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Direct pulp capping with autologous bone marrow derived stem cells in dogs

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Abstract

Bone-marrow derived stem cells (BMSCs) can differentiate into several mesenchymal cell lines that are suitable for bone and dental tissue engineering. This study was aimed to assess the efficacy of cell therapy in direct pulp capping (DPC) of canine teeth using autologous BMSCs along with collagen/hydroxyapatite hybrid scaffold in terms of the quantity and quality of calcified bridge formation. The teeth were randomly divided into three groups of DPC with mineral trioxide aggregate (MTA), hydroxyapatite/collagen hybrid scaffold alone and BMSCs with hydroxyapatite/collagen hybrid scaffold. DPC was performed under general anesthesia in cavities prepared on the buccal surfaces of mandibular and maxillary premolars of the same dogs from which, stem cells had been isolated. All cavities were then restored with light-cure resin modified glass ionomer cement. Histomorphometric assessments after 12 weeks showed formation of dentinal bridge following DPC with BMSCs and MTA. The efficacy of MTA for calcified bridge formation following DPC was significantly higher than that of BMSCs plus hybrid scaffold. According to the present study, we concluded DPC using BMSCs and hybrid scaffold did not provide clinically noticeable results in canine patients.

Introduction

Pulp exposure following complicated crown fractures, iatrogenic injury and caries could result in irreversible damage to the pulp and tooth if left untreated. Crown fracture is the most common endodontic problem in dogs. Complicated crown fractures involve the enamel and dentin. Dental caries are multifactorial disease caused by the formation of microbial biofilm on tooth surfaces. Although the incidence of caries in dogs is lower than in people (about 80.00% of population of most countries), it does occur and is observed in 5.25% of adult dogs in one or more teeth. Usually, these dogs are affected by bilateral symmetrical lesions.[1-3], Vital pulp therapy, standard root canal therapy or extraction is recommended When vitality of pulp tissue is compromised. Treatment by extraction is a good option when the practitioner does not have requirements for endodontic therapy and referral centers available or when the owner has financial considerations. Standard root canal therapy by removing dental pulp and sealing the apex is another good choice but may not be suitable in a young canine patient with thin walled and immature teeth that have open apices. In this situation the open apex cannot seal adequately and continues growth of dentin which is prevented by removing the pulp tissue and may resulted in further weakening of the tooth.[1,2] Vital pulp therapy preserves vitality of dental pulp by eliminating any existing bacteria and use of biocompatible dental materials to seal the area and prevent bacterial reentry. Thus, the ability of the clinician to preserve the remaining pulp tissue and properly cap it during vital pulp therapy plays an important role in outcome of treatment.[4] In the recent years, several methods have been suggested for preservation of pulp vitality in teeth.[5] In case of deep lesions causing reversible pulpitis, a more invasive procedure such as pulp capping or pulpotomy may also be required.[6] During direct pulp capping (DPC), exposed pulp is protected with a suitable material to minimize additional

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damage to pulp tissue and the exposed tissue is allowed to heal by reparative dentin or formation of a dentinal barrier.[7] Direct pulp capping is cheaper and faster than root canal therapy and is better accepted by patients.[8]

Infection control and biocompatibility of pulp capping agents are important factors, which determine the outcome of pulp capping treatment.[9] Calcium hydroxide [Ca(OH)2] is extensively used for DPC and used to serve as the gold standard of pulp capping agent for a long period of time.[10] However, poor quality of the formed dentinal bridge and inadequate seal of dentinal walls are among the causes of failure of calcium hydroxide as pulp capping agent.[11] Also, alkaline pH of calcium hydroxide can cause necrosis around the injured tissue.[10] Mineral trioxide aggregate (MTA) was later introduced for DPC as an alternative to calcium hydroxide.[12,13] The ability of MTA to induce reparative dentin has been well documented.[7,14] Compared to calcium hydroxide, MTA results in faster dentin formation with a higher quality and lower degradation potential.[10] However, long setting time and difficult handling are among the major drawbacks of MTA.[10,12] Several other dental materials such as dentin adhesives,[15] hydroxyapatite,[16] bioactive glasses,[17] bioactive cements, calcium silicate-based cements,[18,19] Biodentine[10,13] and bioceramics[10,19,20] have also been used to induce the formation of dentinal barrier in DPC.

