



## Tooth Repair by Treated Dentin Matrix Alone and Impregnated with Bone Marrow Mesenchymal Stem Cells in Rabbits

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### ABSTRACT

**Purpose:** This study aimed to see if treated dentin matrix paste (TDMP) alone and treated dentin matrix (TDM) powder combined with stem cells derived from bone marrow (BMMSCs) could induce the formation of calcific barrier after direct pulp capping procedures. **Materials and methods:** In this study 18 posterior teeth in 9 New Zealand white rabbits were involved and simple randomization design was used. All of the experimental teeth were subjected to direct pulp capping procedures. Eighteen teeth were divided into 3 groups, each one consisted of 6 teeth. In group I, calcium hydroxide (CH) was used, while TDMP and TDM powder impregnated with BMMSCs were used in group II and group III respectively. Modified glass ionomer was used as a sealing restoration. After four weeks, animals were euthanized and experimental teeth were demineralized and processed for histological examination to see if a hard tissue barrier was formed. **Results:** TDMP presented the best results where a continuous reparative dentin bridge with homogenous tubular structure was formed. On the other hand, CH stimulated the formation of non-continuous irregular hard tissue masses while seeding BMMSCs on TDM powder resulted in defect closure with a bone like material and dentin like islands were formed inside the adjacent pulp. **Conclusion:** pulp capping with TDMP without adding stem cells from other sources like bone marrow is more effective in producing a high quality dentin bridge through its effect in stimulating the resident cells inside the pulp.

### INTRODUCTION

The dentin–pulp complex which is made up of dentin and pulp is responsible for protecting and sustaining the entire tooth<sup>(1)</sup>. Dentin consists of approximately 70% inorganic component (hydroxy apatite crystals), 20% organic matrix (collagen fibrils and ground substance),

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and 10% water by weight <sup>(2)</sup>. One of the most common bacterially generated illnesses is dental caries, resulting in the deterioration of enamel and dentin. If left untreated, the destruction will mostly lead to irreversible pulpal tissue damage <sup>(3)</sup>.

When pulp infection is in its early stages, direct and indirect pulp capping is mandatory to keep the pulp vitality through the formation of tertiary dentin<sup>(4)</sup>. Pulp capping materials such as calcium silicate-based cement (CSC) and calcium hydroxide, have recently been used as natural tissue substitutes through releasing  $\text{Ca}^{2+}$  and alkaline products to mediate mineralized tissue formation <sup>(5)</sup>. Currently available pulp capping materials are thought to have a reparative rather than a regenerative biological response. This is because of the fact that it only occasionally restores the integrity of the dentin and maintains the vitality of the pulp by catalysing tissue regeneration <sup>(6)</sup>.

The gold standard in the therapy of pulp inflammation is CH, although its high pH (pH 12.5) causes pulp tissue necrosis if they come into direct contact <sup>(7)</sup>. Also Long-term use of CH can cause physical changes and insufficient reparative dentin formation due to dentin bridge discontinuity, resulting in a necrosis area known as a “tunnel defect,” prompting dentists to look for another substitutes, such as natural-based ingredients <sup>(8)</sup>.

Appropriate scaffolding material should be non-cytotoxic, bioactive, and capable of supporting cell proliferation, differentiation, and cellular organization into a tissue structure <sup>(9)</sup>. Dentin matrix is the main bulk of the tooth so it comprises growth factors, bioactive molecules, signaling molecules, and transcription factors <sup>(9)</sup>. TDM is a natural scaffold for dental tissue engineering which is extracted from dentin matrix itself. It was found that TDM could promote odontogenesis because it contains a considerable number of odontogenic bioactive proteins and components <sup>(10,11)</sup>.

Stem cells are nonspecialized, undifferentiated cells that divide indefinitely, have the ability to self-

renew, can generate complex tissues and organs, and can differentiate to produce specific types of cells. In recent years, dentistry has begun to investigate the possible use of stem cells and tissue engineering in the repair and regeneration of dental structures. This therapeutic concept is known as “regenerative dentistry” <sup>(12,13)</sup>.

