Comparative Biological Properties and Mineralization Potential of 3 Endodontic Materials for Vital Pulp Therapy: Theracal PT, Theracal LC, and Biodentine on Human Dental Pulp Stem Cells

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SIGNIFICANCE

This *in vitro* study supports the use of Theracal PT over its predecessor Theracal LC as a potential alternative to Biodentine for vital pulp treatment because of its improved cytocompatibility on human dental pulp stem cells.

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ABSTRACT

Introduction: The aim of this study was to assess the biological properties and mineralization potential of the new Theracal PT (Bisco Inc, Schaumburg, IL) compared with its predecessor Theracal LC (Bisco Inc) and the hydraulic silicate–based cement Biodentine (Septodont, Saint-Maur-des-Fossés, France) on human dental pulp stem cells (hDPSCs) *in vitro*. **Methods:** Standardized sample discs were obtained for each material ($n = 30$) together with 1:1, 1:2, and 1:4 material eluates. Previously characterized hDPSCs were cultured with the different materials in standardized conditions, and the following assays were performed: a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, a wound healing assay, Annexin-V-FITC and 7-AAD staining (BD Biosciences, San Jose, CA), reactive oxygen species production analysis, cell adhesion and morphology evaluation via scanning electron microscopy and immunofluorescence, quantification of the expression of osteo/odontogenic markers via real-time quantitative reverse-transcriptase polymerase chain reaction, and alizarin red S staining. Statistical significance was established at $P < .05$. **Results:** All of the tested dilutions of Theracal LC exhibited a significantly higher cytotoxicity and reactive oxygen species production ($P < .001$) and a lower cell migration rate than the control group (hDPSCs cultured in growth medium without material extracts) at all of the measured time points ($P < .001$). Both 1:4 Theracal PT and Biodentine-treated hDPSCs exhibited similar levels of cytocompatibility to that of the control group, a significant up-regulation of at least 1 odontogenic marker (Biodentine: dentin sialophosphoprotein ($P < .05$); Theracal PT: osteonectin and runt-related transcription factor 2 [$P < .001$]), and a significantly higher mineralized nodule formation ($P < .001$). **Conclusions:** The newly introduced TheraCal PT offers an improved *in vitro* cytocompatibility and mineralization potential on hDPSCs compared with its predecessor, TheraCal LC, and comparable biological properties to Biodentine. *(J Endod 2021;47:1896–1906.)*

KEY WORDS

Bioactivity; calcium silicate–based cements; cytotoxicity; resin modified; vital pulp therapy

Vital pulp therapy (VPT) comprises a series of conservative procedures that rely on the intrinsic reparatory mechanisms of the dentin-pulp complex[1]. Upon external noxae, such as trauma or carious lesions, this tissue complex will undergo a series of inflammatory stages that, if uncontrolled, could eventually lead to cellular death or necrosis[2]. During this dynamic inflammatory process, the dentin-pulp complex remains

resilient toward microbial damage, maintaining a regenerative potential even when indicative symptoms of irreversible pulpitis are present[3]. Accordingly, the maintenance of pulp vitality via VPT procedures regained interest as a more conservative alternative to root canal treatment in cases of pulpitis[4].

The removal of irritants and nonviable tissue via chemical-mechanical disinfection, added to the placement of materials as pulp cappers, has shown promising results in terms of the asymptomatic maintenance of pulp vitality and the restoration of physiological dental function[5]. These materials require a series of specific biological properties in order to provide a favorable medium for repair. Because they will be placed in indirect contact (indirect pulp capping) or direct contact (direct pulp capping, partial pulpotomy, or coronal pulpotomy) with pulp tissue, such materials should exhibit at least an adequate cytocompatibility toward its cellular component[6].

Among the cellular component of pulp tissue, human dental pulp stem cells (hDPSCs) play a crucial role in the process of pulp repair and, consequently, in VPT[7]. Since their isolation and characterization, research regarding hDPSCs revealed their mesenchymal nature and their ability to differentiate into odontoblastlike cells involved in the process of tissue neoformation[8]. Therefore, to assess the adequacy of a specific material for VPT, its cytocompatibility toward hDPSCs should be previously elucidated.

Nevertheless, materials used in VPT are expected to not only express adequate cytocompatibility but also bioactive properties and a favorable influence on cellular plasticity. Regarding bioactivity, these materials should favor the deposit of a hydroxyapatitelike superficial layer and consequently form a mineralized attachment to the dentin substrate[9]. Parallelly, a positive effect on cellular plasticity would imply favoring the osteo/odontogenic differentiation of hDPSCs[10].

The aforementioned properties have been exhibited by a subgroup of bioactive materials, namely hydraulic calcium silicate–based cements (HCSCs), in numerous *in vitro*, *ex vivo*, and animal studies[11,12]. These materials have also shown high success rates in clinical studies when used in VPT procedures[13]. Among them, Biodentine (BD; Septodont, Saint-Maur-Des-Fossés, France), a tricalcium silicate–based cement presented in a powder-liquid format, has been extensively studied. Together with the Portland cement–based material mineral trioxide aggregate (MTA), it is being used as the reference hydraulic silicate–based cement[14].

The desirable properties of silicate-based materials led to the development of new material compositions, such as resin-modified calcium silicate–based materials[15]. Among them, Theracal LC (ThLC; Bisco Inc, Schamburg, IL) was introduced as a light-curing material for VPT, combining the desirable properties of the silicate-based component and the superior handling of resin[15,16]. Since its introduction, ThLC has been extensively studied both *in vitro* and *in vivo*, exhibiting mixed results. In fact, various authors recommended limiting its use to indirect pulp capping[17–19].

Most recently, a new dual-cured resin-modified calcium silicate–based material under the name of Theracal PT (ThPT, Bisco Inc) has been introduced[20]. According to its manufacturer, it is primarily indicated for pulpotomies and can also be used for indirect and direct pulp capping. However, its biological properties have not been compared with those exhibited by HCSCs.

Accordingly, the aim of the present study was to perform an *in vitro* analysis of the biological properties and mineralization potential of ThPT compared with its predecessor ThLC and the hydraulic silicate–based cement BD on hDPSCs. The null hypothesis was established as the absence of difference between the tested materials with regard to their cytocompatibility, influence in cell plasticity, and mineralization potential on hDPSCs.

