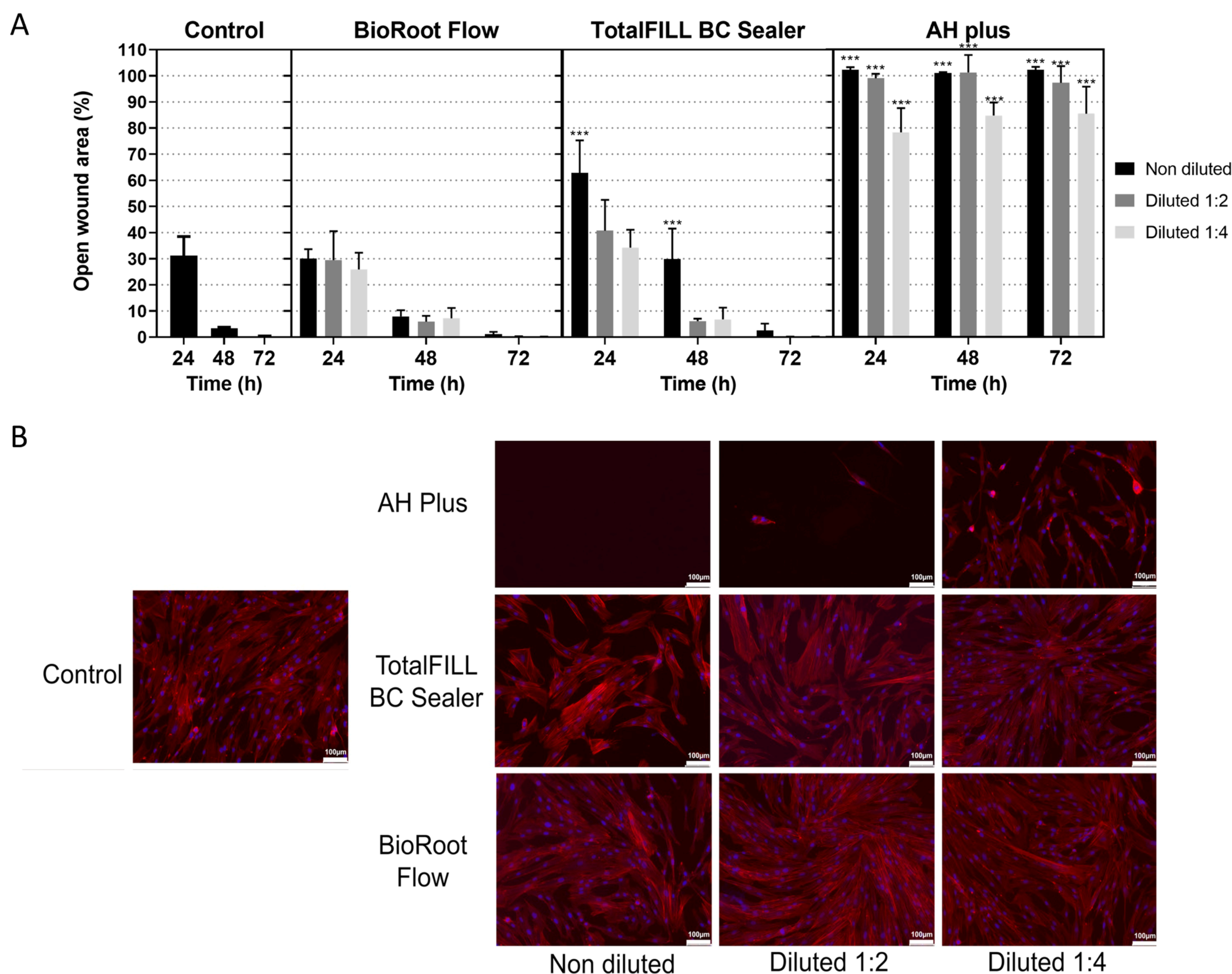

Fig. 2. A) Outcomes of cell migration assays at 24, 48, and 72 hours for hPDLSCs cultured with the test or control groups. Asterisks mark significant deviations from the control group: ** p< .01; *** p <.001. B) SEM images showcasing the surface adhesion and morphology of hPDLSCs at magnifications of 100x, 300x, and 1500x.

et al., 2023; Sanz et al., 2021b).