To the best our knowledge, there is a limited number of studies focused on the effects of stem cells in reparative dentinogenesis and dentin formation during direct pulp capping, especially in dogs.[21,22] As Scaffolds are necessarily required in tissue engineering for optimal formation of tissue and interaction of cells and due to ability of scaffolds to deliver stem cells and/or growth factors,[23] we hypothesized fabrication of a hybrid scaffold using collagen, tricalcium phosphate and hydroxyapatite highly could enhance mechanical and osteoinductive properties.[24] Thus, collagen/hydroxyapatite hybrid scaffold was used in the current study. This study was conducted to investigate whether autogenous bone-marrow derived stem cells (BMSCs) along with the hybrid scaffold was effective for dental hard tissue formation in dogs.

Materials and Methods

Animals. This animal study was conducted in accordance with the guidelines for the care and use of laboratory animals.[25] This study was approved in the ethics committee of Tehran University of Medical Sciences (ethical code: IR.TUMS.REC.1394.927) and local ethics committee for animal experiments in Faculty of Veterinary Medicine, University of Tehran. The animals were kept for two weeks after transportation to become acclimatized. Animal well-being was monitored by examining activity, general appearance, appetite and weight during the study. Four healthy 18 - 24 months old male mixed breed dogs, weighting 21.00 ± 1.80 kg were included in this study. Thirty-nine healthy canine and premolar teeth were selected throughout complete oral examination. The teeth were randomly selected and assigned to three study groups (13 teeth in each group): A) MTA (DPC with MTA), B) Collagen/hydroxyapatite hybrid scaffold and C) BMSCs and the hybrid scaffold. For each of interventions, dogs were anesthetized using intravenous injection of ketamine (5.00 mg kg^{-1}; Alfasan, Woerden, The Netherlands) and diazepam (0.27 mg kg^{-1}; Caspian Tamin, Tehran, Iran). Then, endotracheal intubation was done and anesthesia was maintained by inhalation of 1.50 - 1.80% isoflurane (Baxter healthcare, Aibonito, Puerto Rico). Pre-operatively, tramadol (2.00 mg kg^{-1}; DarouPakhsh, Tehran, Iran) and meloxicam (0.20 mg kg^{-1}; Razak, Tehran, Iran) was administered subcutaneously for pain control. Also, cefazolin (22.00 mg kg^{-1}; Loghman, Tehran, Iran) was injected intravenously as prophylactic antibiotic.

Isolation and culture of BMSCs. The dogs were positioned in lateral recumbency after anesthesia. Pelvic region was clipped and prepared aseptically. A small 5.00 mm incision was made on ileac crest and about 5.00 mL of bone marrow was aspirated from the ileum using bone marrow biopsy needle in a syringe containing sterile heparin. Then, scaling and prophylaxis were performed for teeth. The concentration gradient technique was used for isolation of BMSCs. Briefly, extracted bone marrow was centrifuged in lymphocyte preparation media at 400 *g* for 30 min. The intermediate layer containing stem cells was isolated and centrifuged for 5 min at 400 *g* for concentration and purification. Mononuclear bone marrow cells were cultured in 85.00% Dulbecco's modified Eagle's medium (Gibco, New York, USA) including 2.00 mM L-glutamine (Gibco), 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin (solution 100X; BioWest, Nuaillé, France) and 15.00% fetal bovine serum (FBS; Gibco) and incubated at 37.00 ˚C and 5.00% CO_2 with 95.00% relative humidity. Culture medium was replaced 48 hr after the primary culture and every two or three days thereafter. After formation of mesenchymal stem cell colonies and reaching 80.00 - 90.00% confluence, colonies were passaged. After reaching 80.00% confluence in third passage, cells were detached from the bottom of flasks using trypsin and isolated by centrifugation at 400 *g* for 5 min. They were then suspended again in 0.20 mL of culture medium for injection to the site.

Flowcytometric detection of cell surface antigens CD34, CD44 and CD105 and differential potential. Fluorescence absorbance cell sorting (FACS) analysis were performed using standard protocols and to evaluate cell surface expression of the CD34, CD44 and CD105. Briefly, the isolated cells at passage three were washed several times with phosphate buffered saline (PBS; BioWest) and then detached. After centrifugation, cells were re-suspended in PBS containing FBS and incubated with

CD34, CD44 and CD105 antibodies for 30 min. The labelled cells were washed again, stained and resuspended in PBS and then analyzed by flowcytometry. Data analysis was done by FlowJo™ Software (Treestar, San Carlos, USA). Also, the cells were cultured in osteogenic and adipogenic differentiation medium for three weeks. Subsequently, to verify osteogenic and adipogenic differentiation, the cells were fixed with paraformaldehyde 4.00% and stained with Alizarin red and oil red O solution, respectively.

