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ORIGINAL ARTICLE

Biological effects of a premixed calcium silicate pulp-capping material containing dimethyl sulphoxide as a vehicle: In vitro and in vivo study

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Abstract

This study aimed to compare the biological performance of a hydraulic calcium silicate cement (EndocemMTA Premixed) containing dimethyl sulphoxide (DMSO), developed to improve handling properties, in comparison with ProRoot mineral trioxide aggregate and Biodentine. Human dental pulp cells were used for in vitro evaluation of cytocompatibility, cell migration and cell–material interactions. Odontogenic differentiation was assessed using a 3D culture model designed to simulate the clinical environment. Pulp capping was performed in vivo on rat maxillary molars, and reparative dentin formation and pulpal inflammatory responses were evaluated using micro-computed tomography (μCT) and histological analyses. In vitro, all investigated materials exhibited comparable cytocompatibility, cell migratory behaviour and odontogenic marker expression, with no significant differences among study groups. The μCT analysis demonstrated significantly greater reparative dentin formation in all experimental groups compared with the control, with Biodentine producing a higher dentin volume than EndocemMTA Premixed. Histological evaluation revealed no significant differences among the experimental groups with respect to pulpal inflammation or dentin bridge continuity. These findings suggest that the incorporation of DMSO into premixed formulations would not adversely affect cytocompatibility or pulp healing. Moreover, the 3D model used in this study might serve as a clinically relevant platform for evaluating pulp–material interactions.

KEYWORDS
dental pulp, dentin, endodontics

INTRODUCTION

Preservation of pulp vitality represents a fundamental objective of contemporary minimally invasive endodontics, as maintenance of the natural defence and regenerative capacity of the dental pulp is critical for long-term tooth survival [1]. Despite many recent advances in restorative and bioactive bioceramic materials, the clinical predictability of vital pulp therapy remains highly material-dependent. Within this current paradigm, direct pulp capping has been widely adopted as a conservative therapeutic approach, aimed at sealing the exposed pulp tissue with suitable bioactive materials that would promote healing of the dentin–pulp complex [2]. However, the clinical success of direct pulp capping remains yet highly dependent on the biological, mechanical and handling characteristics of the pulp-capping material.

Hydraulic calcium silicate cements (hCSCs), including mineral trioxide aggregate (MTA), are widely used for vital pulp therapy due to their sealing ability even in the presence of moisture and adequate biocompatibility [3, 4]. An expanding body of experimental and clinical evidence has consistently reported the favourable biological and physicochemical performance of hCSCs, including their ability to promote reparative dentin formation, maintain pulp vitality and provide durable sealing over the exposed pulp surface. Notably, recent reviews have further confirmed that hCSCs demonstrate high success rates in vital pulp therapy, supporting the clinical translation of their favourable laboratory-based biological and physicochemical properties [1, 5]. However, their clinical performance remains influenced by practical limitations, particularly handling characteristics and material consistency during placement. Among these limitations, handling variability during chairside mixing has been identified as a clinically relevant source of inconsistency in material performance, leading to the development of premixed hCSC formulations designed to improve handling reproducibility and delivery [6].

It is well accepted that premixed systems improve clinical usability by eliminating on-site mixing and providing enhanced plasticity and flowability [6, 7]. Nevertheless, to achieve long-term stability, the cement powder in premixed formulations must be suspended in a practical, nonaqueous vehicle, with polyethylene glycol (polyethylene oxide: excipient in many pharmaceutical products) and propylene glycol (propane-1,2-diol: safe under the intended use) being the commonly used carriers to date [8, 9]. Despite the increasing clinical use of premixed hCSCs, the biological consequences of incorporating alternative nonaqueous vehicles remain insufficiently understood, particularly in the context of direct pulp capping, where material–tissue interactions directly influence healing outcomes.

In recent years, increasing attention has been directed toward alternative vehicles that may not only stabilize premixed formulations but also improve material–tissue interactions and biological performance. Among these candidates, dimethyl sulphoxide (DMSO) has attracted interest due to its well-documented ability to enhance tissue permeability and molecular diffusion. Very recently, a premixed hCSC utilizing DMSO as a vehicle has been introduced (EndocemMTA Premixed; Maruchi). To the best of our knowledge, this is the first pulp-capping material formulated with DMSO as a nonaqueous carrier. DMSO is a virtually nontoxic polar aprotic solvent, often used for electrophilic substitution reactions. It is with well-documented abilities to enhance dentin wettability, improve material penetration into dentinal structure and facilitate dentin adhesive reactions, while yet maintaining cellular compatibility at low concentrations [10–12]. Although DMSO has been widely used as a penetration enhancer in biomedical applications, its biological behaviour when incorporated as a vehicle in pulp-capping materials remains insufficiently characterized, particularly in the context of direct exposure to pulp tissue. Therefore, systematic biological evaluation is required to determine whether the incorporation of DMSO alters pulp healing responses compared with established materials. In the present formulation, DMSO was selected as a nonaqueous vehicle to maintain premixed stability and improve handling consistency, while potentially enhancing wetting and diffusion at the dentin interface, properties previously reported for DMSO in dental adhesive and biomaterial systems. However, potential limitations include dose-dependent cytotoxicity, uncertainties in release behaviour and the currently limited clinical evidence supporting its long-term safety. To understand its feasibility in endodontics, its application as a vehicle in direct pulp-capping materials necessitates careful and systematic biological validation under clinically relevant conditions.

A comprehensive biological evaluation of bioceramic materials presents certain methodological challenges, as they are insoluble in water and cannot be highly concentrated in a solution, thereby limiting conventional dose-dependent analyses. Moreover, most previous in vitro studies have relied on 2D cell culture models only, which fail to adequately reproduce the complex cell–cell and cell–matrix

interactions present in vivo [13]. Three-dimensional culture systems would provide a more robust and physiologically relevant microenvironment and be better suited for assessing advanced biological reactions, such as cell differentiation and tissue-like organization. Nevertheless, in vitro models alone are insufficient to determine whether a true reparative dentin bridge is formed at the pulp–material interface, necessitating complementary in vivo animal studies [14, 15]. Accordingly, an integrated experimental strategy combining in vitro and in vivo models is required for reliable biological assessment of novel pulp-capping bioceramic materials. Indeed, recent international guidelines on the biological evaluation of dental biomaterials recommend a stepwise, standardized testing strategy that may include complementary in vitro and in vivo assessments, depending on the intended clinical application and the results of preliminary testing [16, 17].

