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RESEARCH ARTICLE

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Premixed calcium silicate-based ceramic sealers promote osteogenic/cementogenic differentiation of human periodontal ligament stem cells: A microscopy study

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Abstract

To evaluate the effects of premixed calcium silicate based ceramic sealers on the viability and osteogenic/cementogenic differentiation of human periodontal ligament stem cells (hPDLSCs). The materials evaluated were TotalFill BC Sealer (TFbc), AH Plus Bioceramic Sealer (AHPbc), and Neosealer Flo (Neo). Standardized discs and 1:1, 1:2, and 1:4 eluates of the tested materials were prepared. The following in vitro experiments were carried out: ion release, cell metabolic activity 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell migration, immunofluorescence experiment, cell attachment, gene expression, and mineralization assay. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test ($p < .05$). Increased Ca^{2+} release was detected in TFbc compared to AHPbc and Neo ($^{*}p < .05$). Biological assays showed a discrete cell metabolic activity and cell migration in Neo-treated cell, whereas scanning electronic microscopy assay exhibited that TFbc group had a better cell adhesion process of substrate attachment, spreading, and cytoskeleton development on the niche-like structures of the cement than AHPbc and Neo. The sealers tested were able to induce overexpression of the CEMP-1, ALP, and COL1A1 genes in the first days of exposure, particularly in the case of TFbc ($^{***}p < .001$). All materials tested significantly increased the mineralization of hPDLSCs when compared to the negative control, although more pronounced calcium deposition was observed in the TFbc-treated cells ($^{***}p < .001$). Our results suggested that TFbc promotes cell differentiation, both by increasing the expression

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of key osteo/odontogenic genes and by promoting mineralization of the extracellular matrix, whereas this phenomenon was less evident in Neo and AHPbc.

Research Highlights

- TFbc group had a better cell adhesion process of substrate attachment, spreading, and cytoskeleton development on the niche-like structures of the cement than AHPbc and Neo.
- The sealers tested were able to induce overexpression of the CEMP-1, ALP, and COL1A1 genes in the first days of exposure, particularly in the case of TFbc.
- All materials tested significantly increased the mineralization of hPDLSCs when compared to the negative control, although more pronounced calcium deposition was observed in the TFbc-treated cells.

KEYWORDS
bioactivity, bioceramics, biocompatibility, cytotoxicity, endodontic sealer

1 | INTRODUCTION

The biological interactions between root canal sealers and the surrounding tissues are important in ensuring the biocompatibility of these materials (Sanz et al., 2021). Biocompatibility refers to the ability of a material to interact with the surrounding tissues without causing any adverse effects (Peters, 2013).

Root canal sealers have been shown to have both direct and indirect biological interactions with the surrounding tissues (Jung et al., 2022). Direct interactions occur when the sealer comes into contact with the tissues, whereas indirect interactions occur through the release of chemicals or ions from the sealer. Direct biological interactions between root canal sealers and the surrounding tissues can include inflammatory responses, cytotoxicity, or tissue irritation. These interactions can be influenced by the composition and properties of the sealer, as well as the method of application and the anatomy of the root canal system (Jin et al., 2021; Santos et al., 2019). Indirect biological interactions between root canal sealers and the surrounding tissues can include the release of calcium ions, hydroxyl ions or other substances that may promote tissue healing and regeneration. In this regard, bioceramic sealers have been shown to release calcium and phosphate ions, which can promote the formation of new mineralized tissue (Aminoshariae et al., 2022). Ex vivo studies on bioceramic sealers demonstrated the formation of mineralized tissues when cells were cultured in contact to these materials (Alchawoosh et al., 2023; Saber et al., 2023).

To ensure the biocompatibility of root canal sealers, it is important to use materials that are biocompatible and have minimal toxic effects on the surrounding tissues. Clinical studies and laboratory tests can help to evaluate the biocompatibility of root canal sealers and ensure their safe use in dental procedures. In this context, human periodontal ligament stem cells (hPDLSCs) were used as target cells in this study. TotalFill BC Sealer is a bioceramic-based sealer known for its exceptional sealing properties and biocompatibility. The composition of TotalFill BC Sealer consists of a combination of bioceramic powders (tricalcium silicate, dicalcium silicate, calcium phosphate monobasic, and calcium hydroxide) and a liquid component, which, when mixed together, form a flowable paste that can be applied within the root canal system to promote a biological sealing (Castro-Jara et al., 2023). AH Plus Bioceramic Sealer (AHPbc) is a new premixed calcium silicate-based ceramic sealer designed to provide optimal clinical results by combining the advantages of premixed sealers with the bioactive properties of bioceramics (Souza et al., 2023; Zamparini et al., 2022). AHPbc offers superior sealing capabilities, biocompatibility, antimicrobial activity, and excellent physical properties (Kwak et al., 2023). Neosealer Flo has recently been introduced into clinical endodontics. Based on a previous report, this premixed sealer is composed of tricalcium silicate, dicalcium silicate as bioactive components, and calcium aluminate, calcium aluminium oxide (grossite), tricalcium aluminate, and tantalite as radiopacifier. However, to the authors' knowledge, the biological properties of the new Neosealer Flo against hPDLSCs have not been elucidated.

Therefore, the study aimed to evaluate different biological properties of these three premixed sealers such as ion release, cell viability, cell migration, cell adhesion, and osteo/periodontal differentiation. The current study was designed to test the null hypothesis that there is no difference in biocompatibility between the three materials.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The protocol for hPDLSC isolation was approved by the Ethics Committee of the University of Murcia (IRB number: 3686/2021). Ten individuals between the ages of 18 and 23 donated their molars from which hPDLSCs were isolated and agreed to their use in this study after giving informed consent. This work does not contain any patient

data or images that could be used to identify study participants. After aseptic removal, dental pulp tissues were immersed in 0.25% trypsin in 4 mL of EDTA (Life Technologies, Carlsbad, CA, USA) for 30 min at 37°C. The cells were forcibly pipetted out of the solution after neutralization with 4 mL of medium, and they were then filtered through a cell strainer (70 µm; Corning, Corning, NY, USA). The hPDLSCs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 µg/mL) (all from Gibco, Billings, MT, USA), and 100 mM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) in a 37°C incubator with 5% CO_2 (Thermo Forma 3110, Thermo Fisher Scientific, Waltham, MA, USA). The hPDLSCs in passages 3–5 were used in this study and were characterized as mesenchymal stem cells in previous studies performed by our research group (Lopez-Garcia et al., 2019; López-García et al., 2020).

2.2 | Characterization of hPDLSCs

The immunophenotypic characterization of the hPDLSCs was performed according to the recommendations of the International Society for Cell & Gene Therapy (ISCT), using the hMSCs Phenotyping Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 1×10^5 cells were resuspended in 100 mL of phosphate-buffered saline (PBS) containing 1% FBS and the cocktail of fluorescence conjugated specific monoclonal antibodies cocktail (CD14-PerCP, CD20-PerCP, CD34-PerCP, CD45-PerCP, CD73-APC, CD90-FITC, and CD105-PE), or their specific isotype monoclonal antibodies. Labeled hPDLSCs were then acquired using a BD Fortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and later analyzed using FlowJo analysis software (Ashland, OR, USA).