Mesenchymal stem cells (MSCs) are regarded as the most promising resource for cell-based therapy due to their immuno-modulatory properties, self-renewal and multidirectional differentiation potential. Additionally, MSCs can “home” to inflammation and tumor sites <sup>(14,15)</sup>.

Among the MSCs, BMMSCs are gaining popularity in regenerative medicine. Furthermore, the use of autogenous BMMSCs for tissue regeneration prevents immunologic rejection; consequently, replacing dental stem cells with BMMSCs to create dentin is important for tooth regeneration research<sup>(16,17)</sup>.

Ultimately, this study aimed to evaluate the inductive effect of TDM powder (scaffold) on hard tissue formation when coupled with BMMSCs as a kind of non-dental stem cells, and to assess the odontogenic ability of TDMP when used as a direct pulp capping material in vivo.

## MATERIALS AND METHODS

### Animals:

Nine adult male New Zealand white rabbits weighing 2 kilograms were used in this study. This study was performed at Faculty of Veterinary Kafr-Elsheikh University. Rabbits were maintained under optimum conditions of good ventilation and temperature in the animal house. The animals were supplied natural diet and drinking water adlibitum through the whole experimental period which lasted for 4 weeks.

All animals were maintained according to the standard ethical guide lines of Institutional Animal

Care and Use Committee. Approval by the Research Ethics Committee of the Faculty of Dental Medicine for girls Al- Azhar University was obtained with code (REC-B1-21-02).

This study involved 18 teeth in 9 rabbits (i.e. 2 teeth in each rabbit). The rabbits were randomly divided into 3 groups, each group included 3 rabbits. In group I, the pulp was directly capped with calcium hydroxide ( $n = 6$ )<sup>(18)</sup>, in group II, the pulp was directly capped with TDMP ( $n = 6$ )<sup>(19)</sup> while in group III: the pulp was directly capped with TDM powder impregnated with BMMSCs ( $n = 6$ )<sup>(20)</sup>.

#### **Treated dentin matrix preparation:**

Non infected teeth (impacted third molars or premolars extracted from patients for orthodontic purposes) were used. A stainless steel file was used to eliminate dental pulp and predentin through an access opening, while periodontal ligament tissue and cementum were scrapped away using a curette<sup>(21)</sup>.

The teeth were then treated for 10 minutes with 17% ethylene diamine tetra-acetic acid (EDTA; Sigma, USA), 10 minutes with 10% EDTA, and 5 minutes with 5% EDTA. The teeth were then kept for 72 hours in sterile phosphate buffered saline (PBS) containing 100 units/ml penicillin (Hyclone, USA) and 100 mg/ml streptomycin (Hyclone, USA). They then were rinsed in an ultrasonic cleaner for 20 min, and finally stored in  $\alpha$  minimum essential medium (MEM) at 4°C<sup>(22)</sup>. Thereafter, TDM was incubated for 48 h in  $\alpha$ -MEM at 37°C and 5% CO<sub>2</sub>. Then the medium was obtained, filtered (membrane size = 20  $\mu$ m) and stored at -20°C. The TDM that resulted was ready for grinding finely<sup>(23)</sup>.

Dulbecco's modified Eagle medium (DMEM) was added to the materials with a ratio of 20 g materials per 100 ml DMEM, and incubated for 7 days at 37°C. After filtration through a 0.22 mm filter, the resultant liquid was collected for further investigation<sup>(24)</sup>. TDM powders and aqueous TDM extracts were mingled in a 1:1 volume ratio, resulting in Treated Dentin Matrix Paste (TDMP).