Preparation of collagen/hydroxyapatite scaffold. The hybrid scaffold was accomplished using the methods from a previous study.[26] Briefly, water mixtures of collagen (Integra Life Sciences, Plainsboro, USA) and hydroxyl-apatite (Sigma Aldrich, Gillingham, UK) were prepared using distilled water. Fabrication of scaffolds were achieved by freeze drying technique. The prepared layers were cut into small pieces and then re-attached each other by 10.00% collagen solution. Finally, the composite scaffold were placed in glutaraldehyde (Sigma Aldrich) solution to increase the strength.

Pulp capping of teeth. Under general anesthesia, DPC was performed in selected healthy teeth. The teeth were cleaned with 0.20% chlorhexidine solution (Iran Najo pharmaceuticals, Tehran, Iran) and class V cavities were prepared on the buccal surfaces of premolars 1.00 mm above the gingival margin using round bur under sterile 0.90% saline irrigation. The cavities were deepened at the center until dental pulp shadow was observed. Next, sharp tip of a probe was used to mechanically expose dental pulp. Slight bleeding was controlled by sterile cotton pellets and saline. The teeth were divided into three groups (n = 13) using blocked randomization. Pulp capping procedure was continued as follows: Group A: Exposed pulp was directly capped with MTA (ProRoot MTA; Dentsply, York, USA) mixed with sterile saline. Group B: Exposed pulp was directly capped with the scaffold. Group C: Undifferentiated BMSCs were injected at the defect site using a Hamilton syringe (Hamilton Co., Reno, USA) and the hybrid scaffold was placed over the defect. All cavities were then restored with light-cure resin modified glass ionomer cement (Ionolux Voco GmbH, Cuxhaven, Germany), (Fig. 1).

Preparation of histological specimens and histomorphometric assessment. After twelve weeks, the dogs were euthanized by intravenous administration of thiopental sodium (Rotexmedica, Trittau, Germany). Specimens were harvested and the teeth were fixed in 10.00% buffered formalin for one week and decalcified in 10.00% nitric acid. The teeth were then longitudinally sectioned in buccolingual direction close to the defect site. Paraffin blocks were prepared and serially sectioned at the defect site to prepare 5.00 µm-thick slices for histological assessment. The slides were stained with Hematoxylin and and Eosin (H & E), and evaluated under a light microscope (E400; Nikon, Tokyo, Japan). The pathologist was blinded to the group allocation of specimens and type of pulp capping agent used. Calcified bridge formation, continuity of bridge, type of bridge, vitality, degree of inflammation and odontoblastic differentiation were the parameters evaluated histologically by modification of grading systems introduced previously (Table 1).[27-30] Formation of dentinal bridge thickness was measured by calculating the mean of a minimum of three cross-sections using Histo-Morpho Meter Software (version 1.0; Shahid Beheshti University of Medical Sciences, Tehran, Iran).

Statistical analysis. The data were analyzed using SPSS Software (version 24.0; IBM Corp., Armonk, USA). The frequency and percentage of degree of pulp vitality, continuity and uniformity of formed bridge, type of formed bridge, inflammation and odontoblastic differentiation were calculated and reported in the three groups. The variables were compared in the three groups using the Kruskal Wallis test. Pairwise comparison of the groups was carried out using the Dunn's test. A $p < 0.05$ was considered statistically significant.