2.3 | Material extracts and preparation

Neosealer Flo (Neo; Avalon Biomed, Houston, TX, USA), AH Plus Bioceramic Sealer (AHPbc; Maruchi, Gangwon-do, South Korea), and TotalFill BC Sealer (TFbc; Innovative BioCeramix Inc., BC, Canada) were tested in this study (Table 1). A total of 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 left to set for 48 h (Sanz, López-García, et al., 2022). Each disc was placed in a separate well of a 24-well plate and then immersed in fresh growth medium for 24 h at 37°C. The extraction process was carried out in general accordance with ISO 10993-5. Different dilutions (1:1, 1:2, and 1:4 v/v) of these extraction media were then prepared using fresh complete DMEM medium to study the effect of the concentration of each material.

2.4 | Ion release analysis

Samples of the above dimensions ($n = 3$) were prepared and stored at 37°C in 100% humidity for 24 h. After, each sample was suspended in 5 mL deionized water for 24 h and the collected solution was analyzed by inductively coupled plasma-optical emission spectrometry (ICP-MS; Agilent 7900, Stockport, United Kingdom). The proportion of aluminium (Al), silicon (Si), sulfur (S), calcium (Ca), strontium (Sr), barium (Ba), and tungsten (W) released from each material was calibrated against pure deionized water. Analyses were performed independently in triplicate ($n = 3$).

2.5 | Mitochondrial activity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays were used to determine the viability of hPDLSCs in the presence of premixed sealers. Briefly, 5×10^3 cells per well were seeded in 96-well plates, allowed to adhere for 24 h and then treated with different dilutions (1/1, 1/2, and 1/4) of the sealers for 24, 48, and 72 h. The medium was then replaced with 5 mg/mL MTT reagent, and standard culture conditions were followed for 4 h. In these assays, mitochondrial activity is measured by the ability of mitochondrial dehydrogenases to convert the MTT tetrazolium salt into a colored formazan compound. Finally, the absorbance was measured at 570 nm using a microplate reader (ELx800, Bio-Tek Instruments, Winooski, VT, USA) after cell lysis in isopropanol containing 0.4 M hydrochloric acid solution (200 µL per well). Three independent experiments were carried out for each sample and condition.

TABLE 1 Materials tested.

Materials	Manufacturer	Composition	Lot number
NeoSEALER flo	Mfg.by NuSmile, Ltd 3315 West 12th St Houston, TX 77008, USA +1.713.861.0033	Tricalcium silicate, dicalcium silicate, bioactive components, and calcium aluminate, calcium aluminium oxide, tricalcium aluminate, and tantalite	2022041205
AH Plus Bioceramic Sealer	Maruchi, Taejanggongdan-gil, Wonju-si, Gangwon-do, Korea	Zirconium dioxide, tricalcium silicate, dimethyl sulfoxide, lithium carbonate, and thickening agent	KS210719
TotalFill BC Sealer	Innovative BioCeramix Inc. 101–8218 North Fraser Way Burnaby, BC V3N 0E9, Canada	Zirconium oxide, tricalcium silicate, dicalcium silicate, and calcium hydroxide	22002SP

2.6 | Cell migration

The migratory capacity of hPDLSCs cultured with the premixed sealers was determined using in vitro wound healing assays. The hPDLSCs were seeded in 12-well plates (2×10^4 hPDLCs per well), and a vertical scratch was made using a 100 µL sterile pipette tip. Then, microscopy images were acquired at 0, 24, 48, and 72 h and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) (Collado-Gonzalez et al., 2018). Each experimental condition was performed in triplicate for each material and analyzed in three independent experiments.

2.7 | Cell attachment

The hPDLSCs were cultured on the surface of the materials for 72 h. The culture medium was then removed and the cells were washed with PBS solution. The adherent cells on the samples were then fixed with 3% glutaraldehyde for 30 min at 4°C. The samples were dehydrated with hexamethyldisilazane (Sigma-Aldrich, St. Louis, MO, USA) and different concentrations of ethanol at room temperature. After fixation, cell attachment was observed by scanning electronic microscopy (SEM) after gold sputtering onto the samples. Images were taken at 100×, 300×, and 1500× magnification (Rodriguez-Lozano et al., 2021). Each experimental condition was performed in triplicate for each material and analyzed in three independent experiments.

2.8 | Immunocytochemistry

To evaluate possible changes in hPDLSC morphology after exposure to undiluted ion-releasing materials, immunocytochemistry experiments were performed. Briefly, hPDLSCs were grown on coverslips at 37°C for 72 h. The hPDLSCs were then fixed in 4% formaldehyde solution (Merck Millipore, Darmstadt, Germany) for 10 min, and blocked with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. After, the coverslips were stained with AlexaFluor™594-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) or PBS in the control group. The nuclei were then stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (ThermoFisher, Waltham, MA, USA). Finally, the coverslips were analyzed by confocal microscopy (Leica, Wetzlar, Germany) (Rodriguez-Lozano et al., 2021). For each material, each experimental condition was performed in triplicate and subjected to three separate analyses.

2.9 | Gene expression analysis

The gene expression levels of human alkaline phosphatase (*ALP*), collagen type 1 (*Col1A1*), cementum attachment protein (*CAP*), cementum protein 1 (*CEMP1*), amelogenin X (*AMELX*), ameloblastin (*AMBN*), runt-related transcription factor 2 (*RUNX2*), bone sialoprotein progenitor (*BSP*), and osteocalcin (*BGLAP*) were analyzed in cells treated with the different sealers after 14 days ($n = 3$) by quantitative polymerase chain reaction (qPCR) as previously described (Rodriguez-Lozano et al., 2021). Briefly, cells were detached and washed, and total RNA was isolated (Purelink RNA Mini Kit, Invitrogen, ThermoFisher, Waltham, MA, USA). Subsequently, cDNA synthesis was performed by RT-PCR using iScript RT Supermix (Bio-Rad, Hercules, CA, USA). The relative gene expression values obtained by qPCR analysis for each gene compared to the expression of the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene were calculated using the $2^{-\Delta\Delta CT}$ method. Cells cultured in basal growth media were used as negative control, and cells cultured in a commercial medium to induce osteogenic differentiation (StemMACS OsteoDiff Media, Miltenyi Biotec, Bergisch Gladbach, Germany) were used as positive control. For each sample, each experimental condition was performed in triplicate and analyzed in three independent experiments.