MATERIALS AND METHODS

Material Sample Preparation

Five-millimeter-diameter and 2-mm-high sample discs ($n = 30$) were obtained for the tested materials (ie, ThPT [batch no.: 2000002968], ThLC [batch no.: 2000001054], and BD [batch no.: B25359]) by means of sterile (ultraviolet radiation, 15 minutes) cylindrical rubber molds and stored in an incubator (37ºC, 48 hours) to ensure complete setting. BD was mixed by following its manufacturer's instructions. Both ThPT and ThLC are presented in a premixed format and were injected into the rubber molds and photocured (1200 mW/cm^2 output for 20 seconds at a 2-mm distance) using a light-emitting diode curing light (Bluephase 20i; Ivoclar Vivadent, Schaan, Liechtenstein). Light intensity was monitored by means of a MARC Resin Calibrator (BlueLight Analytics, Halifax, Canada).

One:one, 1:2, and 1:4 material eluates were then extracted under sterile conditions as described by previous studies in the field[21]. Dulbecco modified Eagle medium (DMEM; Gibco BRL, Burlingame, CA) was used as an extraction vehicle and stored for 24 hours at 37°C and 5% CO_2 in a humid atmosphere. Following the indications of the ISO 10993-5 guidelines[22], a material surface area/medium volume ratio of approximately 1.5 cm^2/mL was obtained.

Dental Stem Cell Isolation, Culture, and Characterization

The cell extraction protocol was previously approved by the Human Research Ethics Committee from the University of Murcia (reference no. 2199/2018). Human dental pulp was obtained from the pulp chamber and root canals of extracted teeth by means of a barbed broach. Teeth were obtained from 18- to 30-year-old healthy donors and were extracted for orthodontic or periodontal reasons. Once extracted, human dental pulp was rinsed with Hank's Balanced Salt Solution (Gibco BRL) and digested using 3 mg/mL collagenase A (Sigma-Aldrich, St Louis, MO). The resultant cells were cultured in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco), 1% L-glutamine, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco BRL) and incubated under controlled conditions (37°C, 5% CO_2, humid atmosphere). For subsequent assays, cells from passage 4 onward were used.

Stem cell characterization was performed under the International Society of Cellular Therapy guidelines[23] to confirm their mesenchymal phenotype. Cell surface antigens were assessed using fluorophore-conjugated antibodies via flow cytometry (FACS Calibur Flow Cytometer; Becton Dickinson, Franklin Lakes, NJ). The following antibodies from the Human MSC Phenotyping Cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany) were used: CD73-APC (clone AD2), CD90-FITC (clone DG3), CD105-PE (clone 43A4E1), CD34-PerCP (clone AC136), CD20-PerCP (clone LT20.B4), CD14-PerCP (clone TÜK4), and CD45-PerCP (clone 5B1). Additionally, the resultant characterized hDPSCs were cultured in different media (osteogenic/adipogenic/chondrogenic) (Miltenyi Biotech) to confirm their trilineage mesenchymal differentiation following the methodology from similar studies[24]. Both the mesenchymal phenotype and trilineage differentiation of the cells used in this study were confirmed by a previous study performed by our research group[20].

Cytotoxicity Evaluation (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide Assay)

hDPSCs cultured in growth medium without material extracts (negative control group) and with 1:1, 1:2, or 1:4 ThPT, ThLC, or BD extracts (test groups) were assessed for cytotoxicity. To do so, cell metabolic activity was measured by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[25]. Material extracts were placed in direct contact with the hDPSC culture, and an MTT reagent (Sigma-Aldrich) was added for 4 hours as specified by the manufacturer's instructions; 100 μL/well dimethylsulfoxide (Sigma-Aldrich) was then added once a purple precipitate was observed. Covered plates were kept in the dark for 2–4 hours. Cell metabolic activity was assessed at 24, 48, and 72 hours of culture. The absorbance per well at 570-nm wavelength was recorded using a microplate reader (ELx800; Bio-Tek Instruments, Winooski, VT).

Cell Migration Evaluation (Wound Healing Assay)

To evaluate cell migration, hDPSCs were seeded into 24-well plates (2×10^5 cells/well) and cultured in normal growth medium for 24 hours. Then, the medium was replaced with a serum-free medium, and cells were cultured for an additional 24 hours. For the wound healing assay, a scratch wound was made using a sterilized pipette tip, and hDPSCs were exposed to the following experimental conditions: growth medium without material extracts (control group) or complete growth medium with 1:1, 1:2, or 1:4 ThPT, ThLC, or BD extracts (test groups). Images were obtained using a phase-contrast microscope at 0, 24, 48, and 72 hours. The percentage of open wound area at the different time points was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Cell Apoptosis/Necrosis Assay (Annexin-V-FITC and 7-AAD Staining)

hDPSC viability was quantified after 72 hours of culture at 37°C in complete growth medium (control) or in complete growth medium treated with 1:1, 1:2, or 1:4 dilutions of the tested material eluates. To assess cell viability, Annexin-V-FITC and 7-AAD staining (BD Biosciences, San Jose, CA) were applied according to the manufacturer's instructions. Within 1 hour of staining, specimens were analyzed by flow cytometry (FACS Calibur Flow Cytometer). Cell viability was assessed as a percentage and categorized as follows: viable (double negative), early apoptotic (Annexin-V-FITC positive, 7AAD negative), and late apoptotic and necrotic (Annexin-V-FITC negative/7-AAD positive or double positive, respectively).

Reactive Oxygen Species Release

The levels of reactive oxygen species (ROS) released by 1:1, 1:2, and 1:4 dilutions of the tested materials cultured with hDPSCs were determined by flow cytometry and compared with a negative control (hDPSCs in normal growth medium). To do so, hDPSCs were resuspended in 1 mL complete growth medium and stained with a general oxidative stress indicator—5 μmol/L CM-H_2DCFDA (Invitrogen, Carlsbad, CA)—for 30 minutes at 37°C. Then, the cells were rinsed twice and assessed by flow cytometry as described earlier.