Given that the vehicle component plays a pivotal role in determining the physicochemical behaviour and biological performance of premixed hCSCs, the incorporation of DMSO introduces a clinically relevant variable whose biological effects remain insufficiently understood. Accordingly, because the incorporation of DMSO as a vehicle may alter material–tissue interactions and its biological effects in premixed hCSCs remain insufficiently characterized, the objective of this study was to evaluate the cytocompatibility, inflammatory responses, odontogenic differentiation and reparative dentin formation of a newly developed DMSO-based premixed hCSCs using complementary in vitro and in vivo models, in comparison with clinically established pulp-capping materials, including ProRoot MTA (Dentsply Tulsa) and Biodentine (Septodont). The null hypotheses were that (1) EndocemMTA Premixed exhibits cytocompatibility and differentiation-inducing effects comparable to those of ProRoot MTA and Biodentine in vitro, and (2) no significant differences exist among the pulp-capping materials with respect to reparative dentin formation or pulpal inflammation in an in vivo rat model.

MATERIAL AND METHODS

In vitro experiments

Primary culture of human dental pulp cells (hDPCs)

Ethical approval for the use of human teeth was obtained from the Institutional Review Board of Kosin University Gospel Hospital (KUGH 2023-11-014-001). After written informed consent was obtained from all donors, healthy third molars were extracted for clinical indications unrelated to pulpal pathology from patients of the age from 20 to 40.

The teeth were sectioned immediately after the extraction, and the pulp tissue was aseptically separated and obtained. The pulp tissue was gently rinsed using phosphate-buffered saline (PBS; HyClone Laboratories), and the cells were obtained using the explant outgrowth method [18]. Briefly, the tissue was minced into small fragments and cultured in minimal essential medium-α (MEM-α; HyClone Laboratories) supplemented with 10% foetal bovine serum (FBS; HyClone Laboratories), 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen) under 5% CO_2 at 37˚C. Then, the cells were subcultured, and passages 3–5 were selected to minimize phenotypic alterations associated with extended subculturing, as required for in vitro cytocompatibility assays, including cytotoxicity, cell migration, morphological evaluation and immunocytochemistry.

Preparation of material extracts

Three calcium silicate cements were compared and contrasted, and their compositions are listed in Table 1. Each pulp-capping material was carefully mixed according to the manufacturer's instructions and placed in disc-shaped molds (5 mm diameter and 1 mm height) to set. The specimens were allowed to set for 24 h at 37˚C in 100% humidity in an incubator. After setting, the specimens were surface-decontaminated with ultraviolet-C light for 1 h. Then, the discs were immersed in MEM-α at a ratio of 0.5 cm^2/ml and incubated at 37˚C for 24 h. The extracts were subsequently filtered through a 0.22-μm sterile syringe filter to remove debris particles and were then used for the experiments. A lower surface area-to-volume ratio than that recommended by ISO 10993-12 standards was intentionally used to minimize excessive cytotoxicity and enable a consistent and controlled comparative evaluation of cytocompatibility among materials under defined experimental conditions [19].

Cell viability assay

Cells were seeded in 24-well plates (SPL Life Sciences) at a density of 3×10^5 cells/well and cultured in growth medium for 24 h. Each group consisted of seven wells per condition ($n = 7$). The plates with cells were then exposed to undiluted extract, and no extract renewal was performed during the incubation period. The cells cultured in an extract-free medium served as the control. At Days 1, 2 and 3, the cell viability (cell metabolic activity) was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT solution (0.5mg/mL in PBS, 200 μL/well) was added and incubated for 2 h. DMSO (200 μL/well) was then added to

TABLE 1 Materials used in this study, their manufacturers and chemical compositions.

Material	Manufacturer	Reported composition by the manufacturer
EndocemMTA Premixed	Maruchi	Zirconium dioxide 40%–60%
		Calcium silicate 40%–60%
		Calcium aluminate <1%
		Calcium sulphate <1%
		Dimethyl sulphoxide 10%–20%
		Thickening agent <3%
ProRoot MTA	Dentsply Tulsa	Portland cement (tricalcium silicate, dicalcium silicate and tricalcium aluminate) 60%–90%
		Bismuth oxide 10%–40%
Biodentine	Septodont	Tricalcium silicate 80.1%
		Calcium carbonate 14.9%
		Zirconium oxide 5%
		Calcium chloride
		Soluble polymer as an aqueous liquid

Abbreviation: MTA, mineral trioxide aggregate.

dissolve formazan crystals. The plates were shaken using the built-in microplate shaker until the crystal dissolved, and the solution in each well was transferred to a 96-well tissue culture plate. Absorbance at 540nm was measured using a microplate reader (SPECTROstar Nano; BMG Labtech).

Cell migration assay

Cell migratory behaviour was evaluated using a scratch wound-healing assay. Cells (1×10^6) were seeded in 24-well plates (SPL Life Sciences) and cultured for 24 h. Seven wells were analysed per group in each experiment ($n = 7$). Then, the cells were serum-starved (1% FBS, 12 h) prior to scratching to minimize the contribution of cell viability. A linear scratch was created across the confluent cell layer. The scratch width was standardized by using the same 200-μL pipette tip held perpendicular to the plate by the same operator. Next, the cells were treated with undiluted pulp-capping material extracts for 12 h without renewal. The cells cultured in extract-free medium served as the control. Then, the detached cells were gently removed by rinsing with PBS. Images of the scratch area were acquired using a phase-contrast microscope (Olympus) at 0 and 12 h. The wound closure area was normalized to the initial wound area (0 h) and quantified using IMAGEJ software (version 1.53; National Institutes of Health). Wound closure was calculated using the following calculation formula:

$$\text{Wound closure} = (A_0 - A_{12})/A_0 \times 100$$

where A_0 and A_{12} represent the wound area at 0 and 12 h, respectively.

Scanning electron microscopic (SEM) observation of cell morphology

Disc-shaped samples (5 mm diameter and 1 mm height) of each pulp-capping material were placed in 24-well plates, and human dental pulp cells (hDPCs) were seeded directly onto the surface of the samples at 1×10^5 cells/specimen. Cells were cultured in growth medium supplemented with 10% FBS for 24 h. After incubation, cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) for 2 h at 4°C and dehydrated in increasing concentrations of ethanol (in the order of 70%, 80%, 90% and 100%) for 15 min at each concentration. After dehydration, samples were dried using a critical point dryer (EMS850; Electronic Microscopy Sciences) and sputter-coated with gold. Then, the samples were observed under a scanning electron microscope (SEM; SN-3000, Hitachi) at an accelerating voltage of 10 kV. Representative images from multiple randomly selected fields were acquired at various magnifications to evaluate cell attachment and spreading.