2.10 | Alizarin red S staining

In these assays, Alizarin red S staining was used to determine whether hPDLSCs formed mineralized nodules in vitro in the presence of premixed calcium silicate-based ceramic sealers and according to previous studies (Rodríguez-Lozano et al., 2012). The cell seeding step and the experimental groups were the same as for the gene expression assays, including negative and positive controls (basal growth media and OsteoDiff (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively). The culture medium was replaced every 3–4 days. After 21 days of culture, the cells were rinsed twice with PBS and fixed with 10% formalin neutral buffer solution (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 10 min, and then washed twice with PBS and twice with double distilled water (DDW). The cells were then stained with 1 mL/well 40 mM Alizarin red S (Sigma-Aldrich, St. Louis, MO, USA) for 1 h with stirring. The samples were then washed twice with DDW and once with PBS. Finally, the samples were photographed with a light microscope (Olympus CKX41; Olympus, Tokyo, Japan) using a 4 × 10.13 php, UP Lan FL lens. The dye was then extracted from the plate by incubation in 200 µL/well 10% cetylpyridium chloride (Nacalai Tesque Inc., Kyoto, Japan) for 20 min and transferred to a 96-well microplate. Colorimetry was assessed by absorbance at 577 nm using a microplate reader. Three independent samples were performed per treatment and time.

2.11 | Statistical analysis

The experiments used to generate the data presented in this study were performed at least three times. For quantification, data were presented as means and standard deviations. A Q-Q plot was first used to assess the normality in 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 normality and homogeneity of variance requirements, using Graph-Pad Prism v8.1.0 (GraphPad Software Inc., San Diego, CA, USA). Each

dilution was considered as an independent treatment. The symbols *, **, and *** indicate *p*-values less than .05, .01, and .001, respectively.

3 | RESULTS

3.1 | Ion release assays

Increased Ca^{2+} release was detected in TFbc compared to AHPbc and Neo ($p < .05$), while aluminium (Al) and tantalum (Ta) ion release was significantly increased in Neo ($*p < .05$). In contrast, AHPbc showed the highest release of lithium (Li) and sulfur (S) (Table 2).

3.2 | Cell characterization

To confirm the mesenchymal stem cell phenotype of the isolated hPDLSCs, the expression of typical mesenchymal surface markers and hematopoietic markers was analyzed by flow cytometry assays. More than 95% of the hPDLSCs were positive for the mesenchymal markers CD73, CD90, and CD105, and negative for the hematopoietic markers CD14, CD20, CD34, and CD45 (Figure 1).

3.3 | Mitochondrial activity assays

The metabolic activity of cells treated with the different ceramic sealers was compared with that of untreated cells (negative control) for 72 h. MTT assays revealed a significant reduction in mitochondrial activity after exposure to Neo extracts at all dilutions and times evaluated compared to the negative control ($***p < .001$), except for 1/2 and 1/4 dilutions at 24 h. Conversely, cells treated with 1/2 and 1/4 dilutions of TFbc sealer showed a mitochondrial activity similar to the negative control group. Finally, AHPbc-treated cells showed a discrete mitochondrial activity compared to the control group ($***p < .001$), despite evidence of cell proliferation (Figure 2).

3.4 | Migration assays

Cell monolayers were wounded with a scraper and allowed to heal in the presence or absence of material extracts. TFbc-treated cells showed similar behavior to that of the untreated group (control) at all time points in the wound healing assay, and in agreement with the MTT assays, the cells cultured with undiluted Neo showed reduced

TABLE 2 Assessment by ICP-MS of calcium silicate-based sealers.

Sample name	7Li [He] conc. [ppm]	27Al [He] conc. [ppm]	28 Si [He] conc. [ppm]	34 S [He] conc. [ppm]	43Ca [He] conc. [ppm]	90 Zr [He] conc. [ppm]	181 Ta [He] conc.[ppm]
Neosealer Flo	0.01 ± 0.01^{A}	1773.78 ± 0.03^{AB}	2.53 ± 0.02^{AB}	29.24 ± 0.02^{AB}	3.08 ± 0.00^{AB}	1.87 ± 0.01^{AB}	2.16 ± 0.02^{AB}
AH Plus Bioceramic Sealer	6946 ± 0.01^{AC}	185.01 ± 0.03^{AC}	24.72 ± 0.02^{AC}	6020.8 ± 0.03^{AC}	29.68 ± 0.02^{AC}	153 ± 0.02^{BC}	0.04 ± 0.00^{A}
TotalFill BC Sealer	1.43 ± 0.00^{C}	6.68 ± 0.01^{BC}	58.84 ± 0.01^{BC}	10.52 ± 0.01^{BC}	65.77 ± 0.01^{BC}	64.98 ± 0.00^{AC}	0.08 ± 0.00^{B}

Note: Superscript uppercase A indicates significant differences ($*p < .05$) between Neosealer Flo and AH Plus Bioceramic sealer. Superscript uppercase B indicates significant differences ($*\underline{p} < .05$) between Neosealer Flo and TotalFill BC sealer. Superscript uppercase C indicates significant differences ($*p < .05$) between AH Plus Bioceramic Sealer and TotalFill BC sealer.
Abbreviation: ICP-MS, inductively coupled plasma-optical emission spectrometry.

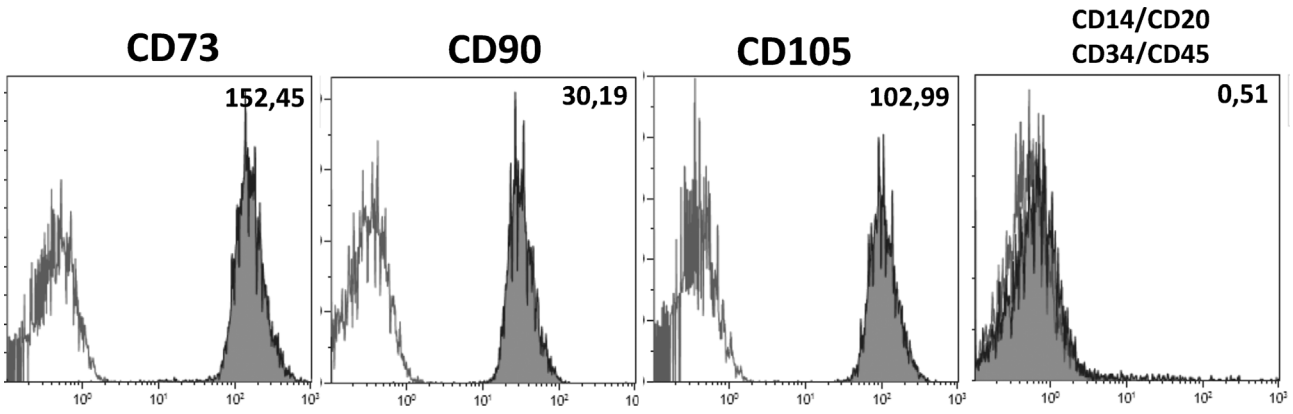

FIGURE 1 MSC immunophenotype analysis of human periodontal ligament stem cells (hPDLSCs) by flow cytometry. hPDLSCs were cultured in complete medium for 96 h at 37°C. The expression of the MSC surface markers CD73, CD90, and CD105, as well as the expression of the hematopoietic markers CD14, CD20, CD34, and CD45 were then determined. The mean fluorescence intensity values obtained in each experimental condition are shown. Representative histograms obtained from three independent experiments are shown.