Cell Morphology and Adhesion (Scanning Electron Microscopic Visualization)

The response (ie, morphologic variations and adhesion rates) of hDPSCs to the direct contact with the surface of the tested materials was assessed under scanning electron microscopy. The previously prepared sample discs ($n = 15$) were randomly divided into 3 groups, 1 for each material ($n = 5$). The surface of the sample discs were seeded with hDPSCs and cultured in normal growth medium for 72 hours. Then, cells were fixed with 3% glutaraldehyde (Sigma-Aldrich) in fetal bovine serum for 30 minutes. Samples were subsequently dehydrated under a graded ethanol series and treated with hexamethyldisilazane (Sigma-Aldrich) for 5 minutes. Finally, the samples underwent a gold sputter coating and visualized under a scanning electron microscope (100×, 300×, and 1500× magnifications).

Cell Cytoskeleton Staining (Immunofluorescence)

To assess any variation in cellular morphology, structure, and organization of the actin cytoskeleton of hDPSCs under exposure to the different material eluates, a qualitative description of immunofluorescence images of phalloidin-stained cells was performed. In brief, hDPSCs were seeded onto glass coverslips, left to adhere, and cultured in complete growth medium (control) or in complete growth medium treated with 1:1, 1:2, or 1:4 dilutions of the tested material eluates for 72 hours at 37°C. Then, cells underwent the following process:

(1) cells were rinsed twice using prewarmed fetal bovine serum at 37°C,

(2) they were fixed in 4% formaldehyde solution (Merck Millipore, Darmstadt, Germany) for 10 minutes,

(3) they were made permeable with 0.25% Triton X-100 solution (Sigma-Aldrich) for 5 minutes, and

(4) their cytoskeleton and nuclei were stained with AlexaFluor 594–conjugated phalloidin (Invitrogen) and 4,6-diamidino-2-phenylindole dihydrochloride (ThermoFisher Scientific, Waltham, MA), respectively. Lastly, immunofluorescence images were obtained and observed under a confocal microscope (Leica TCS SP2; Leica, Wetzlar, Germany).

Cell Osteo/Odontogenic Marker Expression (Real-time Quantitative Reverse-transcriptase Polymerase Chain Reaction)

The odontogenic differentiation of hDPSCs cultured with the tested materials was evaluated by means of the expression of odontogenic markers via real-time quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). Test groups consisted of hDPSCs (2×10^4 cells/well $n = 3$) seeded onto 12-well plates containing undiluted material-conditioned medium (1:1) and cultured for 14 days. Considering the negative results exhibited by ThLC in the cytocompatibility assays, only BD and ThPT were included in this assay. The negative control consisted of hDPSCs cultured in unconditioned culture medium (DMEM). A positive control was also included as a reference, consisting of an osteo/odontogenic medium (OsteoDiff Media, Miltenyi Biotec). The following sequence of primers for odontogenic markers were used (forward/reverse):

- Alkaline phosphatase (ALP): 5′-TCAGAAGCTCAACACCAACG-3′/5′-TTGTACGTCTTGGAGAGGGC-3′
- Collagen type 1: 5′-CCCGGGTTTCAGAGACAACTTC-3′/5′-TCCACATGCTTTATTCCAGCAATC-3′
- Osteonectin (ON): 5′-GCATCAAGCAGAAGGATA-3′/5′-AATAGTTAAGTTACAGCTAAGAAT-3′
- Dentin sialophosphoprotein (DSPP): 5′-GCATTTGGGCAGTAGCATGG-3′/5′-CTGACACATTTGATCTTGCTAGGAG-3′
- Runt-related transcription factor 2 (RUNX2): 5′-TCCACACCATTAGGGACCATC-3′/5′-TGCTAATGCTTCGTGTTTCCA-3′

The 2-ΔΔCT method was used to calculate the relative gene expression[26]. The expression of the housekeeping gene

glyceraldehyde 3-phosphate dehydrogenase was used as a reference, with the following sequence of primers (forward/reverse): 5′-TCAGCAATGCCTCCTGCAC-3′/5′-TCTGGGTGGCAGTGATGG-3′.

Cell Mineralization Assay (Alizarin Red S Staining)

Alizarin red S staining was used to analyze the formation of calcified nodules as a means to describe the mineralization potential of hDPSCs cultured with the tested materials. To do so, hDPSCs were left to proliferate in 12-well plates (2×10^4 cells/well, $n = 3$). Once confluent, cells were transferred onto undiluted material-conditioned medium and cultured for 21 days. Then, specimens were rinsed with fetal bovine serum and fixed with 70% ethanol for 1 hour. Samples were subsequently stained with 2% Alizarin Red S Solution (Sigma-Aldrich) for 30 minutes in dark conditions at room temperature and solubilized using 10% cetylpyridinium chloride monohydrate solution (Sigma-Aldrich). Finally, the absorbance was measured at 570 nm using a Synergy H1 multimode microplate reader (BioTek, Winooski, VT). The same control groups (positive and negative) used for the RT-qPCR analysis were used for this assay.

Statistical Analysis

Three separate measurements were performed for each of the dilutions (1:1, 1:2, and 1:4) of the tested materials for each assay. The homogeneity of variance and normal distribution of the data were confirmed. Consequently, a parametric analysis was performed with 1-way analysis of variance followed by the pair-wise Tukey post hoc test using Graph-Pad Prism v8.1.0 (GraphPad Software, San Diego, CA). The assay involving alizarin red S staining was statistically analyzed by 2-way analysis of variance. Data are presented as the mean ± standard deviation. Statistical significance was established at $P < .05$.

RESULTS

MTT Assay

The MTT assay results of hDPSCs exposed to different dilutions of the tested materials after 24, 48, and 72 hours of culture are presented in Figure 1*A*. All of the tested dilutions of ThLC exhibited a significantly higher cytotoxicity than the control group at all of the measured time points ($P < .001$). The same pattern was observed in the 1:1 and 1:2 ThPT-treated groups ($P < .001$). However, hDPSCs treated with 1:4 ThPT did not present significant differences with the control group after 24 and 48 hours of culture. At 72 hours of culture, a significantly higher cytotoxicity was observed ($P < .05$). Both the 1:2 and 1:4 BD-treated groups exhibited similar results to the control group at all of the measured time points without significant differences. The treatment of hDPSCs with nondiluted BD resulted in a significantly higher cytotoxicity than the control group after 48 and 72 hours of culture ($P < .001$). However, at 24 hours of culture, the difference observed was not significant.