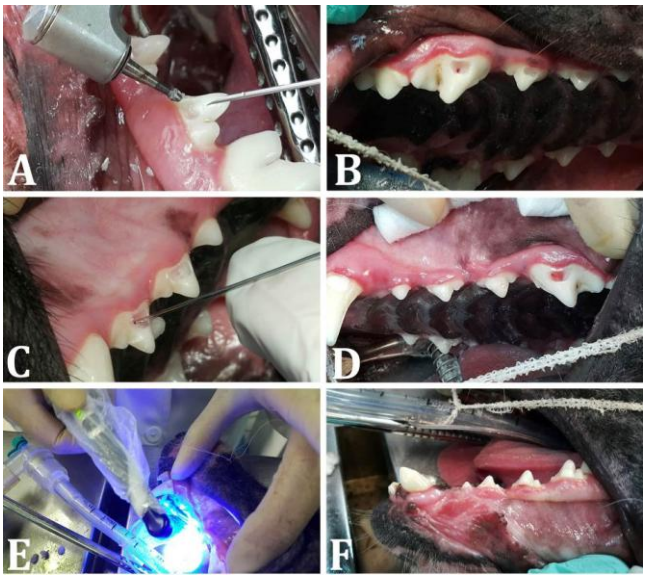

Fig. 1. A) Class V cavity was prepared on the buccal surface of a mandibular third premolar using a round bur with low speed under saline irrigation. **B)** Pinpoint exposure of the pulp by sharp tip of a probe. **C)** After bleeding control, BMSCs were injected at the defect site using a Hamilton syringe. **D)** Collagen/ hydroxyapatite hybrid scaffold was placed at the site. **E)** Glass ionomer cement was light cured at the defect site. **F)** Final restoration of premolar tooth.

Results

Flowcytometric analysis. The FACS analysis exhibited positive expression of CD44 and CD105 cell surface markers, which indicated the cells were of mesenchymal origin. Also, the expression of CD34 was negative, which indicated absence of hematopoietic stem cells among the isolated cells.

Table 1. Parameters evaluated in histological assessment.

Parameters	Scores	Characterization
Calcified bridge formation[30]	0	No formation of calcified bridge.
	1	Formation of calcified bridge.
Continuity and structure of formed calcified bridge[31]	0	Absence of bridge: no formation of calcified bridge or a hard tissue wall around the defect site.
	1	Interrupted bridge: formation of hard tissue only around the exposed site.
	2	Continuous bridge: complete formation of dental hard tissue bridge.
Type of formed dentinal bridge[32]	0	No formation of dentinal bridge.
	1	Formation of osteodentin bridge.
	2	Formation of dentinal bridge with abnormal tubular pattern.
	3	Formation of dentinal bridge with normal tubular pattern.
Pulp vitality[30]	0	No sign of necrosis.
	1	Signs of pulp necrosis.
Inflammation[33]	0	Normal tissue and no inflammation: presence of 0-1 inflammatory cell in microscopic field.
	1	Mild: presence of 2-5 inflammatory cells in microscopic field.
	2	Moderate: presence of 6-15 inflammatory cells in microscopic field.
	3	Severe: presence of more than 15 inflammatory cells in microscopic field.
Odontoblastic differentiation[30]	0	Presence of odontoblastic cell layer.
	1	No differentiation and absence of odontoblastic cell layer.

Differential to osteoblast and adipocytes. The ability of cells to differentiate into osteogenic and adipogenic lineage as stem cells was observed. After 21 days, BMSCs were differentiated to adipocytes with lipid droplets within cells cytoplasm that shown by oil red O staining (Fig. 2A). They were also differentiated to osteoblast like cell with nodular structure of mineralized matrix after 21 days which were shown by Alizarin red staining (Fig. 2B).

Histological observation. A total number of 32 specimens were analyzed for histological evaluation of exposure sites of the teeth. The other seven teeth were non-evaluable due to damages to samples during harvesting or sectioning of specimens. Hematoxylin & Eosin staining revealed calcified barrier and continuous and complete dentinal bridge formation in nearly all specimens of MTA group. In some cases, tubular dentin as well as odontoblastic differentiation was not observed, and only osteodentin bridge were formed. There were cases which revealed formation of dentinal bridge with normal tubular pattern. Moderate to severe inflammation was not observed in any specimens of this group and there was just a mild (score 1) inflammation in some specimens. The pulps were vital and no signs of necrosis were seen.

In scaffold group specimens, dentinal bridge formation and calcified barrier were absent. Mild to severe (score 1 to 3) inflammation were seen in all cases and signs of pulp necrosis were dominant (Fig. 3A). Observations revealed formation of calcified barrier, which was interrupted and made of osteodentin, in half of teeth after direct pulp capping using BMSCs along with the hybrid scaffolds. Some samples revealed moderate to severe (scores 2 and 3) inflammation. Odontoblastic-like cells were not observed in this group. Also, signs of necrosis were not seen and the pulp were vital (Fig. 3B).