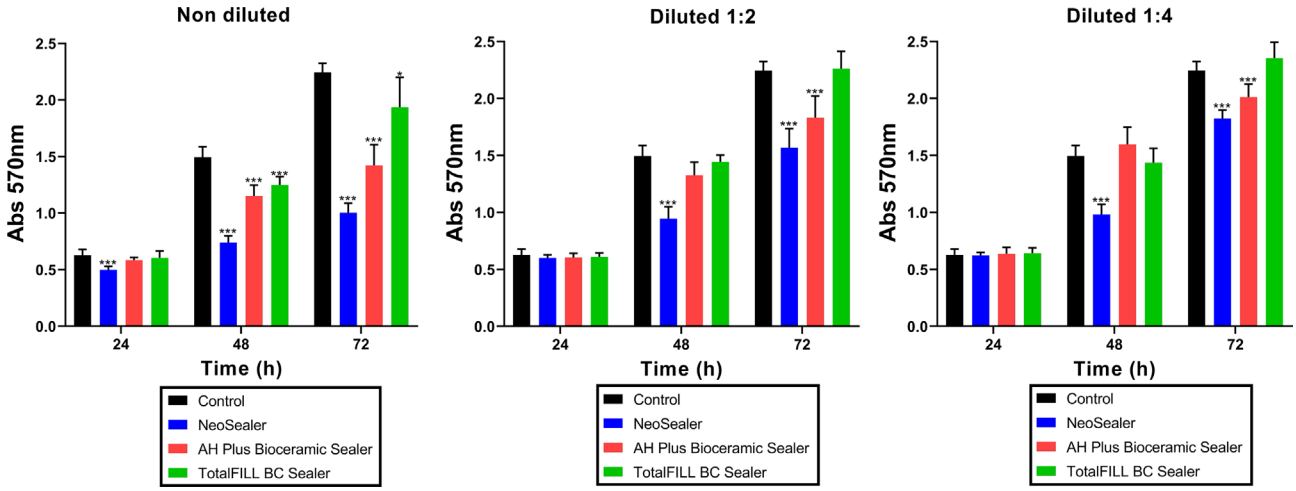

FIGURE 2 MTT assay results after 24, 48, and 72 h of culture of hPDLSCs with the tested groups or the control group. Asterisks indicate significant differences from the control group: *$p < .05$; **$p < .01$; ***$p < .001$, respectively.

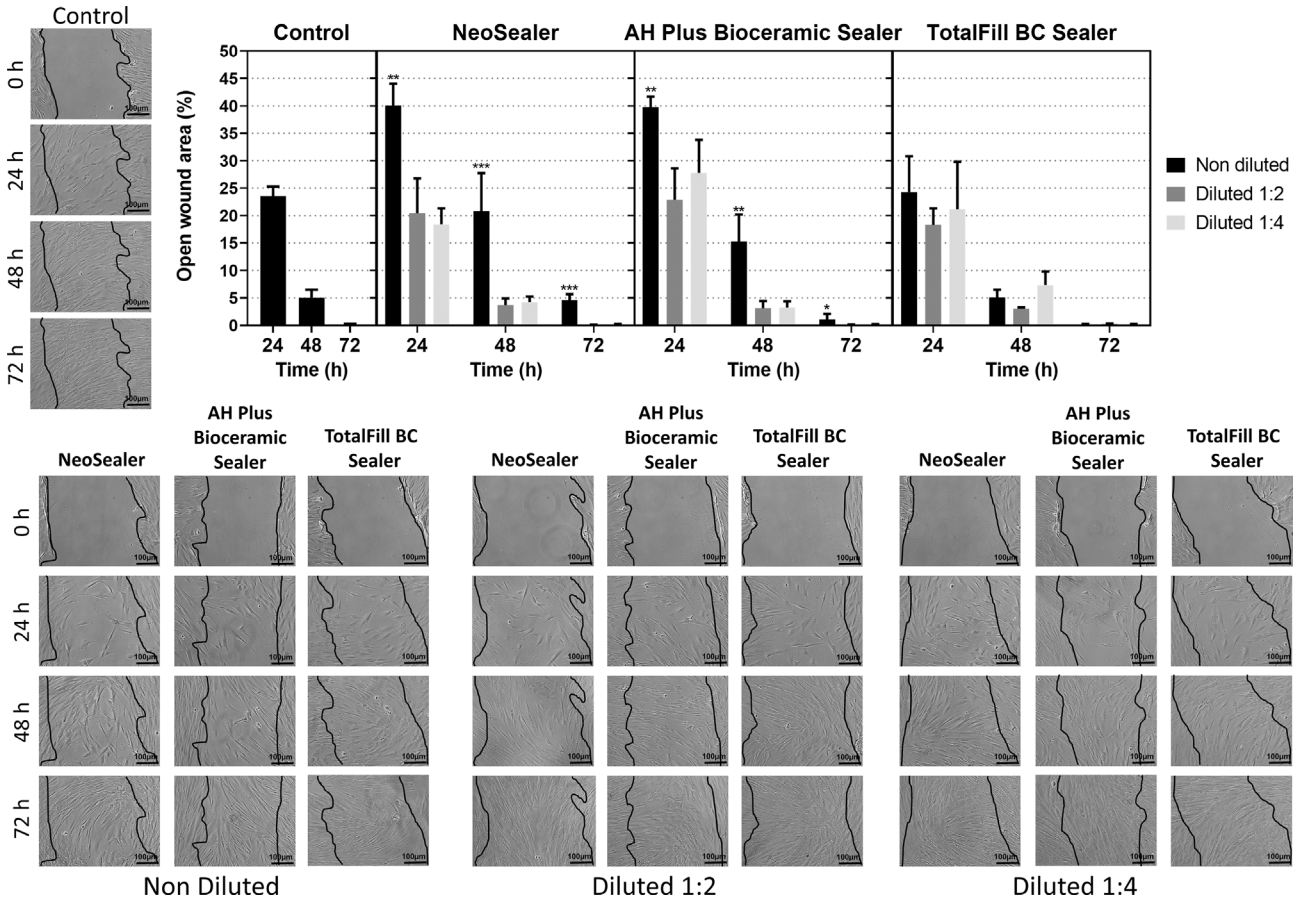

FIGURE 3 Cell migration assay results after 24, 48, and 72 h of culture of human periodontal ligament stem cells with the tested groups or the control group. Asterisks indicate significant differences from the control group: **$p < .01$; ***$p < .001$, respectively.

wound closure compared to the control group at all time points (***$p < .001$). Remarkably, significant differences were observed in the cells treated with AHPbc compared to the untreated cells (**$p < .01$; Figure 3).

3.5 | Cell attachment

SEM analyses at 300×magnification showed that the TFbc group had a better cell adhesion process of substrate attachment, spreading, and

cytoskeleton development on the niche-like structures of the cement than AHPbc and Neo, which showed a moderate number of cells and less cytoplasmic interaction between cells (Figure 4).