3D culture model and immunocytochemical staining

To approximate key aspects of the clinical direct pulp-capping environment, where freshly mixed cements directly contact pulp–matrix complexes, a customized 3D culture model was developed. A cylindrical structure (6 mm diameter and 9 mm height) with 0.5 mm side channels for the nutrient flow and a 2.5 mm opening on the top surface was designed using MESHMIXER software (Autodesk) and fabricated with a 3D-printer (Sonic; Phrozen) at 50 μm resolution. The dimensions were

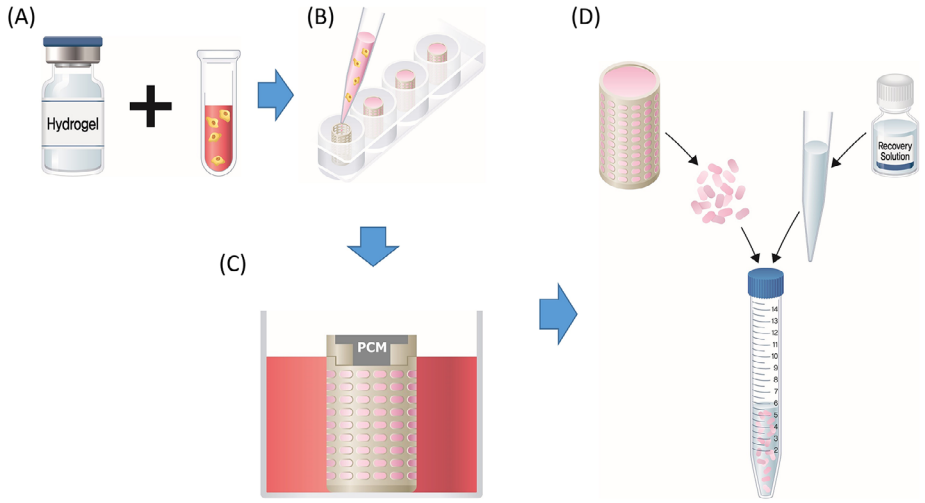

FIGURE 1 Schematic description of materials and methodology using the 3D culture model: (A) Cell suspension was mixed with hydrogel; (B) the hydrogel-cell mixture was transferred to the 3D model and allowed to incubate for 15 min, medium was added to cover the structures, and the cells were incubated for 2 wk; (C) freshly mixed hydraulic calcium silicate cements (hCSCs) were applied to the lid hole, and hCSC had direct contact with the hydrogel-cell mixture; and (D) after culturing the 3D structures for 3 wk, the cells were mixed with recovery solution and centrifuged to collect the cell pellet. PCM, pulp-capping material.

designed to allow sufficient cell loading, nutrient diffusion and direct material contact.

The cells were suspended in MEM-α containing 10% FBS and mixed at a 2:1 ratio with VitroGel Hydrogel Matrix (TheWell Bioscience), resulting in the final cell density of approximately 2×10^6 cells/ml within the hydrogel (Figure 1A). The hydrogel–cell mixture was carefully loaded into the 3D culture scaffolds placed in 24-well plates (Figure 1B), gelled for 15 min and subsequently covered with MEM. Next, the constructs were cultured for 2 wk with medium changes every 3 d. Then, freshly mixed materials were placed in direct contact with the top opening of the constructs and cultured for an additional period of 3 wk (Figure 1C). The medium was refreshed regularly to support cell viability during the long-term culture.

After 3 wk, the constructs were rinsed twice with PBS. Encapsulated hydrogels were dissociated using VitroGel Cell Recovery Solution (The Well Bioscience) at 37˚C, followed by centrifugation at 100 *g* for 5 min to collect the cells (Figure 1D). Cells were cytospun onto New Silane III microslides (Muto Pure Chemicals) at 800 *g* for 10 min, fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100. After blocking with 5% bovine serum albumin for 1 h, the cells were incubated overnight at 4˚C with antibodies against β-actin (Cell Signalling), dentin sialophosphoprotein (DSPP; Santa Cruz Biotechnology) and dentin matrix protein-1 (DMP-1; Takara Bio). Detection was performed using the VECTASTAIN Elite ABC-HRP kit (Vector Laboratories) and DAB peroxidase substrate (Vector Laboratories). Negative controls were prepared by omitting the primary antibodies. Stained cells were observed microscopically, and the number of positive cells was quantified. In brief, three randomly selected microscopic fields per slide were analysed, and a minimum of three independent slides per group were evaluated using identical threshold settings in IMAGEJ.

In vivo animal experiments

Direct pulp capping

All procedures involving animals were conducted in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines. Ethical approval of the animal study was obtained from the Institutional Animal Care and Use Committee of Jeonbuk National University Hospital (JBUH-IACUC-2021-9-1). Fourteen 8-wk-old male Wistar rats (300 g) were used, providing a total of 28 maxillary first molars. Each tooth was considered an experimental unit, with bilateral molars obtained from the same animal. Teeth were randomly allocated to each group using a randomization table, with the bilateral molars assigned to different groups whenever possible: Control, EndocemMTA Premixed, ProRoot MTA and Biodentine. The control group was included to evaluate the natural pulp response without the use of capping materials.

General anaesthesia was induced by intraperitoneal injection of ketamine (100 mg/kg; Yuhan) and xylazine (20 mg/kg; Bayer). After disinfection with 75% ethanol, Class I cavities were prepared under a dental operating microscope (Global Surgical). The pulp exposure was achieved with a sterile #15 K-file (Mani), and haemostasis was achieved by gently applying a saline-moistened cotton pellet for approximately 1 min. Freshly mixed pulp-capping materials were applied directly onto the exposed pulp tissue, but no capping material

was applied in the control group. Next, all cavities were restored with a universal adhesive (Single Bond Universal, 3 M ESPE) and light-cured composite resin (Filtek Z350 XT, 3 M ESPE). Postoperative care was provided in accordance with institutional guidelines. After 4 wk, the animals were euthanized by carbon dioxide inhalation in accordance with institutional animal care guidelines, and the maxillae containing the treated teeth were dissected and fixed in 4% paraformaldehyde for 48 h. The specimens were subsequently processed for radiological and histological analyses.