Human PDLSCs have multipotent properties that allow them to proliferate, migrate to injury sites, and differentiate into cementoblast-like cells that promote the formation of new mineralized bone/ cement-like tissue (Abedian et al., 2020; Tatullo et al., 2019). When used in endodontic treatments, calcium silicate-based cements come into direct contact with periapical tissues. Therefore, evaluation of the cytotoxicity and bioactivity of these materials on hPDLSCs is crucial for the repair of these tissues (Lopez-Garcia et al., 2023).

In the present study, the cytotoxicity of the materials was assessed using the MTT assay, which determines the number of viable hPDLSCs based on their mitochondrial activity. Our MTT analysis showed that undiluted concentrations of AHP were associated with a significant reduction in mitochondrial activity compared to the untreated cells (*** $p < .001$). This finding aligns with prior research showing the cytotoxic effects of this sealing compound, particularly when it is in its initial, non-hardened state, owing to the release of formaldehyde or bisphenol-A during the curing process(Nguyen et al., 2023; Souza et al., 2023). In agreement with our results, another study reported that Endosequence BC Sealer and TFbc had better cell viability rates at 72 hours than AHPbc using human periodontal cells (Kwak et al., 2023b).

In terms of cell migration, it has been described that the wound healing closure in the scratch assay involves proliferation and migration capacity (Nguyen et al., 2023). In fact, biological interactions between biomaterials and cells can cause cellular degeneration and delay wound healing (Sanz et al., 2021a). In this study, BRF-treated cells showed behavior similar to that of the untreated group (control) at all times in the wound healing assay. In contrast, cells cultured with undiluted AHP decreased wound closure compared to the control group. Previous works have demonstrated that bioceramics improve the wound healing and tissue regeneration environment by modulating macrophage function, thereby increasing the production of cytokines with anti-inflammatory activities and reducing pro-inflammatory cytokines (Alchawoosh et al., 2023; Guo et al., 2023).

Previous studies have reported that cell spreading and adhesion to a material surface are the initial stages of cellular function, which plays a crucial role in regulating intercellular signal transduction and cell differentiation (Ahmed et al., 2014; D'Anto et al., 2010). Thus, in our study, cell attachment was performed using SEM, which revealed cellular filopodia anchoring the cells to the BRF and TFbc surface, and the low presence of these cells on the AHP. Calcium plays an essential role in fibroblast adhesion, and increased cell attachment is associated with Ca^{2+} release from endodontic sealers. As shown in Table 1, the presence of Ca^{2+} in the composition of TFbc and BRF may explain this phenomenon. Phalloidin/DAPI staining clearly showed that hPDLSCs exhibited abundant cellular extensions in the presence of TFbc. Previous reports have indicated that the cytoskeleton can be affected by exposure