3.6 | Immunocytochemistry

The cytoskeletal integrity of hPDLSCs after exposure to different extracts of materials was assessed using phalloidin staining. Images obtained after 72 h of culture showed a high confluence of cells with an increase in F-actin stress fibers and focal adhesion complexes in the presence of TFbc and AHPbc-treated groups (Figure 5). Only with undiluted Neo showed a slight rearrangement of the actin filaments compared to the control.

3.7 | Gene expression analysis

The osteogenic and cementogenic potential of the tested materials on hPDLSCs was investigated by analyzing the expression of the osteogenic and cementogenic related genes *ALP*, *Col1A1*, *RUNX2*, *CEMP-1*, *CAP*, *BSP*, *AMELX*, *AMBN*, and *BGLAP* at 3, 7, 14, and 21 days using qPCR. As shown in Figure 6, the early expression (at 3 days) of *ALP*, *Col1A1*, *and CEMP-1*, was significantly higher in the TFbc-treated cells than in the others, whereas in the APHbc-treated cells there was an overexpression of *RUNX2*, *Col1A1*, *BSP*, *AMELX*, *AMBN*, and *BGLAP* (*$p < .05$). Notably, in the Neo-treated cells the overexpression of CEMP-1, CAP, *RUNX2BSP*, *AMELX*, *AMBN*, and *BGLAP* was more pronounced at the late time points (14–21 days).

3.8 | Alizarin red staining

Mineralization potential was measured using a colorimetric assay based on Alizarin red staining (Figure 7). This assay showed that all materials tested significantly increased the mineralization of hPDLSCs when compared to the negative control (i.e., hPDLSCs cultured in growth medium without any material) after 21 days (***$p < .001$). Interestingly, more pronounced calcium deposition was observed in the TFbc-treated cells (***$p < .001$). However, no differences were observed between Neo and AHPbc.

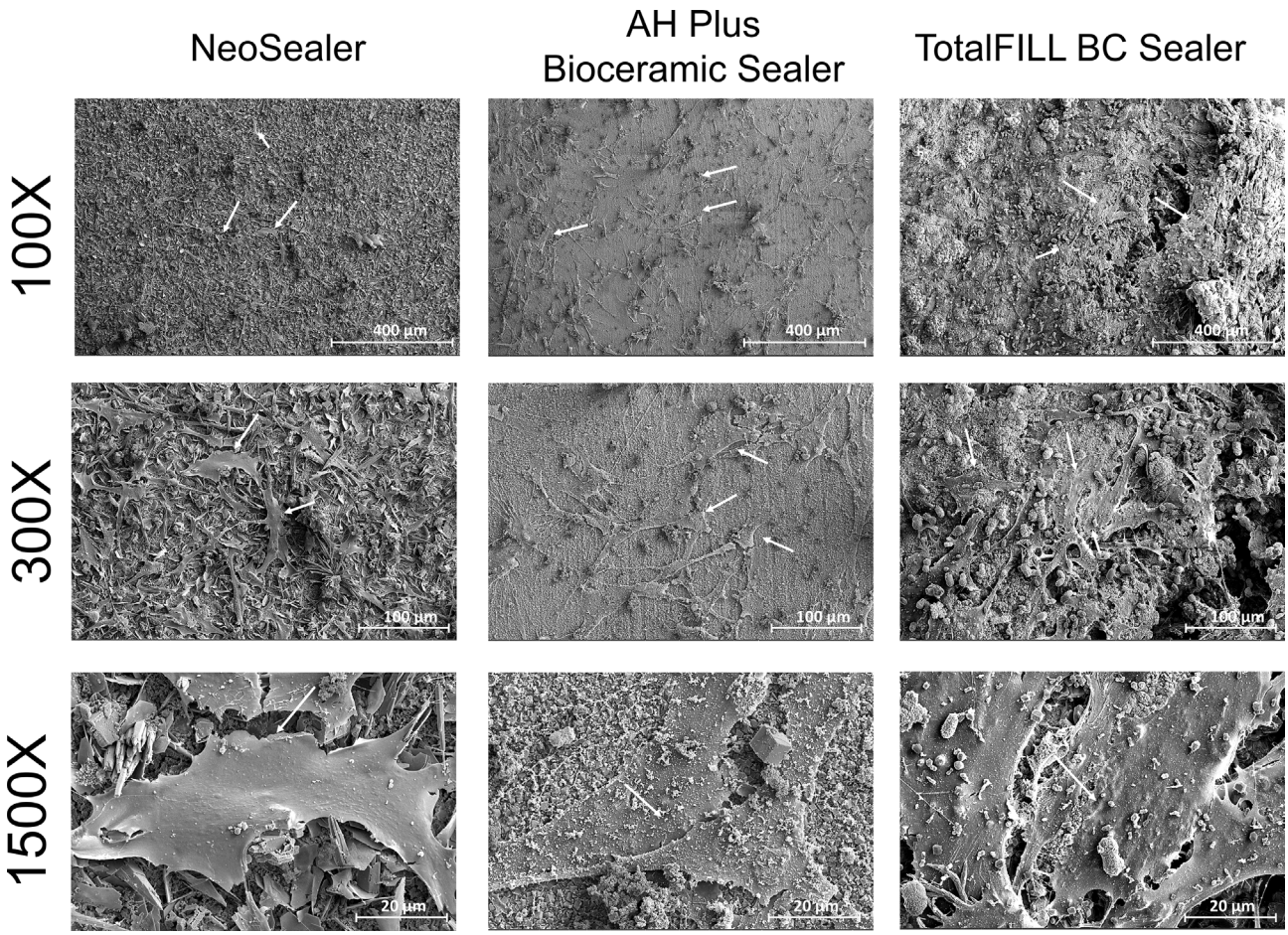

FIGURE 4 Representative scanning electronic microscopy images assessing the superficial adherence and morphology of human periodontal ligament stem cells. Magnification: 100×, 300×, and 1500×.