Micro-computed tomography

The fixed specimens were scanned in distilled water to prevent dehydration and minimize imaging artefacts, using a micro-computed tomography (μCT) system (Skyscan 1076, Bruker MicroCT) with a voxel size of 10 μm under the following conditions: 100 kV/100 μA, 1 mm aluminium filter, 0.6˚ rotation step, and 360˚ rotation. Images were reconstructed using NRECON software (Bruker MicroCT). Standard reconstruction parameters provided by the manufacturer were used. The volume of reparative dentin, newly formed radiopaque area over the exposure site, was measured using CTAN software (ver. 1.13, Bruker MicroCT). A consistent grayscale threshold was applied to all specimens for the segmentation of reparative dentin, and the regions of interest selection was performed with reference to histological sections to ensure anatomical consistency. All measurements were performed by the same examiner.

Histological analysis

Following μCT, the specimens were decalcified in 10% EDTA for 6 wk and embedded in paraffin. Decalcification was performed at room temperature with regular EDTA changes, and completion was confirmed mechanically. Serial sections (4 μm) were cut in the mesiodistal plane and stained with haematoxylin–eosin. Representative sections passing through the centre of the pulp exposure site were selected for evaluation. Two blinded examiners evaluated the sections according to modified criteria proposed in a previous study [20] (Table 2). The examiners were blinded to the treatment groups during evaluation, and discrepancies between examiners were resolved by re-examination, and the stricter score was adopted to avoid overestimation of tissue response.

Statistical analysis

The sample size was determined using G*POWER software (version 3.1; University of Düsseldorf). A power analysis for an F-test (effect size = 0.8) was conducted to determine the required sample size. A large effect size was assumed due to the exploratory nature of the study. Statistical analyses were conducted using SPSS (ver. 23; IBM). The reparative dentin volume from μCT analysis was tested for normality using the Shapiro–Wilk test, followed by one-way analysis of variance and Dunnett's post hoc test to compare each experimental group with the control group. Nonparametric data other than μCT analysis were analysed with the Kruskal–Wallis test with the Bonferroni correction. Parametric data are presented as mean ± standard deviation, and nonparametric data as median (interquartile range). A p-value <0.05 was considered statistically significant. In the animal study, each tooth was treated as an experimental unit, and the potential interdependence of bilateral teeth from the same animal was taken into account during statistical interpretation.

RESULTS

In vitro findings

Cell metabolic activity of hDPCs exposed to hCSC extracts was evaluated using the MTT assay. All groups maintained comparable levels of cell metabolic activity throughout the experimental period ($p > 0.05$) (Figure 2A). Scratch wound-healing assays demonstrated comparable wound closure behaviour among the study groups. As shown in Figure 2B–F, cell migration was not significantly influenced by the presence of hCSC extracts ($p > 0.05$). SEM analysis of cells attached to the material specimens revealed that after 24 h, the cells exhibited well-spread and elongated morphology comparable to that of the control group (Figure 3). Notably, numerous long, thin cytoplasmic processes characteristic of a fibroblast-like morphology were also observed.

Immunocytochemical analyses utilizing the 3D culture model (Figure 4) revealed that cell viability, as indicated by β-actin staining, which represents comparable cytoskeletal integrity and cell presence, did not differ significantly among the groups ($p > 0.05$). The DSPP and DMP-1 expression was detectable in all groups, suggesting that none of the assessed pulp-capping materials adversely affected the odontogenic marker expression ($p > 0.05$).

In vivo findings

The volume of the reparative dentin bridge formed after direct pulp capping was quantitatively assessed using μCT. At 4 wk, all teeth treated with hCSCs exhibited substantial reparative dentin formation (Figure 5). No significant

TABLE 2 Criteria used for the histologic evaluation of the pulps treated with direct pulp capping.

Grade	Reparative dentin formation	Inflammatory cell response
1	Heavy: hard tissue deposition as a complete and continuous dentin bridge	Absent or very few inflammatory cells
2	Moderate: hard tissue formation as an incomplete and discontinuous dentin bridge	Mild: inflammatory cells only next to dentin bridge or area of pulp exposition
3	Slight: a layer of scattered and foggy hard tissue deposition	Moderate: the presence of abscesses or inflammatory cells is observed in one-half of the coronal pulp
4	No hard tissue deposition	Severe: pulp necrosis or inflammatory cells in more than one-half of the coronal pulp