#### **Bone marrow mesenchymal stem cells (BMMSCs):**

MSCs were isolated from the bone marrow of mature male albino rats' femurs and tibias. Briefly, sterilized PBS was used to drain bone marrow from rats' femurs and tibias and resuspended with high glucose DMEM (Gibco, Carlsbad, CA), 10% fetal bovine serum (FBS, Gibco) was added. MSCs from bone marrow were grown in a T-25 flask (Greiner culture flasks, Sigma-Aldrich Co.) at 37°C with 5% CO<sub>2</sub> in tissue culture incubator (Thermo Scientific, Barrington, IL). Non adherent cells were taken away after 48 hours. Remaining cells were considered as MSCs due to their adherence to plastic and expression of CD29, CD 73 and CD90, but not CD31 (hemopoietic stem cell marker) and CD34 which belongs to platelet endothelial cell adhesion molecules. The cells were detached with trypsin (Trypsin-EDTA 1X, Gibco) when they reached 80% confluence. MSCs after passages 3 were used in the study.

#### **Cell seeding on TDM scaffold:**

A total of ( $1 \times 10^6$ ) of BMMSCs (third passage) were suspended in 1ml complete medium (DMEM + 10% FBS + 1% penicillin), then the suspension was incubated at 37°C and 5% CO<sub>2</sub> for 24 hours. After 24 hours the medium was discarded, then 100 mg of the TDM powder was added to the cells and a new 1ml of complete medium was added to TDM+BMMSCs. Then the combination of cells and scaffold were incubated at 37°C and 5% CO<sub>2</sub> for 48h before implantation. Further, the cells became attached to the TDM scaffold<sup>(25,26)</sup>.

#### **Calcium hydroxide and Sealing restoration:**

As a pulp capping substance, Dycal calcium hydroxide was used (Promedica Urbical calcium hydroxide, Germany) and China Glass Ionomer Cement (modified Glass ionomer) was used as a sealing restoration.

## Procedures

To anaesthetize rabbits, both ketamine (50mg/kg body weight) and xylazine (5 mg/kg body weight) were given intramuscularly (IM) <sup>(27)</sup>. The operative area was disinfected with a 2.5 percent iodine tincture and 70% isopropyl alcohol solution <sup>(28)</sup>. A pinpoint exposure was prepared occlusally with the depth of 1.5 - 2 mm using a sterile low-speed diamond bur size 1 while being irrigated with sterile water <sup>(29)</sup> figure (1).

The exposed pulp was treated with sterile cotton pellets soaked in normal saline to stop bleeding. Calcium hydroxide paste, TDMP and TDM powder with BMMSCs were applied one to each exposure site according to the experimental design <sup>(30)</sup>.



Figure (1) A photomicrograph showing pulp exposure.

## Sample collection

By the end of the experimental period for each group, rabbits euthanization was done by using an over dose of anesthetic solution and their heads were immediately dissected to obtain the teeth. The specimens were processed for routine histo-

logical examination by hematoxylin and eosin stain (H&E).

## Specimen preparation for histological examination

The specimens were fixed for 7 days in a 10% neutral buffered formalin solution and then decalcified for 3 months in a 10% EDTA solution. Paraffin blocks were prepared and approximately 5  $\mu$ m thick slices were obtained through buccolingual sectioning of blocks. After that, H&E stain was used to stain 5-micron thickness sections, which were then examined under a light microscope at various magnifications <sup>(31)</sup>.