Wound Healing Assay

The wound healing assay results of hDPSCs exposed to different dilutions of the tested materials after 24, 48, and 72 hours of culture are presented in Figure 1*B*. All of the tested dilutions of ThLC resulted in a significantly lower migration of hDPSCs compared with the control group at all of the measured time points ($P < .001$). The same occurred in hDPSCs cultured with nondiluted ThPT or BD at 48 and 72 hours ($P < .001$). On the other hand, the treatment with 1:2 and 1:4 ThPT or BD resulted in similar migration rates to the control group at all of the measured time points without significant differences. As an exception, 1:4 BD-treated hDPSCs exhibited significantly higher migration rates than the control group at 24 hours ($P < .05$).

Annexin-V-FITC and 7-AAD Staining

The mean cell viability rates of hDPSCs exposed to different dilutions of the tested materials after 72 hours of culture are presented in Table 1. The treatment with nondiluted BD or ThPT resulted in a similar percentage of viable hDPSCs (91.2% and 86.7%, respectively), whereas the 1:1 ThLC-treated group exhibited a substantially lower cell viability rate (14.7%). The same was observed with 1:2 dilutions of the tested materials as follows: BD (92.9%) > ThPT (88.4%) > ThLC (38.2%). Similar cell viability rates were obtained in the 1:4 BD- and ThPT-treated groups (92.4% and 89.1%, respectively); the 1:4 ThLC-treated group exhibited a higher percentage of viable hDPSCs compared with 1:2 and 1:1 dilutions (82.2% vs 38.2% and 14.7%, respectively) although still lower than BD and ThPT.

ROS Release

Intracellular ROS release in hDPSCs induced by the different dilutions of the tested materials is shown in Figure 2 as the percentage of CM-H_2DCFDA–positive cells compared with the control group. None of the dilutions of the BD-treated groups exhibited significant differences with the control group. However, all of the dilutions of the ThLC-treated groups exhibited a significantly higher percentage of CM-H_2DCFDA–positive cells ($P < .001$), which was lower as the material was more diluted. The ThPT-treated groups exhibited a significantly higher percentage of CM-H_2DCFDA–positive cells when nondiluted ($P < .05$), but no significant differences were found when diluted at a ratio of 1:2 or 1:4.

Scanning Electron Microscopic Visualization

Scanning electron microscopic images at 100×, 300×, and 1500× magnifications of the surface of standardized sample discs of the tested materials seeded with hDPSCs and cultured in normal growth medium for 72 hours are presented in Figure 3*A*. The ThLC samples exhibited a lack of adhered cells and debris, which indicate cellular death. However, on the ThPT samples, a moderate number of elongated cells adhered to the surface were visualized. Lastly, a high number of functionally oriented cells were observed on the surface of the BD samples.

Cell Cytoskeleton Staining

Immunofluorescence images of phalloidin–4,6-diamidino-2-phenylindole dihydrochloride staining of hDPSCs exposed to different dilutions of the tested materials after 72 hours of culture are presented in Figure 3*B*. Both BD- and ThPT-treated groups exhibited a wide spread of hDPSCs with a fibroblastlike spindle-shaped morphology and a high F-actin content. At 1:1 and 1:2 dilutions, BD and ThPT immunofluorescence images were similar to that of the control group. However, at a 1:4 dilution, a higher number of functionally oriented cells were observed in both cases. In contrast, ThLC-treated groups exhibited a scarce number of cells with an aberrant morphology at 1:1 and 1:2 dilutions and a low count of functionally oriented cells at a 1:4 dilution.

Cell Osteo/Odontogenic Marker Expression (RT-qPCR)

The expression of the previously described odontogenic markers exhibited by hDPSCs after a culture period of 14 days with the tested materials relative to the expression of glyceraldehyde 3-phosphate dehydrogenase is shown in Figure 4*A*. The ThPT-treated groups exhibited a significantly higher expression of ON and RUNX2 compared with the negative control group ($P < .001$). Alternatively, a significantly higher expression of DSPP was observed in the BD-treated groups ($P < .05$). Both of the test groups exhibited a significantly lower expression of ALP ($P < .001$), but only the BD-treated