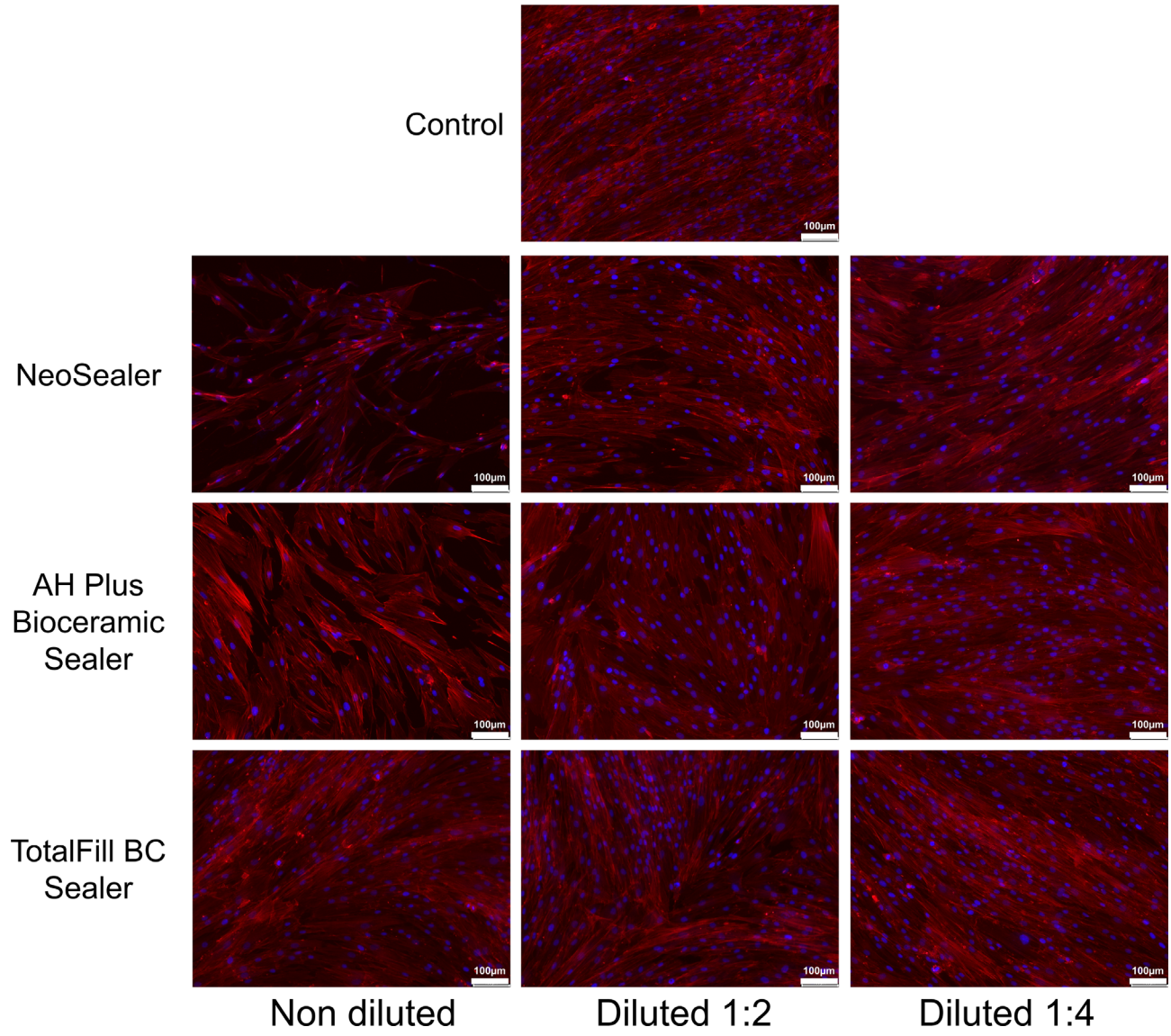

FIGURE 5 Cell cytoskeleton staining results after 72 h of culture of human periodontal ligament stem cells with the tested groups or the control group.

4 | DISCUSSION

New ceramic formulations for clinical use in endodontics are constantly being introduced to the market (Kwak et al., 2023; Shokrzadeh et al., 2023). Due to the interaction between the material and the periapical tissues, a good endodontic sealer should not damage the periapical tissues or prevent the tissue healing process, especially at the cellular level (Sanz et al., 2021). Therefore, the present study aimed to evaluate the osteo/cementogenic potential of different materials by analyzing cell viability, migration, morphology, cell attachment, gene expression, and mineralization assays. Several authors have mainly focused on the study of three materials: Endosequence BC Sealer, Bioroot RCS, and AH Plus, with the latter being the most studied material (Mestieri et al., 2020; Rodriguez-Lozano et al., 2020; Saghiri et al., 2020). However, no information is available on the osteo/cementogenic potential of Neosaler Flo. Therefore, we included three materials in this study: TotalFill BC Sealer, AHPbc, and Neosaler Flo. All premixed sealers tested in this study were incubated as set eluates. This is consistent with most in vitro studies on the biological interaction between calcium silicate-based sealers and dental stem cells (Saber et al., 2023; Sanz et al., 2020).

This study was performed on hPDLSCs, because access to the site where these cells are found is easy and low morbidity, their extraction is highly efficient, they have demonstrated high long-term survival, and the ability to form mineralized bone/cement-like tissue. Inaddition, and their use as a target cell population for the in vitro assessment of the biological properties of endodontic materials is widespread in the literature (Sanz et al., 2021; Tomokiyo et al., 2019; Torii et al., 2015). Furthermore, the use of non-human cells in previous studies with endodontic materials has shown variability in terms of cytotoxicity (Kumar et al., 2023). Based on the results obtained, the