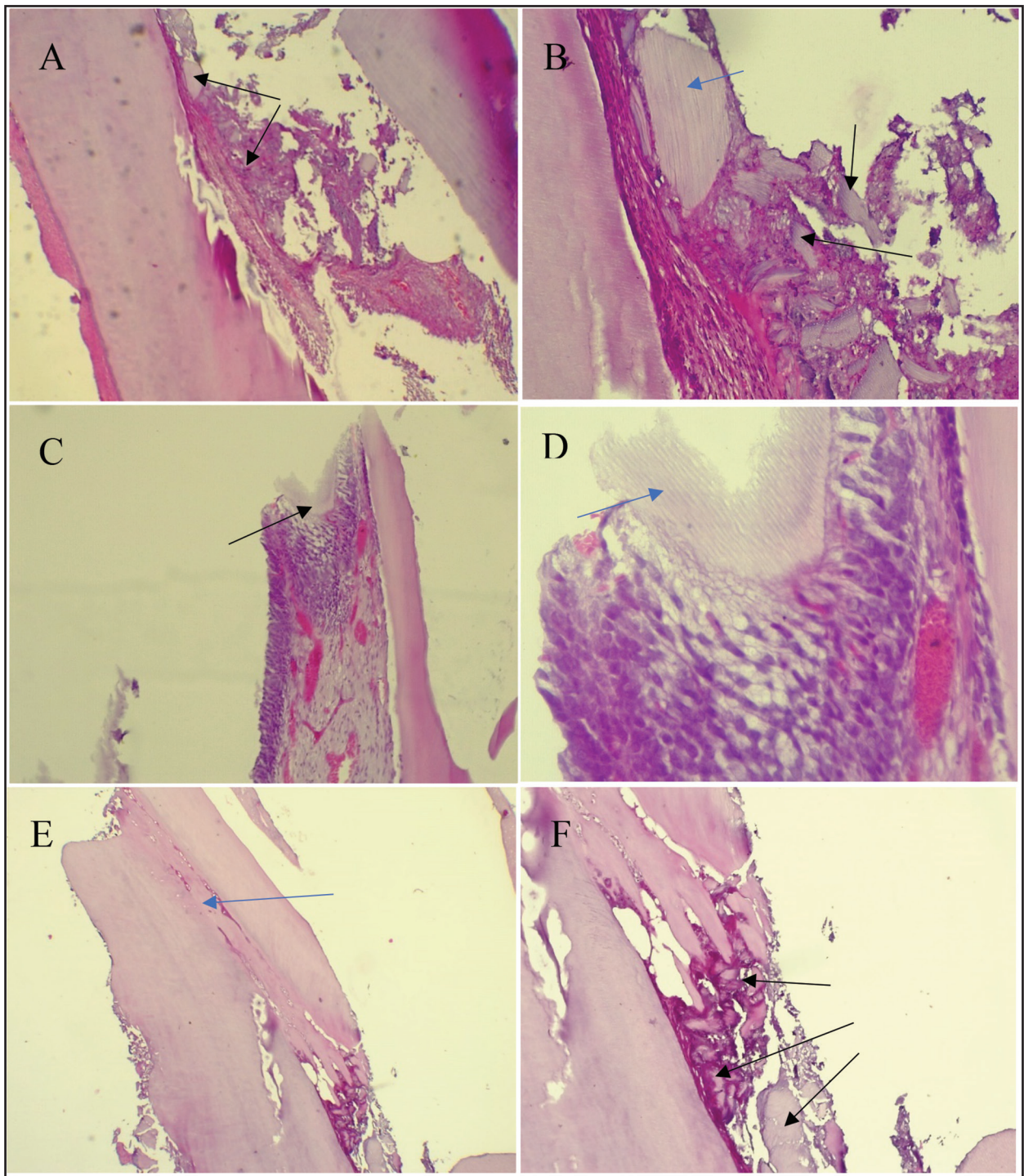
## RESULTS

In calcium hydroxide group, areas of calcification were formed and there was no pulp tissue proliferation into the excavation cavity, the calcific masses were found showed dentinal tubules, marked decrease in the odontoblastic layer, tubular dentin like tissue were observed and area of inflammation and fibrosis (Fig.2:(A, B)).

In treated dentin matrix paste group, histological analysis presented continuous dentin bridge which showed pre-dentin and homogenous dentinal tubules which were the same as primary dentin to a large extent. Odontoblastic differentiation and reparative dentin formation occurred. Some dilated blood vessels were observed (Fig.2:(C, D)).

In TDM and BMMSCs group, hard tissue formation was clearly detected with a remarkable decrease in inflammatory cell infiltration, some of the cells differentiated into osteoblasts-like cells and closed the defect with bone like tissue containing many osseous lacunas and enclosing some soft tissue islands. In the adjacent pulp, irregular calcified masses appeared and dentin islands with tubular structure were observed toward the pulp center (Fig.2:(E, F)).