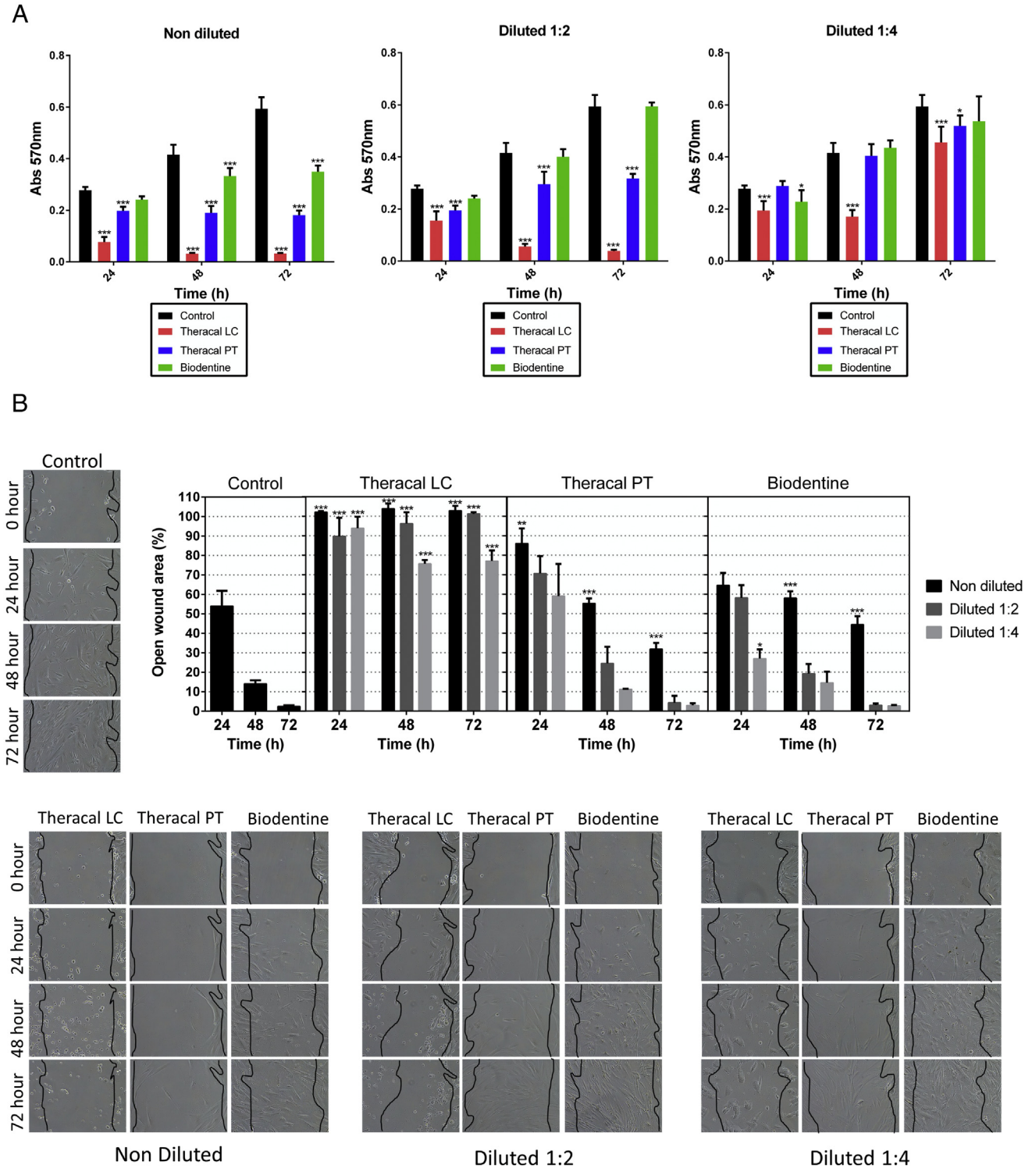

FIGURE 1 – Cytocompatibility and migration assays. (*A*) The results of the MTT assay on the cytotoxicity of 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, and BD after 24, 48, and 72 hours of culture with hDPSCs. Data are expressed as absorbance values at 570 nm compared with the control. $*P < .05$, $***P < .001$. (*B*) The results of the wound healing assay on the migration of hDPSCs cultured with 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, or BD for 24, 48, and 72 hours. The percentages of the open wound area at the different time points are presented graphically, relative to that of the control group. $*P < .05$, $**P < .01$, $***P < .001$.

groups showed a significantly lower expression of collagen type 1 ($P < .05$). Lastly, the reference osteogenic medium, used as a positive control, exhibited a significant up-regulation of ALP, DSPP, and RUNX2 compared with the negative control ($P < .001$).

Alizarin Red S Staining

The mineralization potential exhibited by hDPSCs after a culture period of 21 days with

TABLE 1 - Annexin-V-FITC and 7-AAD Staining Results after 72 Hours of Culture

Groups	Dilution	Percentage of viable hDPSCs			
		Q1	Q2	Q3	Q4
Control	—	0	0.04	4.62	95.3
Biodentine	1:1	0.09	2.18	6.50	91.2
	1:2	0.01	1.75	5.36	92.9
	1:4	0.01	2.11	5.47	92.4
Theracal LC	1:1	0.24	57.8	27.3	14.7
	1:2	0.03	29.4	32.4	38.2
	1:4	0.04	5.13	12.6	82.2
Theracal PT	1:1	0.10	6.81	6.37	86.7
	1:2	0.02	4.31	7.28	88.4
	1:4	0.09	0.02	5.58	89.1

hDPSCs, human dental pulp stem cells; Q1, necrotic (double positive); Q2, late apoptotic (Annexin-V-FITC negative/7-AAD positive); Q3, early apoptotic (Annexin-V-FITC positive, 7AAD negative); Q4, viable (double negative).

the tested materials, expressed as the formation of calcified nodules stained by alizarin red S staining, is shown in Figure 4*B*. Expectedly, the ThLC-treated groups exhibited a significantly lower mineralization potential than the control groups and the other test groups ($P < .001$). However, both the BD- and ThPT-treated groups showed a significantly higher mineralization potential than both the negative and positive control groups ($P < .001$). Interestingly, the difference in the mineralization potential between the BD- and ThPT-treated groups was also significant in favor of BD ($P < .001$).

DISCUSSION

In vitro assays have been widely used in the literature to characterize and establish a "biological profile" of new commercially available endodontic material compositions before the analysis of their performance in animal models or clinical trials[6]. Following this framework, the present study aimed to report the biological properties of ThPT, a new resin-modified calcium silicate–based material, compared with its predecessor, ThLC, and the well-established HCSC BD. The superficial element distribution of the tested materials has been previously described[20,27]. However, this