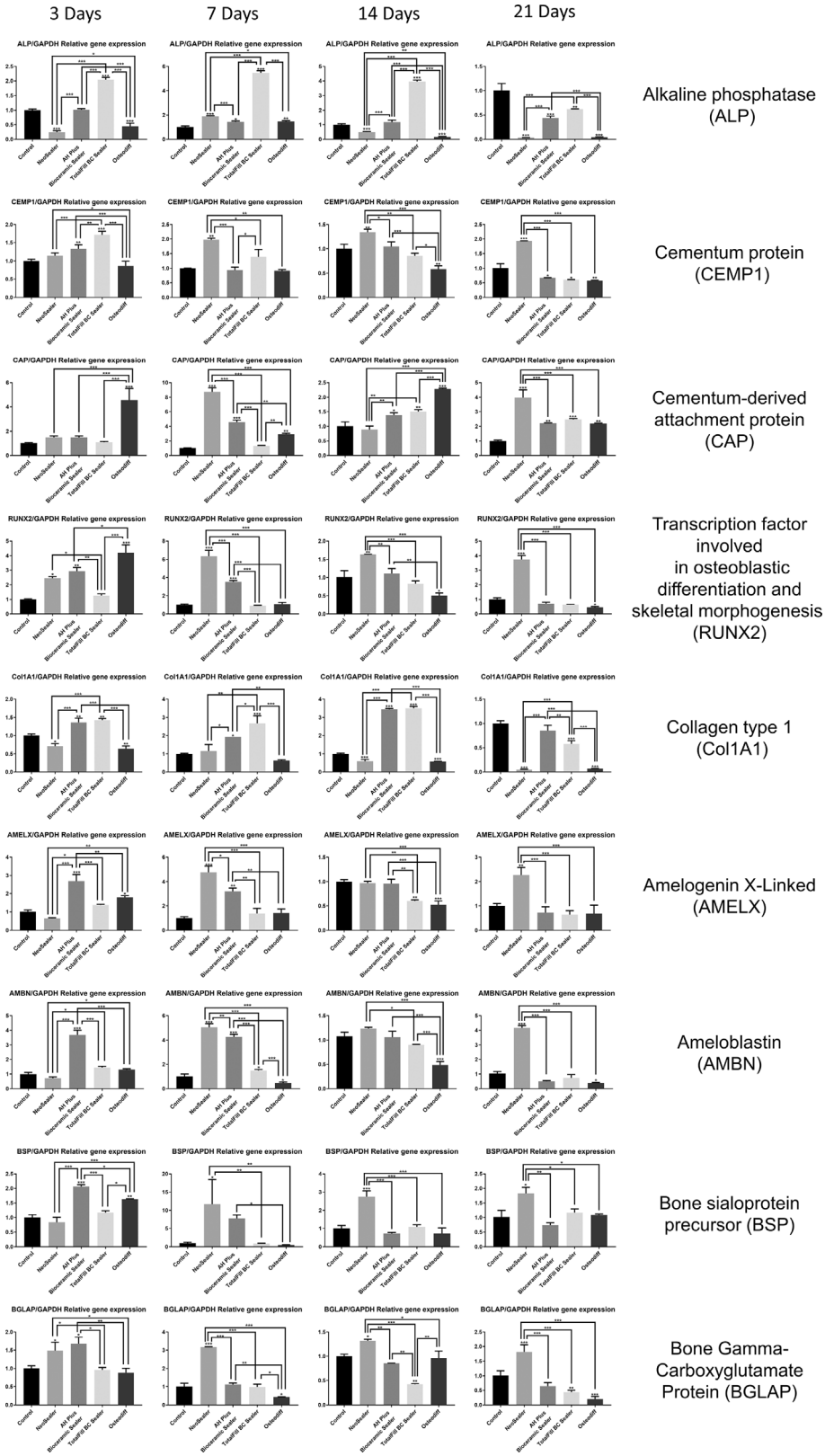

FIGURE 6 RT-quantitative polymerase chain reaction osteogenic marker expression assay results after 3, 7, 14, and 21 days of human periodontal ligament stem cells culture with the tested materials. *$p < .05$; **$p < .01$; ***$p < .001$, respectively.

null hypothesis was rejected as the behavior of the different materials on the hPDLSCs was different in all tests performed.

The metabolic activity assay is based on the idea that most living cells have a stable mitochondrial activity and therefore the increase or decrease in the percentage of living cells is proportional to the mitochondrial activity (Guedes et al., 2021; Sequeira et al., 2018). In this way, the mitochondrial activity of the cells can be measured by calculating the conversion of the MTT tetrazolium salt into formazan

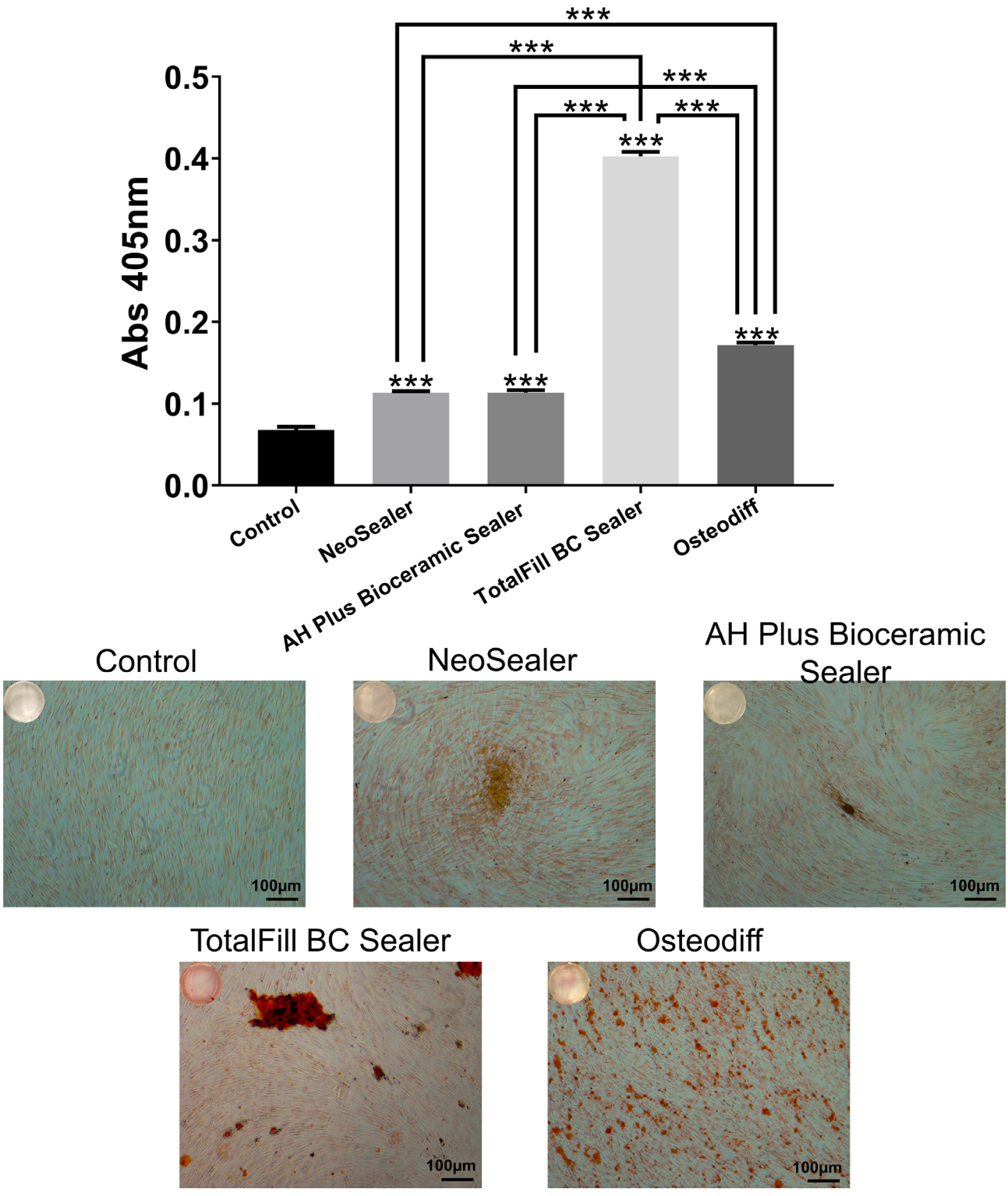

FIGURE 7 Mineralization assay results after 7, 14, and 21 days of human periodontal ligament stem cells culture with the tested materials. *p < .05; ***p < .001, respectively.

crystals. Our MTT analysis showed that undiluted concentrations of Neo were associated with a significant reduction in mitochondrial activity compared to the control (***p < .001). Despite the presumed cytocompatibility of calcium silicate-based sealers, several authors have demonstrated inconsistent results with different calcium silicate-based sealers in terms of cell viability (Lee et al., 2019; Seo et al., 2019). Souza et al. (2023) showed that cells treated with AHPbc and Endosequence BC extract media for 24 and 48 h showed a similar cell viability to untreated cells (control). In contrast, our results showed that AHPbc-treated cells showed a discrete mitochondrial

activity compared to the control group. This discrepancy may be due to the fact that the cell viability experiments were only performed at 24–48 h, which does not allow sufficient exposure time to observe the cellular response to this treatment. Another reason could be attributed to the non-complete set of materials, also confirmed in recent clinical studies, which highlighted the occurrence of a not stable seal in the periapical area (Zamparini et al., 2023). In agreement with our results, another study reported that Endosequence BC Sealer and TFbc had better cell viability rates at 72 h than AHPbc using human periodontal cells (Kwak et al., 2023).