**Figure (2):** A photomicrograph of the pulp exposure area showing the hard tissue formation:

[A-B]:CH group showing areas of calcification (black arrows) with uniform arranged dentinal tubules (blue arrow). [C-D]: TDMP group showing continuous dentin bridge (black arrow) and reactionary dentin with homogenous dentinal tubules (blue arrow). [E-F]: TDM+BMMSCs showing irregular calcified masses appeared and dentin islands with tubular structure were observed toward the pulp center (black arrows) and bone like tissue closing the defect (blue arrow).

## DISCUSSION

Dental pulp is essential for the stability and health of the teeth because it is the only vascularized tissue in the tooth. Damaged pulp tissue can have a substantial impact on the normal physiological functioning and lifespan of a tooth <sup>(32)</sup>.

New Zealand white rabbits were used in this study due to the fact that they have such a limited lifespan, they are relatively inexpensive, their teeth are larger than those of other rodents, making them more appropriate for restorative procedures, and their resemblance to human teeth in tooth structure and jaw <sup>(33,34)</sup>. Furthermore, in a single rabbit, many teeth can be picked for any experiment, minimizing the number of animals utilized in a single study. The use of rabbits in dental research is common particularly to examine the ability of various pulp capping materials on pulp regeneration <sup>(35,36)</sup>.

Maintaining pulp vitality and preserving the dentin-pulp complex is the main purpose of healthy dental pulp capping. Success of dental pulp capping is related to the presence/absence of caries, pulpitis and invasion of bacteria or their by-products<sup>(37)</sup>. Thus far, CH has previously been thought to be the most frequent material for pulp capping. CH could achieve its functions in pulp capping through: (i) Calcium ions stimulate the pulp microvasculature, facilitating the formation of calcite crystals<sup>(38)</sup>, which attract fibronectin, causing cellular adhesion and differentiation; (ii) stimulating expression of ephrinB1 and EphB2 by DPSCs, thereby upregulating the migration, proliferation, differentiation and mineralization of these cells; and (iii) its alkaline pH has an antimicrobial effect<sup>(39)</sup>. Furthermore, according to various reports, CH has various drawbacks: It only sets in a dry environment, the dentinal bridges it creates may have tunnel defects, and it eventually disintegrates <sup>(40)</sup>.

Reactionary dentin genesis has been linked to specific growth factors and bioactive molecules found within the dentin matrix <sup>(41)</sup>. Researches assured that extracellular matrix components linked

to dentin formation and mineralization are found in the TDM, which is taken out from dentin matrix. These include dentin sialoprotein (DSP), dentin matrix protein1 (DMP-1), and transforming growth factor- $\beta$  (TGF- $\beta$ ). TDM can stimulate hard tissue formation when coupled with bone marrow stem cells<sup>(42-44)</sup>.

In the present study, histological analysis of samples taken over a 4-week period revealed the pulp ability in damage response, which manifested itself as pulp tissue inflammation during this time. According to one study, when inflammation has been controlled, cell repair starts with replacing the damaged area by undifferentiated cells then, these cells differentiate into a tissue resembles the previously intact one with three stages of healing process: mild inflammation, cell proliferation closing the injured area, and cell differentiation in the pulp, resulting in the formation of neo-odontoblasts to produce reparative dentin <sup>(45)</sup>.

As shown in results, CH group specimens showed signs of mild inflammation. In addition, there are calcific areas scattered around the exposure site. However, tunnel defects were observed in the dentin bridge resulting in bacterial recolonization, which could fail the pulp treatment procedure. Meanwhile, blood vessels that appeared to be angiectatic were discovered in the pulp capping site <sup>(46)</sup>.