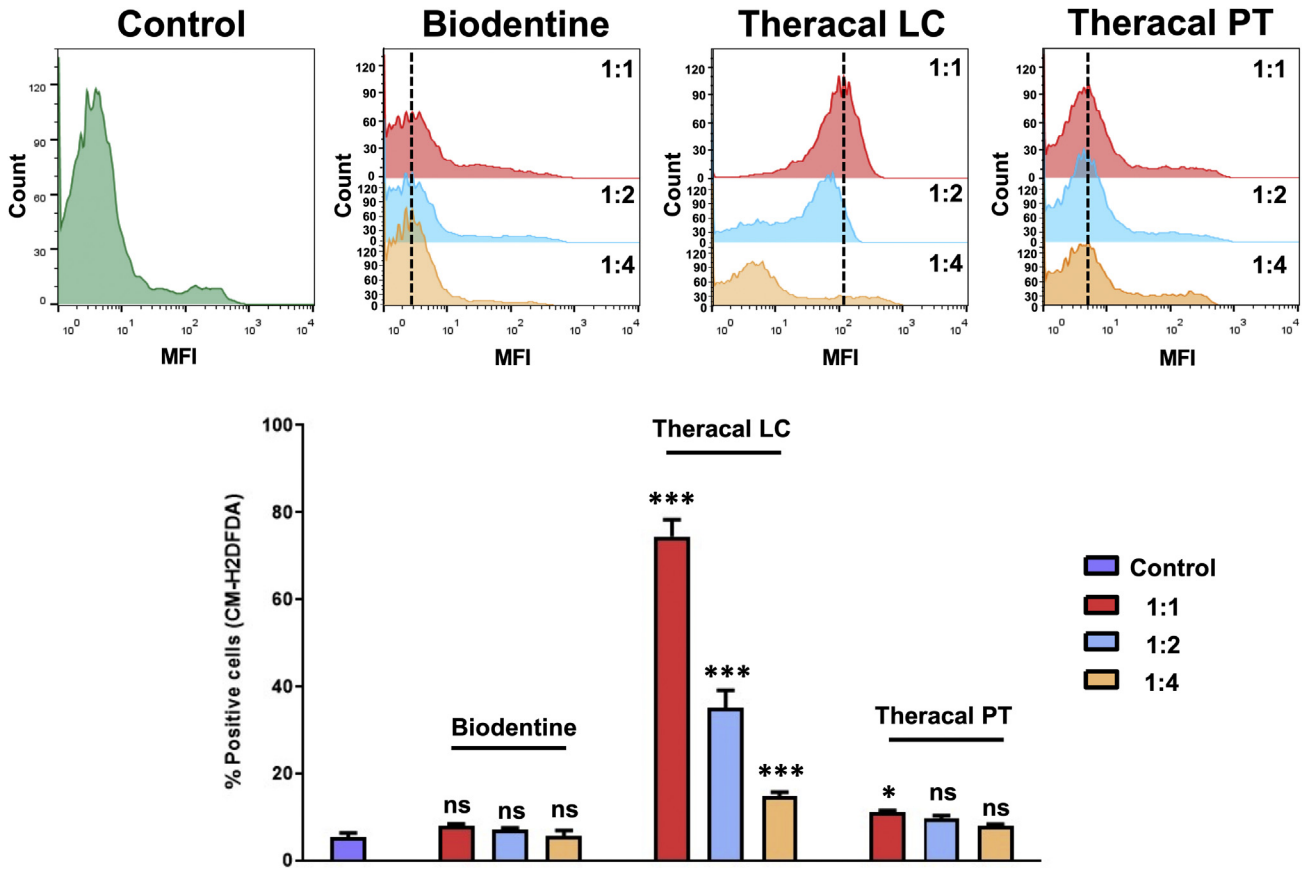

FIGURE 2 – The results of the ROS assay on the percentage of CM-H_2DCFDA–positive cells after culture with 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, or BD compared with the negative control group. *$P < .05$, ***$P < .001$. The histogram's y-axis represents the number of cells × 10e3, whereas the x-axis represents the mean fluorescence intensity (MFI) in logarithmic scale. The dashed line marks the MFI value of each material at 1:1 dilution.