The cell viability results, as an indirect cell proliferation method, were consistent with those obtained in the cell migration experiment. 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., 2021). In this study, TFbc-treated cells showed similar behavior to the untreated group (control) at all times in the wound healing assay, whereas cells cultured with undiluted Neo decreased wound closure compared to the control group. This phenomenon has been demonstrated in a previous work reported by Rodriguez-Lozano et al. (2017) who pointed out that the wound closure area with the appearance of TFbc medium was similar to that of untreated cells.

Several studies have reported that cell adhesion and spreading on a material surface are the initial stages of cellular function, being essential for bone and periapical tissues healing (D'Anto et al., 2010; Rodriguez-Lozano et al., 2017). Therefore, cell attachment was performed using SEM, which revealed the spreading of hPDLSCs on TFbc disc surfaces and the low presence of these cells on the AHPbc and Neo disc surfaces. Calcium plays an important role in fibroblast adhesion and increased cell attachment is associated with Ca^{2+} release from endodontic sealers (Akbulut et al., 2016). As shown in Table 2, the high calcium release in TFbc (Table 2) may explain this phenomenon. Phalloidin/DAPI staining clearly showed that hPDLSCs exhibited abundant cellular extensions in the presence of TFbc. Previous reports have been indicated that the cytoskeleton can be affected by exposure to various materials and directly reflects cell injury (Akbulut et al., 2016). Furthermore, alterations in the cytoskeleton have been considered as a direct indicator in the assessment of cytotoxicity (Sanz, Lopez-Garcia, et al., 2022).

Silicon ions and calcium ions are the active components of calcium silicate-based sealers. ICP-MS analysis showed that TFbc released more silicon and calcium than AHPbc and Neo ($p < .05$). Silicon and related compounds are essential elements responsible for many biological processes and are involved in the regulation of bone metabolism (Wang et al., 2014). Silicon and calcium ions can also promote osteogenesis by influencing the expression of genes that regulate cell cycle induction and progression (Kralovec et al., 2020). Silicon substitution in the crystal structures of calcium phosphate ceramics has been reported to produce superior biological performance (Huang et al., 2009). Furthermore, previous studies have demonstrated that the high content of CaSi is linked to both the ability to release mineralizing ions and the presence of functional groups able to bind ions and trigger the nucleation of apatite (Gandolfi et al., 2011).

Previous studies have been described the incorporation of Ta^{5+} as an alternative radiopacifier, avoiding discoloration and preserving acceptable values of radiopacity (Zamparini et al., 2022). In our study, Ta^{5+} was detected in Neo according to the manufacturer's composition. The differences in ion release may also influence the role of calcium silicates in upregulating the expression of genes related to mineralization by hPDLSCs (Sanz et al., 2021). On the other hand, it has been described that materials containing zirconium oxide, such as TFbc or AHPbc, induced the proliferation of fibroblasts and accelerated the regression of inflammatory reactions (López-García et al., 2020). Thus, the differences in biological activities and ions released in the materials tested may be attributed to radiopacifier percentages and the available quantity of CaSi.

Regarding gene expression, previous reports have highlighted the ability of calcium silicate-based sealers to induce the differentiation of periodontal ligament stem cells into osteoblasts and cementoblasts (Sanz et al., 2020; Xue et al., 2023). The sealers tested were able to induce overexpression of the CEMP-1, ALP, and COL1A1 genes in the first days of exposure, particularly in the case of TFbc. These markers are involved in early differentiation into osteoblasts/cementoblasts (Srinivasan et al., 2015). ALP is a marker whose importance resides in the osteogenic differentiation process, as its enzymatic activity allows the removal of pyrophosphate, a natural inhibitor of tissue mineralization. COL1A1 encodes the major component of collagen type 1: the collagen-enriched extracellular matrix (ECM), which plays a crucial role in the differentiation of osteoblast and cementoblasts. In addition, type I collagen is the most abundant collagen found in bone and teeth (Chang et al., 2014; Suzuki-Barrera et al., 2022). CEMP1 has been shown to be a cementum marker gene and a regulator of hPDLSC commitment into cementoblast-like cells, which is associated with the formation of bone tissue and cementum. In the periapical regeneration situation, cement production is a key factor in the induction of replacement tissue, also called "biological seal," which constitutes the "ideal context" for endodontic treatment repair (Sanz et al., 2021). Therefore, the neoformation of cementum through endodontic materials is considered an optimal process.

Another notable finding of this study is that TFbc promoted a significantly higher mineralization than the control and Osteodiff at 21 days. The reason for such different behavior could be that TFbc revealed a markedly higher nucleation activity with higher calcium release (Zamparini et al., 2019). Mineralization is widely studied in the repair of tissues such as bone and cementum and is also an indication of cemento/osteoblastic differentiation (Shokrzadeh et al., 2023). Previous evidence suggests that calcium silicate-based sealers may enhance osteogenic activity and therefore serve as an ideal component in a sealer intended for biological sealing (Santos et al., 2021). In fact, the high mineralizing activity accelerated root canal mineralization, making the sealer bind well to the canal wall and occlude the dentinal tubules, thus promoting the root canal sealing effect (Sanz et al., 2021). All the data obtained suggest that calcium silicate-based

sealers, particularly TFbc, promote cell differentiation, both by increasing the expression of key osteo/odontogenic genes and by promoting the mineralization of the ECM.

The main limitation of this study was the lack of previous reports on the biological effects of Neosealer Flo. Therefore, the results of the present study were obtained under controlled laboratory conditions, where hPLSCs were cultured together with standardized samples of the materials tested. Clinically, both the tested materials and the cells may be exposed to different factors that may alter their response and behavior, namely changes in pH, temperature, oxygen levels, different manipulation of the materials, host inflammatory response, and further in vitro and in vivo studies are required to confirm the potential of these materials.

5 | CONCLUSIONS

With the limitations of this laboratory study, our results suggested that TFbc promotes cell differentiation, both by increasing the expression of key osteo/odontogenic genes and by promoting mineralization of the ECM, whereas this phenomenon was less evident in Neo and AHPbc.

AUTHOR CONTRIBUTIONS

Sergio López-García: Investigation; methodology. **Sonia Sánchez-Bautista:** Conceptualization; data curation. **David García-Bernal:** Investigation; methodology. **Adrián Lozano:** Writing – original draft. **Leopoldo Forner:** Conceptualization; project administration; formal analysis. **José L. Sanz:** Writing – original draft. **Laura Murcia:** Conceptualization; data curation. **Francisco J. Rodríguez-Lozano:** Conceptualization; formal analysis; project administration; writing – review and editing; validation; supervision. **Ricardo E. Oñate-Sánchez:** Validation; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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