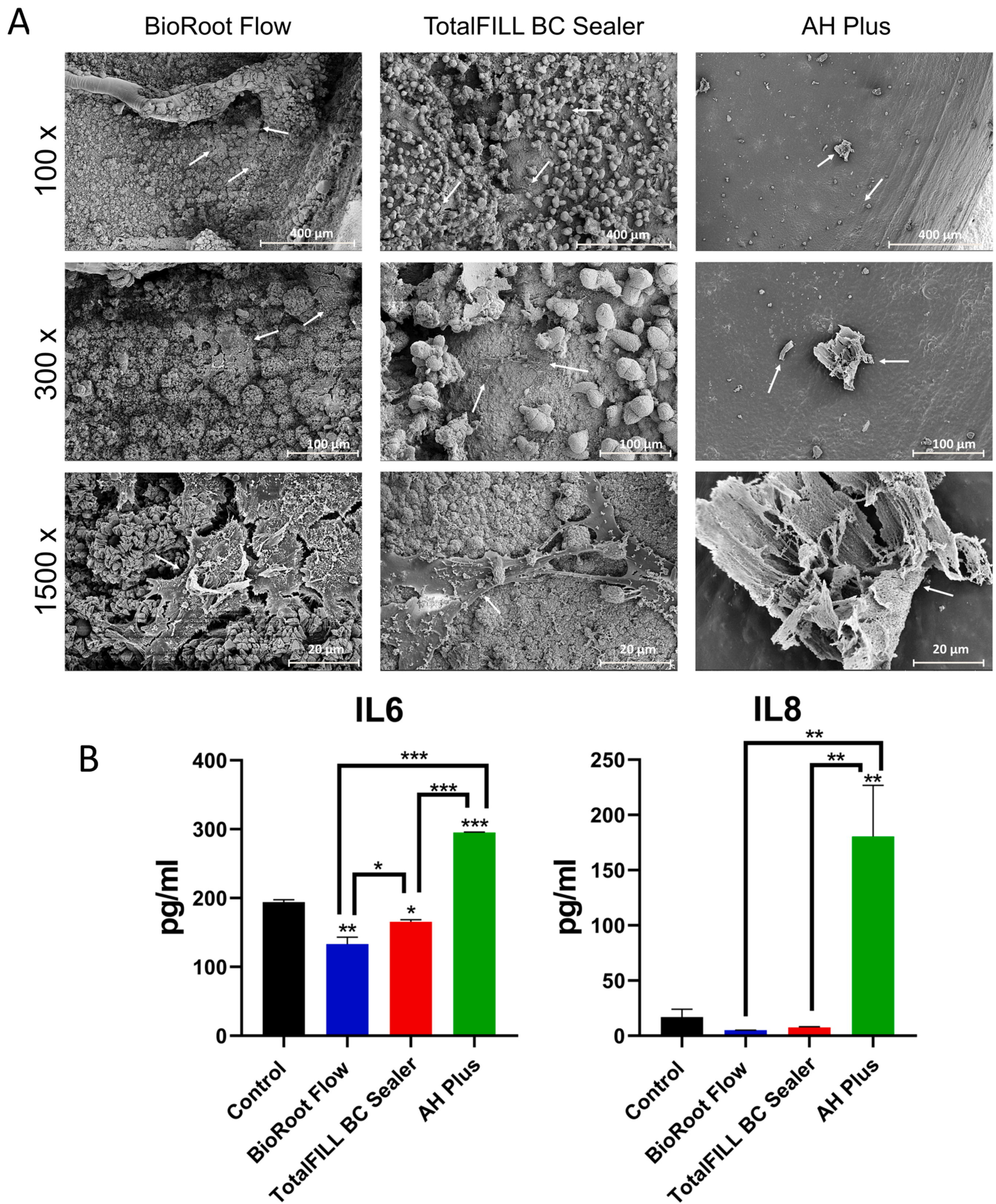

Fig. 3. A) Results of cell cytoskeleton staining after 72 hours of culturing hPDLSCs with test or control groups. B) ELISA results. Asterisks signify significant differences from the control group: ** p< .01; *** p< .001.