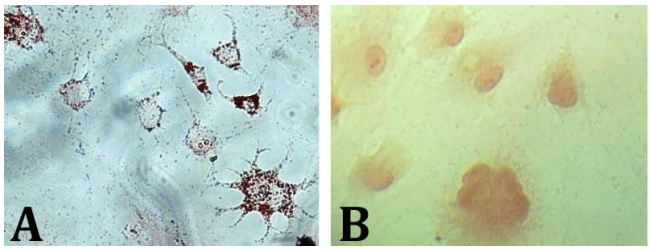

Fig. 2. A) Lipid droplets stained in red by oil red O staining indicate adipogenic differentiation of cells (40×). **B)** Red Clusters stained positive by Alizarin red staining demonstrated osteogenic differentiation potential of cells (40×).

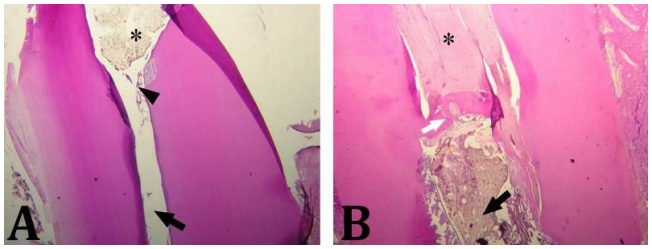

Fig. 3. Premolar tooth after twelve weeks. **A)** After direct pulp capping with hybrid scaffold. Black arrowhead: No bridge formation. Asterisk: Exposure site capped with pulp capping agent. Black arrow: Necrotic pulp (H & E staining, 4×). **B)** After direct pulp capping with BMSCs along with collagen/ hydroxyapatite hybrid scaffold. Asterisk: Exposure site capped with the pulp capping agent. White arrow: Formed calcified bridge, which is interrupted and made of osteodentin. Black arrow: Dental pulp tissue (H & E staining, 10×).

Histomorphometric analysis. Calcified bridge formation was significantly different among all groups. The mean thickness of dentinal bridge was 0.12 ± 0.07 µm in MTA group, 0.06 ± 0.07 µm in BMSCs group and 0.00 µm in scaffold group. The thickness of dentinal bridge formed

in MTA and BMSCs groups was significantly greater than that in scaffold group, and this thickness in MTA group was greater than that in BMSCs group. Significant difference between MTA and other groups were revealed in continuity, type of formed bridge, inflammation and odontoblastic differentiation ($p < 0.0001$). However, the difference between BMSCs and scaffold group was not significant. It means scaffold group was similar to BMSCs group in above parameters. In other words, scaffold and BMSCs had equal performance but lower compared to the MTA group. The difference between scaffold group and other groups in vitality were significant but it was not between MTA and BMSC groups. In other words, MTA and BMSCs groups had the same performance in terms of pulp vitality, and the performance of both in this respect was superior to that of scaffold alone.

Discussion

This study was aimed to assess dental hard tissue induction potential of autogenous BMSCs after DPC. The results of the current study revealed the hypothesis regarding enhancement of mechanical properties or even equality of hard tissue bridge formation following DPC with BMSCs along with collagen/hydroxyapatite scaffold and the scaffold alone was refuted.

Vital pulp therapy is performed after intentional, traumatic, carious or iatrogenic pulpal exposure. The pulp is purposefully exposed as a disarming procedure in which all canines are shortened to the level of the incisors. Also, this procedure may be employed on maloccluded mandibular canine teeth to resolve its penetration to the upper gingiva or palate.[2] The crown fracture - usually involving the canine, maxillary fourth premolar, or mandibular first molar teeth - is the most common endodontic condition in small animal dentistry. Uncomplicated fractures include the enamel solely, or the enamel and dentin. Complicated fractures involve the enamel and dentin resulting in pulp exposure.[1,2] Caries or cavities affects about 5.00% of dogs. The most common type of caries in canine patients are class I and class V caries, which occur on occlusal or on the buccal and labial surfaces, respectively.[2,3]

The severity of injury to the crown, the extent of bacterial contamination, degree of pulp trauma and the duration of pulp exposure have a direct effect on the success of vital pulp therapy. For this reason, the prognosis of maintenance for vital pulp therapy in teeth undergoing crown reduction is excellent in dogs. Prolonged pulp exposure following complicated crown fractures leading to progressive pulpitis and pulp necrosis have a poor prognosis.[1,2] A study indicated that long-term postoperative follow-ups revealed about 88.00%, 41.00% and 23.50% of teeth were vital when treated by vital pulp therapy within two days, seven days or three weeks, respectively.[1] Therefore, vital pulp therapy is just indicated for recent crown fractures and has the best results within 48 hr of the fracture of a mature tooth and should be performed as soon as possible following traumatic pulp exposure. Of course, this time can be extended to two or three weeks after the trauma in an incompletely developed adult tooth in the dog. This procedure is not recommended in the cat.[2]