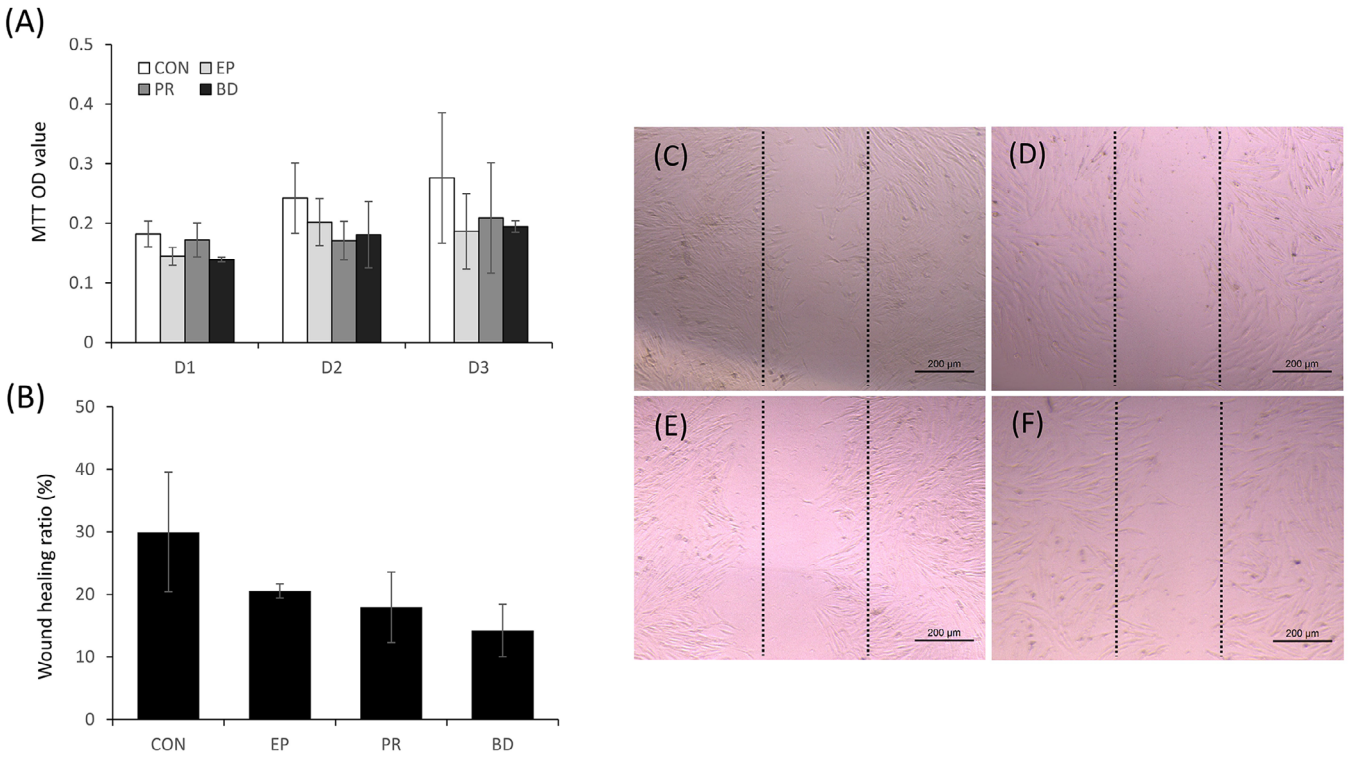

FIGURE 2 Effects of hydraulic calcium silicate cements (hCSCs) on cell viability and migration ability: (A) Evaluation of cell viability of human dental pulp cells (hDPCs) at 24, 48 and 72 h and (B) comparison of cell migration values by material at 12 h. (C–F) Representative images from the cell migration assay: (C) Control, (D) EndocemMTA Premixed, (E) ProRoot mineral trioxide aggregate (MTA) and (F) Biodentine. There was no statistically significant difference among groups. BD, Biodentine; CON, control; EP, EndocemMTA Premixed; PR, ProRoot MTA.

differences were observed between EndocemMTA Premixed and ProRoot MTA groups or between ProRoot MTA and Biodentine groups ($p > 0.05$). However, the Biodentine group exhibited a significantly greater volume of reparative dentin compared with the EndocemMTA Premixed group ($p < 0.05$).

Histological evaluation (Figures 6 and 7) revealed that specimens treated with BD showed no evidence of pulpal inflammation, whereas the EndocemMTA Premixed- and ProRoot MTA-treated groups exhibited either no inflammation or only mild inflammatory responses. In contrast, the control group demonstrated moderate-to-severe pulpal inflammation, which was significantly greater than that observed in all experimental groups ($p < 0.05$). Moreover, all experimental groups exhibited continuous and thick reparative dentin bridges, whereas the control group showed only sparse, cloud-like calcified deposits in some specimens. These histological findings were significantly different between the control and experimental groups ($p < 0.05$).

DISCUSSION

The present endodontic study investigated the biological performance of a newly developed DMSO-based premixed hCSC (EndocemMTA Premixed) for direct pulp capping

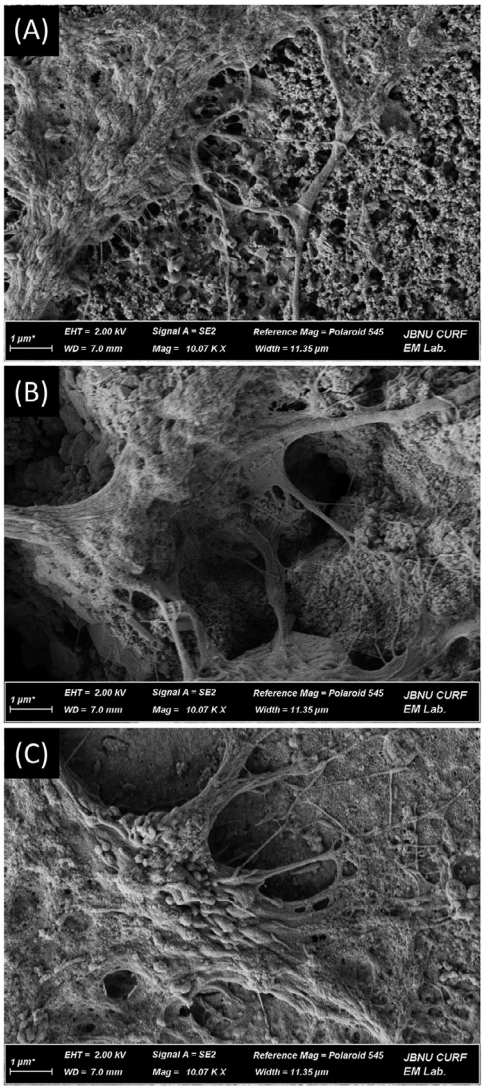

FIGURE 3 Scanning electron microscope (SEM) imaging of cells incubated for 24 h on the surfaces of (A) EndocemMTA Premixed; (B) ProRoot mineral trioxide aggregate (MTA) and (C) Biodentine. Note that well-spread and flattened cells were observed in contact with the surfaces of the three tested pulp-capping materials.

using an integrated experimental in vitro and in vivo approach. Moreover, this study was designed to address unresolved questions regarding whether modification of hCSC formulations, including the incorporation of DMSO as a nonaqueous vehicle intended to enhance handling characteristics, could be achieved without adversely affecting cytocompatibility, odontogenic differentiation or pulpal healing responses. Overall, the current findings demonstrated that EndocemMTA Premixed exhibited biological outcomes comparable to those of established pulp-capping materials, ProRoot MTA and Biodentine, thereby supporting the clinical feasibility of this premixed formulation.

The in vitro experiments showed no significant differences among the tested pulp-capping materials with respect to cell metabolic activity, migratory behaviour or cell morphology. All of this indicates that none of the hCSCs would exert cytotoxic effects on hDPCs. Nonetheless, these findings are consistent with previous reports demonstrating favourable cytocompatibility of calcium silicate–based endodontic materials [21–23]. Importantly, this current study extended conventional cytotoxicity testing by evaluating odontogenic differentiation using a developed 3D culture model (in lieu of 2D) designed to simulate clinical direct pulp-capping conditions.