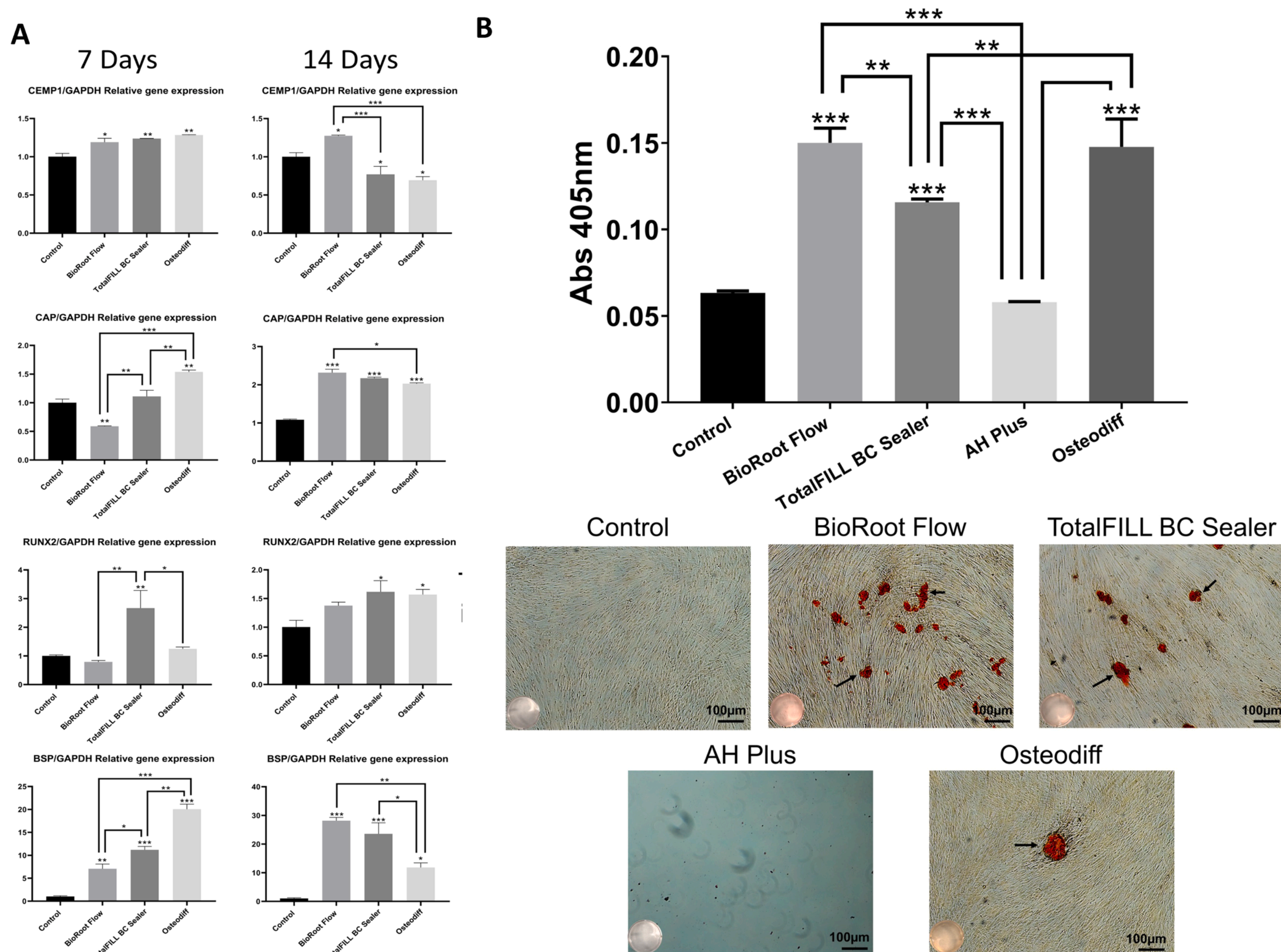

Fig. 4. A) RT-qPCR results for osteo/cementogenic marker expression in hPDLSCs cultured with test materials over 7 and 14 days. Significance levels are indicated: * $p < 0.05$; ** $p < 0.01$; *** $p < .001$. B) Results of mineralization assays after 21 days of culturing hPDLSCs with the test materials. Significance levels: * $p < .05$; *** $p < .001$.

to various materials and directly reflect cell injury (Akbulut et al., 2016). Furthermore, alterations in the cytoskeleton have been considered a direct indicator in the cytotoxicity assessment (Lozano-Guillen et al., 2022).

Furthermore, anti-inflammation is a critical process in tissue repair that can be used to assess the ability of the immune system to clear cell debris and improve cell differentiation (Alchawoosh et al., 2023; Yan et al., 2023). We hypothesized that bioceramic sealers contain high concentrations of Ca^{2+}, rendering the culture medium alkaline, which induces hPDLSCs to attenuate proinflammatory cytokine synthesis. IL-6 is critical in bone remodeling and the activation and differentiation of immune cells and osteoclasts(Yan et al., 2023). Several studies have demonstrated a correlation between upregulated levels of IL-6 and the presence of symptomatic and active endodontic lesions. Furthermore, IL-8 has also been involved in these processes, promoting the inflammatory response and lesion progression. These cytokine interactions, especially IL-6 and IL-8, could significantly influence the healing of endodontic lesions by modulating the immune response and tissue remodeling (Alchawoosh et al., 2023; Castro-Jara et al., 2023). In our study, the AHP group evidenced significantly more proinflammatory cytokines (IL6 and IL8) than BRF and TFbc, which may be attributed to calcium release from bioceramic sealers, promoting anti-inflammatory effects. In the same line, other calcium silicate-based sealers, such as MTA Fillapex and EndoSequence BC have been found to have a down-regulating effect on pro-inflammatory cytokines, specifically TNF-α and IL-6, which inhibits the inflammatory response and helps to promote osteogenic differentiation in MC3T3-E1 cell lines (Lee et al., 2019).