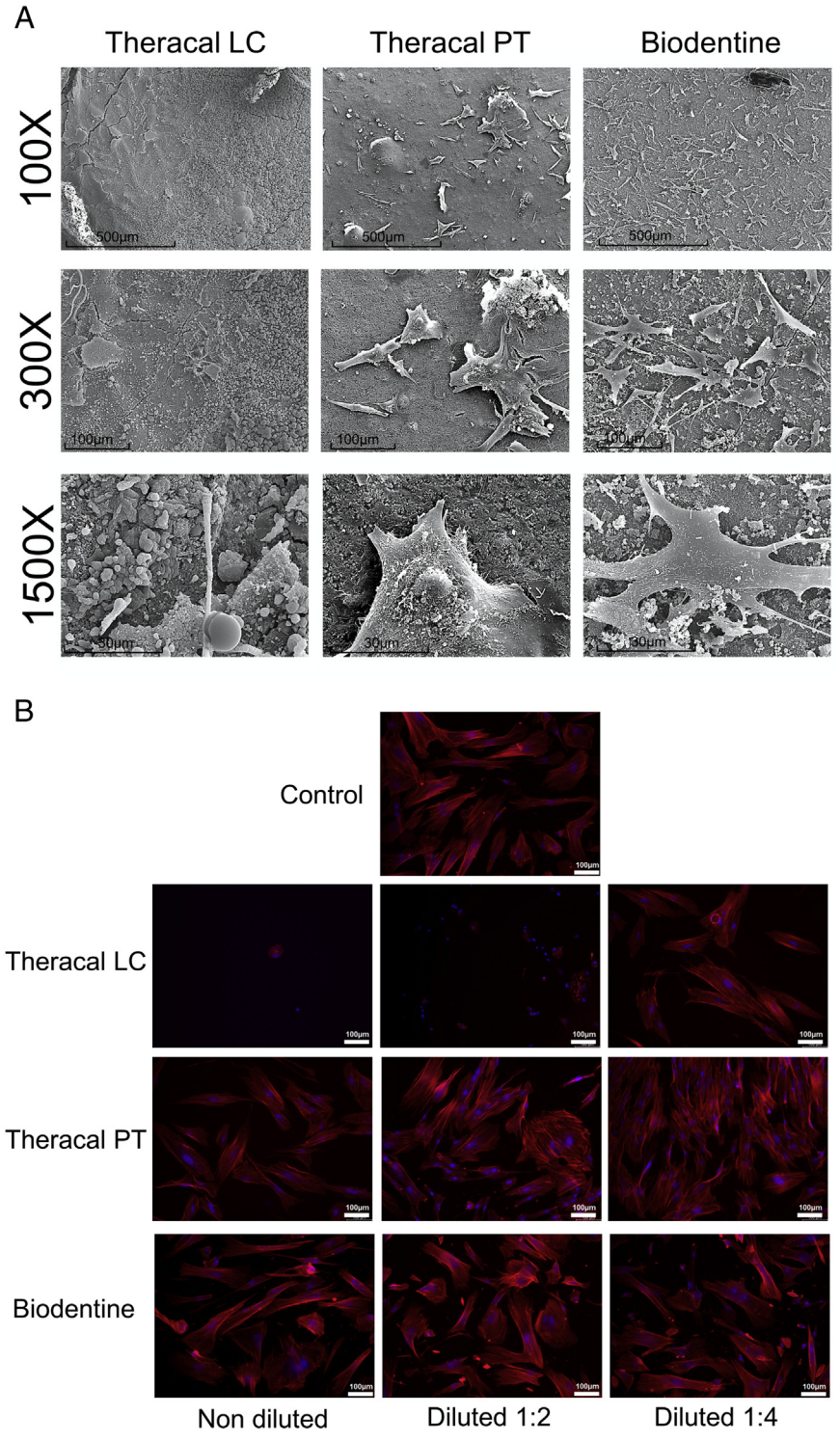

FIGURE 3 – Cell morphology and adhesion assays. (*A*) The results of the scanning electron microscopic visualization assay after 72 hours of culture with 5-mm-diameter and 2-mm-high sample discs of ThPT, ThLC, or BD compared with the negative control group. Magnifications: 100×, 300×, and 1500×. Scale bars: 500 μm, 100 μm, and 30 μm. (*B*) The results of the cell cytoskeleton staining via immunofluorescence after 72 hours of culture with 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, or BD compared with the negative control group. The images shown are representative from $n = 3$ separate experiments. Scale bar: 100 μm.

is the first study to compare the biological properties of ThPT with BD as a reference HCSC.

The comparison of this newly introduced material with ThLC and BD appears suitable because of their shared clinical indications, namely VPT procedures. The selection of BD as a reference HCSC was based on similar studies in the field[17,27], its high clinical success rates[28], and extended use among daily endodontic practice[29]. The traditional reference material, MTA, was also previously compared with ThPT by our research group in order to preliminarily establish the difference between the biological behavior of resin-modified calcium silicate–based materials and Portland cement–based materials *in vitro*[20].

As performed by similar studies[20,27], previously characterized hDPSCs were used in the present study as the target cellular population. These cells, residing within perivascular niches, are recruited into the dentin-pulp complex, both in a healthy and an inflammatory state, and induced to differentiate into odontoblastlike cells through a cascade of molecular events involved in the process of reparative dentinogenesis[2,8,30]. Therefore, by analyzing the biological response of hDPSCs toward the tested materials by means of a wide variety of *in vitro* tests that assess different cellular parameters, their clinical behavior may be potentially anticipated.

Within the biological properties of the tested materials, their cytocompatibility, as expressed by the MTT and wound healing assay and by Annexin-V-FITC and 7-AAD staining, can at least be deemed as adequate. Recurrently, the cytocompatibility of the tested materials appeared to be highest when more diluted. Both the 1:4 BD- and 1:4 ThPT-treated hDPSCs exhibited similar results in the MTT assay as the control group after 48 hours of culture as well as similar migration rates after 72 hours of culture. Furthermore, cell viability rates were the highest when treated with the highest dilution (1:4) of both materials (92.4% and 89.1%, respectively). Altogether, the results from the *in vitro* cytocompatibility assays point toward the absence of cytotoxicity of the lowest dilutions of ThPT and BD toward hDPSCs.

In contrast, all of the tested dilutions of ThLC exhibited significantly higher cytotoxicity and lower migration rates than the control group at all of the measured time points ($P < .001$) and the lowest cell viability rates among the tested materials (82.2%, 38.2%, and 14.7% at 1:4, 1:2, and 1:1 dilutions, respectively). These results are in accordance with a previous study in which the biological properties of ThLC and ThPT were compared with the Portland cement–based material MTA (Angelus, Londrina, PR, Brazil). Similar to the present study, both ThPT and MTA exhibited an absence of cytotoxicity when highly diluted (1:4), whereas a high cytotoxicity was observed in the ThLC-treated groups[20].