Unlike traditional two-dimensional monolayer cultures, the customized 3D model enabled direct contact between freshly mixed pulp-capping materials and pulp-like cell–matrix constructs through a limited exposure area, allowing an evaluation of cellular responses under conditions designed to approximate aspects of pulp exposure. It can be said that this feature represents a methodological refinement over previous 3D culture studies, in which cells were typically cultured on or beneath material surfaces in configurations that do not reflect the actual complex clinical geometry [13, 24]. Within this model, the expression of DSPP and DMP-1 was observed in all experimental groups, indicating that none of the tested materials adversely affected the expression of odontogenic differentiation-associated markers. However, functional mineralization assays were not performed in the present study, and therefore, the observed marker expression should be interpreted as supportive evidence of odontogenic potential rather than definitive functional differentiation.

A central concern addressed in this study was the biological safety of incorporating DMSO as a vehicle in a premixed pulp-capping material. Despite the fact that DMSO has been widely used as a solvent and penetration enhancer in biomedical research, however, its cytotoxic effects at high concentrations are well-documented [25–27]. In the present study, EndocemMTA Premixed contains 10–20 wt% DMSO, not a minute amount, as per the manufacturer-provided formulation data, raising legitimate concerns regarding its potential impact on pulp cells and tissues. Despite these concerns, the current in vitro results demonstrated that EP did not reduce cell viability, alter migratory behaviour or even suppress odontogenic marker expression compared with ProRoot MTA and Biodentine. These findings may be attributed to the physicochemical reactions of EndocemMTA Premixed during setting. As hydration progresses, DMSO is gradually being replaced by water, and its release into the surrounding environment is likely limited by the material's low solubility and rapid setting reaction. Consequently, the effective concentration of DMSO to which pulp cells are exposed may still remain below the cytotoxic thresholds, thereby preserving cellular compatibility. However, it should be noted that the present study evaluated the biological performance of a commercially available DMSO-containing hCSCs, rather than systematically isolating the independent effect of DMSO

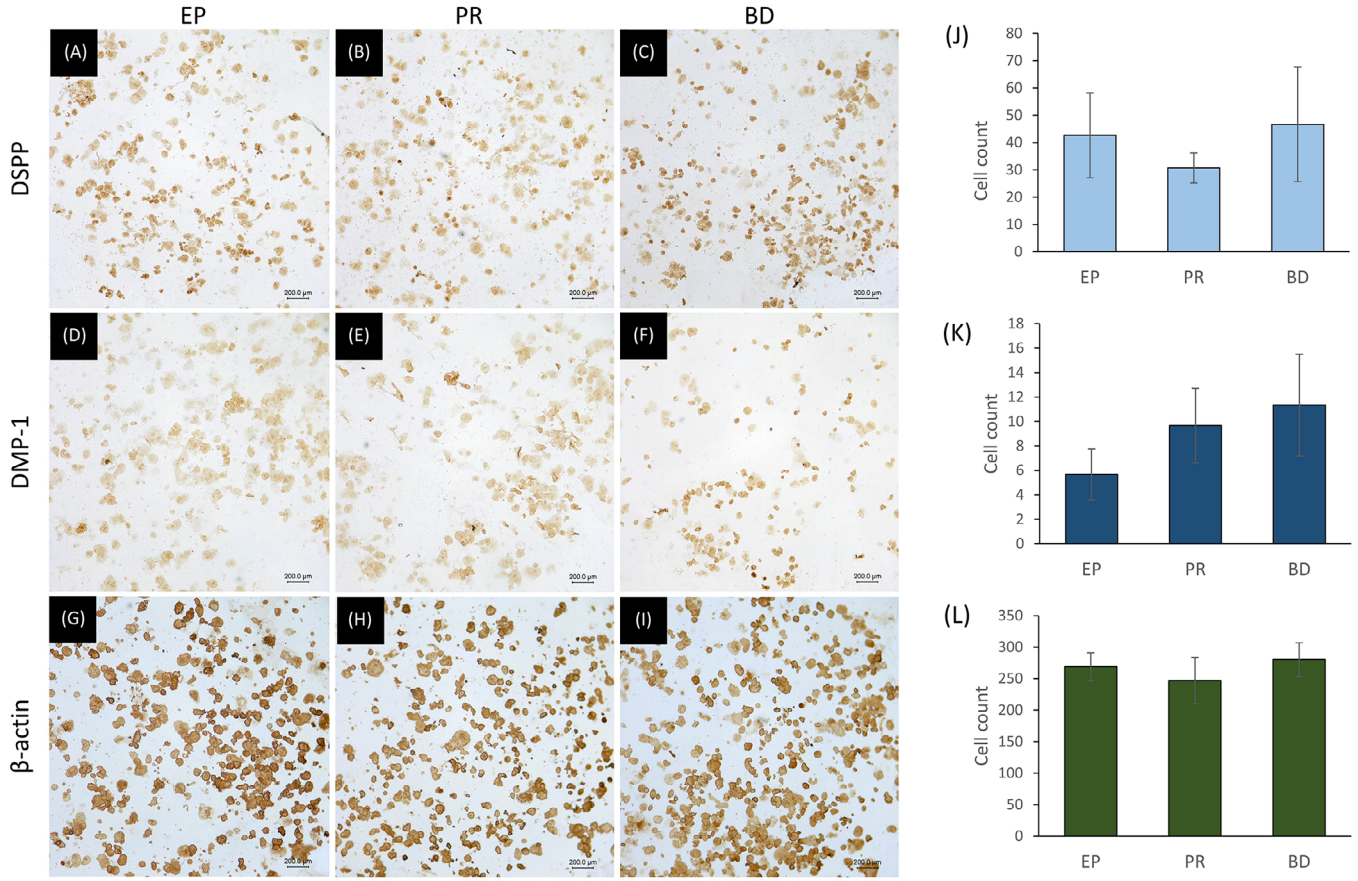

FIGURE 4 Immunocytochemistry of dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1) and β-actin protein exposed to hydraulic calcium silicate cements (hCSCs): (A, B, C and J) results of the DSPP antibody, (D, E, F and K) results of DMP-1 antibody, (G, H, I and L) results of β-actin antibody. There was no statistically significant difference among groups. BD, Biodentine; CON, control; EP, EndocemMTA Premixed; PR, ProRoot mineral trioxide aggregate (MTA).

as a single formulation variable. In other words, the release kinetics and local concentration of DMSO were not directly quantified in the present study. Therefore, conclusions regarding the biological safety of DMSO exposure should be interpreted as indirect and based on observed biological responses rather than direct measurement of DMSO release. Future studies investigating the release behaviour and concentration profile of DMSO during material setting would provide further insight into its biological safety.