The bioactivity of bioceramic sealers on alveolar bone cells has been well documented and shown to promote bone cell mineralization (Saber et al., 2023). Generally, previous similar studies assess the bioactivity of calcium silicate-based sealers from a cellular perspective, via the quantification of differentiation-related markers, and/or from a mineralization potential perspective, via the measurement of calcium deposition (López-García et al., 2019, 2020; Sanz et al., 2021). Following the methodology of such studies, a qRT-PCR and Alizarin Red S staining were performed for such purposes, respectively. The results from the Alizarin Red S staining, namely calcium deposition, act as a validation of the expression of differentiation markers. Alternatively, a Western Blot assay to assess protein expression could also be performed, as seen in previous studies (Xue et al. 2023). In the present study, BRF and TFbc were able to induce overexpression of the BSP, CEMP-1, and CAP. These markers are involved in early differentiation into osteoblasts/cementoblasts (7–14 days) (Han et al., 2015). BSP is a well-known marker that is considered crucial in identifying osteogenic differentiation. On the other hand, CEMP1 is a marker that is specifically used to identify cementoblasts and their progenitors (Li et al., 2019). In the context of periapical regeneration, the production of cementum is a crucial factor in inducing the formation of replacement tissue, also referred to as the "biological seal". This biological seal is considered to be the ideal environment for

endodontic treatment repair, as highlighted by Sanz et al.(Sanz et al., 2021a). Therefore, cementum neoformation by endodontic materials is considered the optimal process for achieving successful periapical regeneration.

Our findings suggest that BRF may have a superior ability to promote mineralization when compared to both the control and TFbc at 21 days. The observed differences in behavior may be attributed to the higher nucleation activity and calcium release exhibited by TFbc (Zamparini et al., 2023). Previous evidence suggests that calcium silicate-based sealers may enhance osteogenic activity and serve as an ideal component in a sealer intended for biological sealing (Santos et al., 2021). The high mineralizing activity accelerated root canal mineralization, allowing the sealer to bind well to the canal wall and occlude the dentinal tubules, thus promoting the root canal sealing effect (Sanz et al., 2021a).

The main limitation of the study was the lack of previous research on the biological effects of BioRoot Flow. The results were obtained in a controlled laboratory environment, and further in vitro and in vivo studies are required to confirm the potential of these materials in a clinical setting. Studies in animal models would also be very useful to assess the influence of new calcium silicate-based material formulations in inflammation recovery and tissue healing processes, as performed previously (Santos et al. 2021).

5. Conclusions

Our laboratory study revealed that BRF can potentially enhance cell differentiation by promoting the expression of essential genes related to bone and cement formation. In addition, BRF was found to facilitate the mineralization of the extracellular matrix. Conversely, the effects of TFbc and AHP on this process were not as pronounced as those of BRF. In addition, BRF and TFbc promoted anti-inflammatory effects.

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CRediT authorship contribution statement

Francisco Javier Rodríguez-Lozano: Visualization, Validation, Resources, Project administration, Funding acquisition, Conceptualization. **Ricardo E Oñate-Sánchez:** Visualization, Validation, Supervision, Conceptualization. **José Luis Sanz:** Writing – review & editing, Writing – original draft, Resources, Project administration, Conceptualization. **Laura Murcia:** Visualization, Validation, Software, Formal analysis. **Sergio López-García:** Validation, Methodology, Investigation, Data curation. **Adrián Lozano:** Visualization, Validation, Resources, Funding acquisition. **Leopoldo Forner:** Validation, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **David García-Bernal:** Software, Methodology, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data Availability

Data will be made available on request.

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