Bone marrow is a rich source of stem cells for regenerative treatments.[31] These cells are uniquely capable of differentiation to many tissues in response to signals following injury.[32-34] Also, they are capable of gene expression and differentiation similar to dental pulp cells and periodontal ligament cells. They can differentiate into special cells such as muscle cells, hepatic cells, brain cells, pancreatic cells and epithelial cells.[35] Several techniques are used for isolation of BMSCs.[36] In this study, concentration gradient technique was used for isolation of BMSCs, which has several advantages. For instance, specimens are not mixed during centrifugation and all cells of the same size, shape and density are separately precipitated by centrifugation.[37] Moreover, the ability of these cells to differentiate into osteoclast-like cells has been previously confirmed.[38] Previous studies revealed that BMSCs also have odontogenic potential and are able to differentiate into odontoblast-like cells as well as express odontogenic genes, therefore contributing to odontogenesis.[39-41]

In the current study, dentinal bridge formation following DPC was evaluated histologically, which is a current routine technique. Although ideal prognosis is obtained by formation of a continuous dentinal bridge at the pulp-dentin border (since it protects the pulp from external stimuli), formation of a hard tissue barrier can be considered a success, as well.[38]

In this study, Image Analyzer Software was used to quantify the formed dentinal bridge, which makes the comparison of results reliable.[27] Histological assessment in our study showed that DPC with MTA resulted in formation of a visible calcified bridge after three months, which was in agreement with the results of previous studies.[14,18] Success of MTA is due to its physical and bioactive properties.[42] In the current study, the efficacy of MTA for calcified bridge formation following DPC was significantly higher than that of BMSCs and the hybrid scaffold.

Although a previous study found no significant difference in use of BMSCs along with hydroxyapatite/ tricalcium phosphate scaffold and MTA in terms of dentinal bridge formation,[21] however, in the current study, the pulp vitality was preserved following DPC with BMSCs in 80.00% of the cases and calcified bridge was formed in only 50.00%, however, the performance of BMSCs was significantly superior to that of scaffold alone for DPC which was in agreement with this work. The difference

between our results and those of Obeid *et al*. may be attributed to different materials and methods used for fabrication of hybrid scaffold.[21]

Success of BMSCs may be related to their regeneration potential. Previous studies showed that BMSCs when implanted into the dental pulp are able to migrate into damaged and injured tissues and differentiate into a dental tissue–specific cell type.[35,39,43-45] Also, previous animal and clinical studies showed that the efficacy of BMSCs may be related not only to replacement of injured cells but also to conduction of regeneration by numerous direct effects on cells in tissue including reinforcement, vascularization and production of growth factors.[46] Considering the sensitivity of stem cells to impaired blood supply and hypoxia,[47] morphological limitation of pulp chamber and small volume of dental pulp in dogs teeth might have limited blood supply to the area and comprised the success of DPC. If class I cavities had been prepared, blood supply by the pulpal floor would have been enhanced.[21] Since pulp horns were extended close to the occlusal surface, class V cavities were prepared on the buccal surfaces of teeth (instead of class I cavities on the occlusal surface) in the present study to prevent restoration failure due to masticatory forces. However, this might have impaired adequate blood supply to stem cells since the buccolingual width of pulp chamber was very small in teeth and there was not enough space posterior to the exposed area for adequate blood supply to stem cells. Adequate blood supply could have been obtained if cavities had been prepared on molar or canine teeth in dogs and superior DPC results could have been obtained. However, due to limitations such as difficult access to molar teeth, difficult decalcification process for histological analysis and limited number of available canine teeth due to ethical considerations, premolar teeth of dogs (four per each quadrant) are used for pulp capping studies.[48,49]

Here, formation of calcified bridge following DPC with BMSCs and the hybrid scaffold was not significantly different, however, DPC with application of MTA showed superior results in terms of formation of calcified bridge.

In conclusion, it appears that DPC with BMSCs did not lead to clinically remarkable results. However, considering the formation of dental hard tissue in a number of samples in our study and the significance of preserving pulp vitality, future studies are required on other aspects of this topic such as application of growth factors, different scaffolds and different cell implantation conditions.

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Conflict of interest

The authors declare that there is no conflict of interest.

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