On the other hand, it is hard to manipulate TDM in the pulp capping process if it was in the form of blocky solid material. So to preserve the bioactivity of TDM, it was further processed into TDMP, which was made up of TDM powders and aqueous extracts of TDM. With its moderate pH and low cytotoxicity, TDMP was found to have better biological compatibility for transplantation than CH<sup>(47)</sup>.

In this research TDMP was fabricated as a new version of pulp capping materials, as the extracellular matrix can be used as a culture substrate for cells to proliferate and differentiate into the original tissue from which it was derived <sup>(48)</sup>. This was presented in the second group through

formation of dentin like tissue with homogenous tubular structure. This promising effect is due to the ability of the matrix to release bioactive agents. So, TDM launches extracellular matrix molecules, such as TGF- $\beta$ , DMP-1, and DSP, that are bioactive for regenerating dentin-pulp complex via inducing dental pulp stem cells <sup>(49)</sup>.

Presence of dentinal tubules is considered one of the significant prognostic criteria for dentin regeneration, also the cytological features of the odontoblasts. This explains why teeth treated with TDMP exhibited a good tendency toward dentin regeneration <sup>(50,51)</sup>. According to one study, this is correct as it was found that orderly tubular thicker dentin bridge was formed when TDMP was used as pulp capping material. These findings could be referred to that TDM has good biocompatibility and bioactivity, is non-immunogenic, has appropriate mechanical characteristics, and is rich in dentinogenetic components <sup>(52)</sup>.

There are some difficulties that must be addressed. Due to the large defect range, TDMP tended to collapse into the pulp tissues, lowering the height of the pulp chamber roof. The flexibility of TDMP is not perfect, making pulp capping an inconvenient process. More studies are needed to investigate if adding excipients to TDM may assist retain the pulp chamber profile while also improving viscosity without losing TDMP's bioactivity <sup>(53)</sup>.

Dental tissue engineering also entails taking stem cells from a patient, inducing them to form a specific lineage on a convenient scaffolding material, and then returning the cell-scaffold construct to the patient <sup>(54)</sup>. Finding convenient seeding cells is a serious point. It must be easy to obtain as Bone marrow stem cells, and be able to differentiate into different cell types. These cell types may be osteoblasts, adipocytes, chondrocytes, or even muscle and neural tissues <sup>(55)</sup>.

BMMSCs may differentiate into dental tissues in the presence of TDM which induces odontogenic differentiation. Moreover, BMMSC differentiation

may secrete a variety of proteins and growth factors that aid in dentin regeneration <sup>(56,57)</sup>. In this study the capacity of BMMSCs when loaded onto TDM as a scaffold to promote hard tissue formation was investigated <sup>(58,59)</sup>.

On the other hand, BMMSCs appeared to have a lesser odontogenic capacity than dental stem cells, so MSC derived from different embryonic origins are not equivalent <sup>(60)</sup>. Furthermore, BMMSCs have osteogenic ability due to the newly formed bone that was observed and TDM could cause BMMSCs to differentiate into osteogenic cells <sup>(61)</sup>.

So in the third group when BMMSCs were seeded onto TDM, some of the cells differentiate into osteoblasts and the histological results revealed a new bone formation closing the defect area and some islands of dentin were formed. As a result, BMMSCs have a greater ability to promote mineralization and osteogenic differentiation, and with TDM induction, they may even be able to create bone-like tissue <sup>(62)</sup>.

## CONCLUSION

From the previously mentioned results, TDMP had a good biocompatibility and induced formation of hard tissues, and it outperformed CH in closing in vivo dentin defects through recruitment dentin regeneration. As a result, TDMP may be a viable pulp capping material, and it is favourable to be used as an alternative material in clinical purposes. While seeding BMMSC on TDM was not worthy in reparative dentin formation.

## RECOMMENDATIONS

Evaluating odontogenic effect of TDM may be beneficial for future studies. Further studies should be carried out to correlate the clinical outcomes of TDM with its biological mechanisms. These informations will be used to develop a new era for pulp capping procedures or even for bone defects closure.



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## Informed consent

For this type of study, formal consent is not required.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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