The in vivo evaluation further confirmed the biological acceptability of EndocemMTA Premixed. All hCSC-treated groups exhibited significantly greater reparative dentin formation and reduced pulpal inflammation compared with the control group, which findings are consistent with previous studies demonstrating the regenerative potential of calcium silicate–based bioceramic materials [28–30]. Histological analyses revealed comparable inflammatory responses and dentin bridge continuity among EndocemMTA Premixed, ProRoot MTA and Biodentine, supporting a partial acceptance of the second null hypothesis. However, although the sample size was determined based on a priori power analysis, the possibility that clinically significant differences between materials were undetected cannot be completely ruled out due to the relatively small number of experimental animals. Therefore, the finding of no statistically significant differences between the experimental groups should be interpreted with caution.

Interestingly, the μCT analysis showed that Biodentine induced a significantly greater volume of reparative dentin bridge than EndocemMTA Premixed, whereas histological scoring did not reveal marked differences among the experimental groups. This discrepancy is likely reflecting methodological differences between, on the one hand, the volumetric μCT analysis and, on the other hand, section-based histological evaluation. Although μCT provides a 3D quantitative assessment of mineralized tissue, the histology captures localized tissue responses and may be influenced by section orientation and sampling limitations. These findings clearly highlight the importance of combining radiological and histological methods for the comprehensive evaluation of reparative dentinogenesis. In addition, importantly, the present findings suggest that although EndocemMTA Premixed supported the

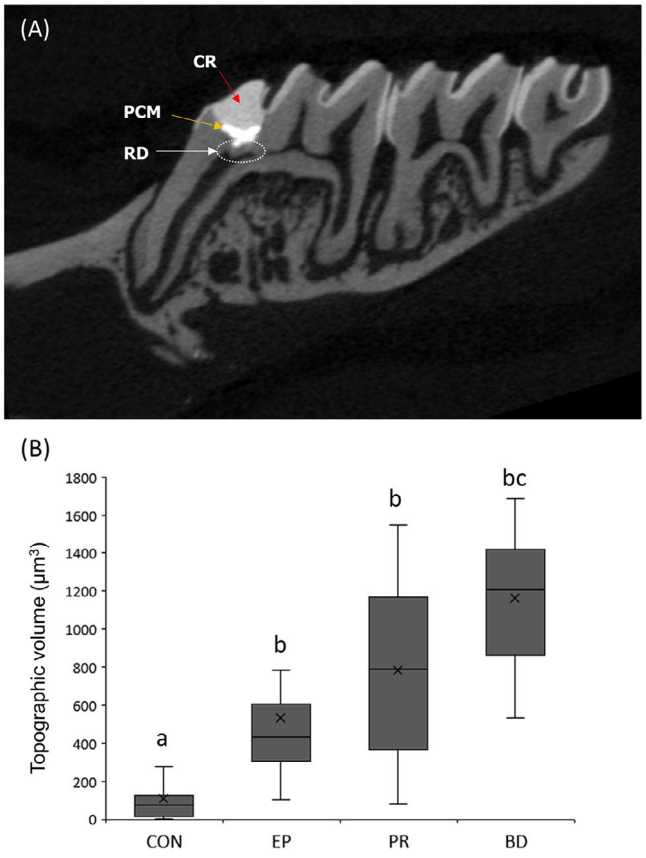

FIGURE 5 Reconstructed micro-computed tomography (μCT) image and the mean volume of newly formed reparative dentin: (A) representative 3D reconstructed μCT image of reparative dentin induced by EndocemMTA Premixed; (B) quantitative comparison of reparative dentin volume. Different superscript letters mean statistically significant differences between groups ($p < 0.05$). BD, Biodentine; CON, control; CR, composite resin; EP, EndocemMTA premixed; PCM, pulp-capping material; PR, ProRoot mineral trioxide aggregate (MTA); RD, reparative dentin.

formation of continuous dentin bridges with favourable histological characteristics, the total volume of mineralized tissue formed within the 4-wk observation period was slightly lower than that observed with Biodentine. The clinical relevance of this quantitative difference remains uncertain and warrants further investigation with longer observation periods.

Several studies have evaluated the reparative dentin formation of Biodentine using μCT [31, 32]. However, most of them have shown results similar to those of existing hCSCs. In particular, to date, no studies have compared the reparative dentin-forming ability with that of premixed hCSCs. Consequently, the results of this study must be evaluated with caution. Nevertheless, in this study, the relatively lower volume of dentin observed in the EndocemMTA Premixed group may be explained by compositional differences among the pulp-capping materials. It is noteworthy that Biodentine contains a relatively higher proportion of tricalcium silicate (approximately 80%) [33], which has been associated with enhanced calcium ion release and mineralization potential [34–36]. In contrast, EndocemMTA Premixed contains a lower proportion of calcium silicate, with additional components, such as zirconium oxide and DMSO, which may modulate and affect the ion release kinetics and ultimately influence the extent of dentin formation. Although published information on the quantitative composition of EndocemMTA is limited, according to manufacturer-reported data, the proportion of calcium silicate in EndocemMTA is approximately 40%–60% (Table 1). Furthermore, the formation of continuous dentin bridges and minimal inflammatory responses observed with EndocemMTA Premixed suggest that these compositional differences did not compromise the quality of pulp healing. Nevertheless, the compositional explanation proposed in the present study should be interpreted as a plausible contributing factor rather than a definitive mechanistic cause of the observed differences in reparative dentin volume.

A key strength and scientific contribution of the current study lies in its use of a multifaceted experimental strategy that integrates advanced in vitro modelling with in vivo validation. The 3D direct pulp-capping model introduced herein provides a clinically relevant platform for investigating pulp-capping material interactions that surpass the limitations of the extract-based 2D assays. However, although this model incorporates structural features relevant to pulp exposure, it represents a simplified experimental system and does not fully replicate the complex biological and mechanical conditions encountered in clinical settings. Furthermore, several limitations should be acknowledged. In fact, the in vitro model lacks vascular and immune components, which play critical roles in pulp healing. Moreover, the in vivo experiments were conducted using healthy, freshly extracted rat first molars, which may not fully represent clinical conditions involving inflamed or carious pulps. That said, future studies incorporating disease models and longer observation periods are warranted to further elucidate the clinical performance of DMSO-based premixed hCSCs. Therefore, the biological responses observed in this study should be interpreted with caution and should not be directly extrapolated to clinical outcomes without further validation.