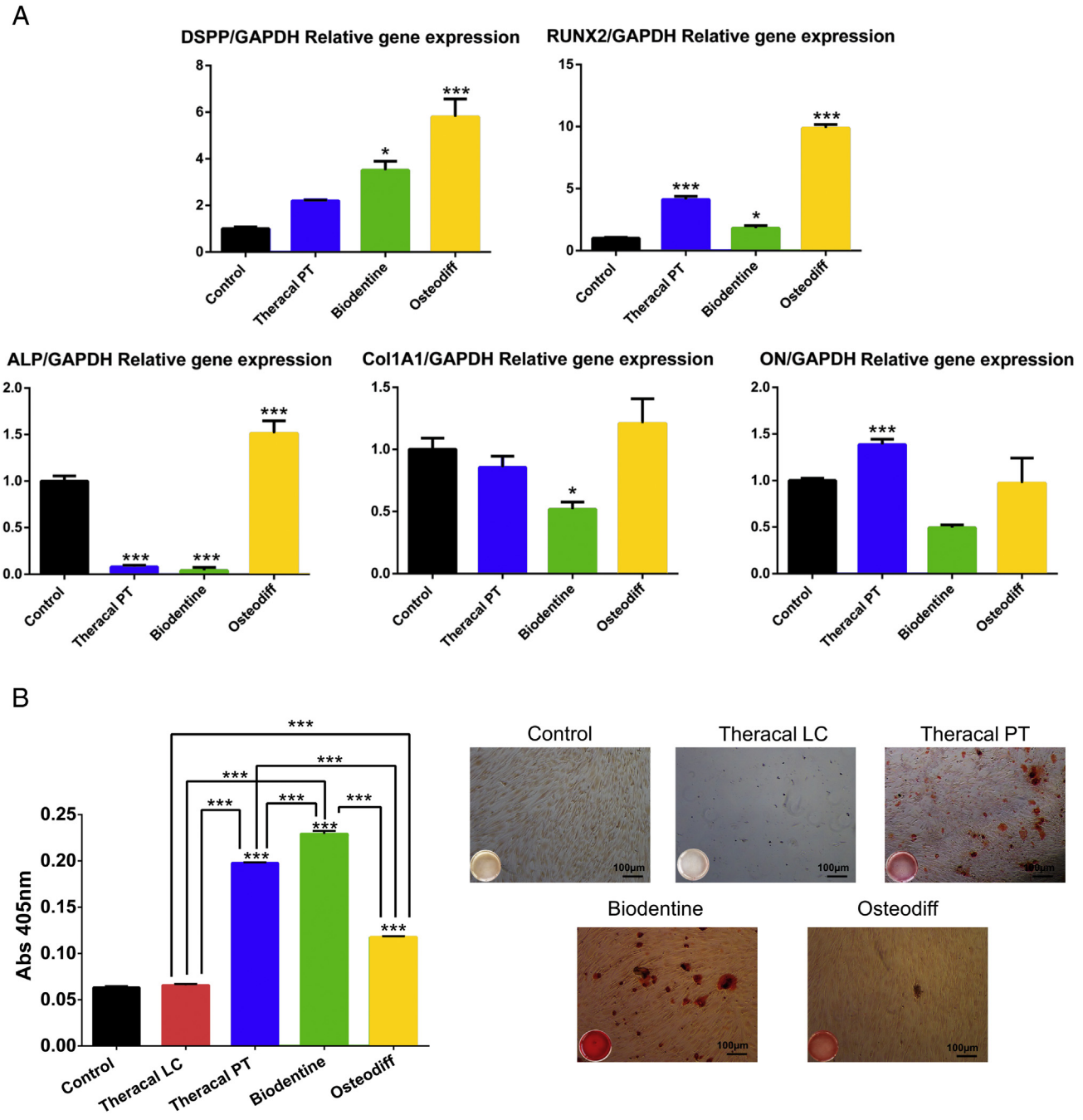

FIGURE 4 – Cell differentiation and mineralization assays. (*A*) The RT-qPCR results on hDPSC expression of osteo/odontogenic markers after 14 days of culture with 1:1, 1:2, and 1:4 eluates of ThPT or BD compared with the negative control group. Data are expressed as the mean ± standard deviation and relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. $*P < .05$; $***P < .001$. (*B*, *left*) Alizarin red S staining results on the mineralized nodule formation of hDPSCs after 21 days of culture with 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, or BD compared with the negative control group and the positive control group. $***P < .001$ (2-way analysis of variance). (*B*, *right*) Representative images of alizarin red S staining. An increase in the red color from the samples indicates a higher fixation to calcium deposits and therefore a higher mineralization.

Coincidental results have also been described with regard to ThLC in studies on stem cells from human exfoliated deciduous teeth[21]. Regarding BD, similar *in vitro* studies in the field have also reported its absence of cytotoxicity toward hDPSCs[31]. This is also supported by a recent clinical study on the efficacy of ThLC, BD, and MTA as pulp capping agents in which ThLC resulted in a lower success rate than BD and MTA[32].

Following this tendency, the culture with the different eluates of ThLC led to significantly higher CM-H_2DCFDA–positive hDPSCs than the control group ($P < .001$), whereas none of the dilutions of BD or 1:2 and 1:4 ThPT exhibited significant differences.

CM-H_2DCFDA is a chloromethyl derivative of H_2DCFDA, which is used as an indicator for ROS in cells[33]. Upon bacterial infection, hypoxia, or low nutrient supply, dental stem cells produce excessive levels of intracellular ROS, which result in oxidative stress. Excessive ROS may lead to the oxidation of cellular DNA, proteins, lipids, and membranes,

among others, and consequently alter the cells' architecture and integrity[34]. Therefore, the adequate levels of ROS exhibited by BD- and ThPT-treated groups further support their use in VPT procedures in which the regenerative potential of hDPSCs with an appropriate stress response is exploited.

Both of the morphologic assays, namely scanning electron microscopic visualization and immunofluorescence staining, expectedly support the aforementioned findings. The lower cytocompatibility exhibited by ThLC translates into the following observable indicators of cellular death: the presence of cell debris, the lack of cellular adhesion, and the absence of functionally oriented cells, whereas the opposite was observed in the ThPT- and BD-treated hDPSCs. The morphologic differences between the ThLC- and ThPT-treated hDPSCs observed in the present study confirm those reported in a previous study performed by our research group[20].

Altogether, the results from the cytocompatibility assays disfavored the inclusion of ThLC in the cell plasticity assay because of its negative influence on hDPSC viability, proliferation, adhesion, and morphology. The decision to exclude ThLC from this assay was further supported by previous studies in the field in which it was also excluded from a cell plasticity assay[20], it was associated with significantly lower rates of stem cells from human exfoliated deciduous teeth viability[21], the ThLC-treated hDPSCs exhibited a significantly lower ALP activity than BD and MTA[31], and it adversely affected the osteogenic differentiation of hDPSCs[35].

Regarding the evaluation of the influence of the tested materials on cellular plasticity, both the ThPT- and BD-treated hDPSCs exhibited a significant up-regulation of at least 1 odontogenic marker compared with the negative control group after a 14-day culture period. The process of reparative dentinogenesis involves a complex intercommunication of a series of cell-signaling transduction pathways, which culminate overexpression of specific markers for cell proliferation, differentiation, secretory activity, and/or inflammatory response[30]. The up-regulation of odontogenic markers expressed by both of the tested materials suggests their favorable influence on hDPSC odontogenic differentiation.

Lastly, the influence of all of the tested materials on hDPSC mineralized nodule formation was assessed via alizarin red S staining. The significantly higher mineralization potential exhibited by both BD and ThPT compared with both the negative and positive control groups highlights their bioactive properties and, consequently, their ability to potentially favor the development of a mineralized layer on their surface when placed in direct contact with pulp tissue in VPT procedures.

Altogether, considering the differences exhibited by the tested biomaterials in terms of their cytocompatibility, influence on cell plasticity, and mineralization potential on hDPSCs led to the rejection of the null hypothesis. However, the lack of evidence regarding the comparative biological properties between ThPT and HCSCs hinders the direct comparison of the results of this study with previous evidence, thus acting as a limitation of the present work. The use of standardized procedures, such as those described in the ISO 10993-5 guidelines[22], together with proven methodologies, such as the 2-ΔΔCT method to calculate relative gene expression[26], ensure that the data are reported with adequate reliability. However, the extrapolation of the results of the present *in vitro* study into the clinical setting requires further support from animal and clinical trials. Nevertheless, the *in vitro* biological properties of the tested materials described in the present work may act as a preliminary assessment of their potential biological behavior.

CONCLUSION

The newly introduced ThPT offers an improved *in vitro* cytocompatibility and mineralization potential on hDPSCs compared with its predecessor, ThLC, and comparable biological properties to the HCSC BD.

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