In summary, the present findings suggest that EndocemMTA Premixed, through its premixed formulation, may facilitate handling while maintaining biological responses comparable to those of established pulp-capping materials under the experimental conditions evaluated. This balance between endodontic clinical convenience and biological performance supports its biological acceptability as a pulp-capping material under the experimental conditions evaluated in this study and underscores the importance of systematic biological validation when introducing novel formulation strategies for clinicians. In addition, the 3D in vitro direct pulp-capping model provides a complementary experimental

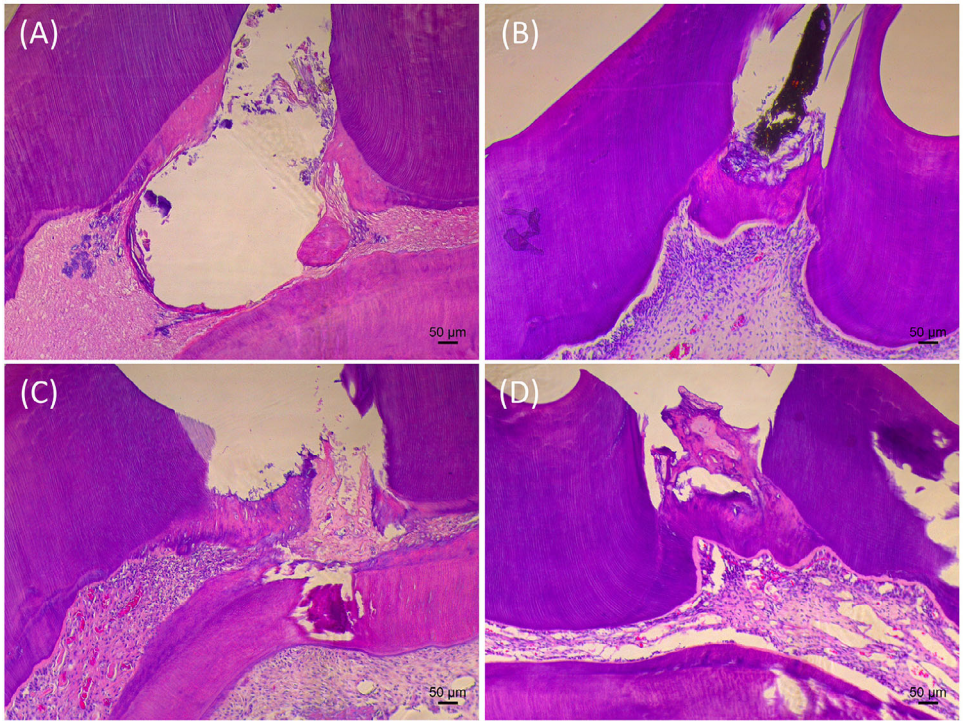

FIGURE 6 Histology of rat pulps at Day 28 stained with haematoxylin–eosin (×100). (A) Control: necrosis of pulp. No mineralized tissue deposition was observed (score 4, 4). (B) EndocemMTA Premixed: only a few scattered inflammatory cells are present. A complete dentin bridge with a regular tubular pattern is observed (score 1, 1). (C) ProRoot mineral trioxide aggregate (MTA): mild inflammation with infiltration of inflammatory cells and blood vessel congestion. An incomplete and discontinuous dentin bridge is formed (score 2, 2). (D) Biodentine: no or presence of a few scattered inflammatory cells. A continuous bridge structure containing dentinal tubules was revealed (score 1, 1).

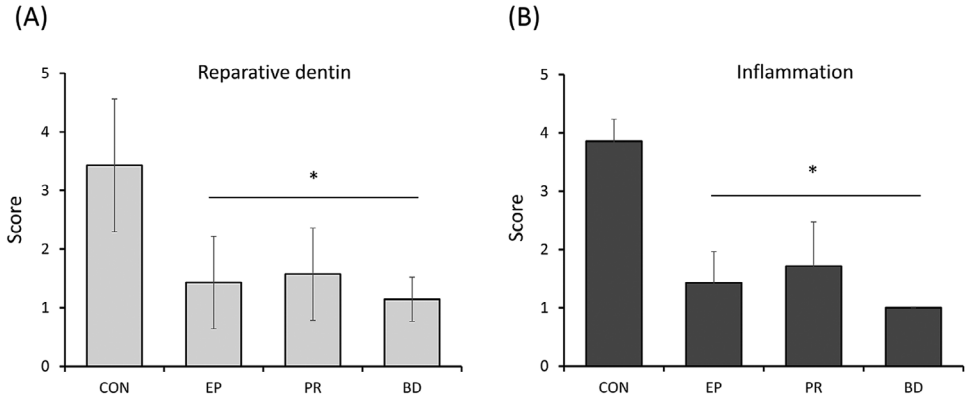

FIGURE 7 Hard tissue formation and inflammation scores were graded based on Table 2: (A) reparative dentin formation; (B) inflammatory cell response. Asterisks indicate statistically significant differences compared to the control group ($p < 0.05$). BD, Biodentine; CON, control; EP, EndocemMTA Premixed; PR, ProRoot mineral trioxide aggregate (MTA).

platform to conventional extract-based assays for evaluating material–cell interactions.

AUTHOR CONTRIBUTIONS

Conceptualization: Min-Ji Choi and Kyung-San Min. **Investigation**: Min-Ji Choi, Eun-Sook Kang and Kyung-San Min. **Writing—original draft**: Min-Ji Choi, Kwang-Won Lee and Kyung-San Min. **Methodology**: Min-Ji Choi, Eun-Sook Kang, Min-Kyeong Kim and Kyung-San Min. **Formal analysis**: Min-Kyeong Kim. **Funding acquisition**: Kyung-San Min. **Validation**: Kenta Tsuchiya and Jukka P. Matinlinna. **Writing—review and editing**: Kenta Tsuchiya, Jukka P. Matinlinna and Kwang-Won Lee. **Supervision**: Kwang-Won Lee.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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