Original Article

Responses of oral-microflora-exposed dental pulp to capping with a triple antibiotic paste or calcium hydroxide cement in mouse molars

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ABSTRACT

Introduction: Responses of oral-microflora-exposed dental pulp to a triple antibiotic paste (TAP), a mixture of ciprofloxacin, metronidazole, and minocycline in ointment with macrogol and propylene glycol, remain to be fully clarified at the cellular level. This study aimed to elucidate responses of oral-microflora-exposed dental pulp to capping with TAP in mouse molars.
Methods: A cavity was prepared on the first molars of 6-week-old mice to expose the dental pulp for 24 h. The exposed pulp was capped with TAP (TAP group) or calcium hydroxide cement (CH group), in addition to the combination of macrogol (M) and propylene glycol (P) (MP, control group), followed by a glass ionomer cement filling. The samples were collected at intervals of 1, 2, and 3 weeks, and immunohistochemistry for nestin and Ki-67 and deoxyuride-5′-triphosphate biotin nick end labeling (TUNEL) assay were performed in addition to quantitative real-time polymerase chain reaction (qRT-PCR) analyses.
Results: The highest occurrence rate of pulp necrosis was found in the control group followed by the CH group at Weeks 2 and 3, whereas the highest occurrence rate of healed areas in the dental pulp was observed in the TAP group at each time point. Tertiary dentin formation was first observed in the dental pulp of the TAP group at Week 2. In contrast, bone-like and/or fibrous tissues were frequently observed in the CH group. qRT-PCR analyses clarified that TAP activated the stem and dendritic cells at Weeks 1 and 2, respectively.
Conclusions: The use of TAP as a pulp-capping agent improved the healing process of oral-microflora-exposed dental pulp in mouse molars.

1. Introduction

Dental pulp is a highly specialized soft connective tissue of ectomesenchymal origin that supports dentin [1,2]. The pulp is responsible for tooth vitality and health, as well as pain sensation, immune defense, and tissue repair/regeneration following tooth injuries [3]. Clinical procedures such as cavity preparation and restorations may influence a series of biological effects through solubilization of several dentin matrix-bound bioactive molecules including transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs) [4–6]. The death of odontoblasts and other pulpal cells may release intracellular contents such as heat-shock proteins at the injury site [7]. In addition, inflammatory cells attracted to the site of injury will secrete cytokines and growth factors such as osteopontin and granulocyte-macrophage colony-stimulating factor (GM-CSF) that modulate the underlying cellular events [8]. Therefore, it is necessary to consider that a variety of signal molecules present in the extracellular milieu at sites of injury play crucial roles in regulating the sequence of events leading to reparative dentinogenesis in the injured pulp tissue. However, the precise interaction among these molecules to induce the differentiation of odontoblast-like cells during pulp repair/regeneration remains to be elucidated [9].

Tooth injuries facilitate the activity of survival odontoblasts or elicit the degeneration of preexisting odontoblasts to induce either reactionary or reparative dentin formation, both of which are categorized as tertiary dentin formation. In the latter case, newly differentiated odontoblast-like cells are thought to be associated with postnatal dental pulp stem cells (DPSCs) that are clonogenic, highly proliferative, and capable of regenerating tissues [10,11]. Although several mesenchymal stem cell populations have been implicated, the precise derivation of odontoblast-like cells remains unclear [12,13]. Recently, discrete sub-populations of mesenchymal stem cells (MSCs) marked by differential gene expression (markers) have been identified in the dental pulp of mouse incisors. This finding suggests that not all but specific cell populations within a niche may be related to tissue repair and growth following tooth injuries [14]. Under same conditions, host protective events triggered by afflicted odontoblasts induce the production of pro-inflammatory mediators, which in turn attract several inflammatory cells at the site of injury [15,16]. For example, HLA-DR-immunopositive cells, or putative dendritic cells (DCs), were shown to be predominantly situated in the sub-odontoblastic layer of human dental pulp, with some located in the odontoblast layer and/or predentin and extending their cytoplasmic processes into the dentinal tubules [17]. In addition, a close spatio-temporal relationship between the immunocompetent cells and odontoblasts has been demonstrated in the animal model for tooth injuries such as cavity preparation and tooth replantation in rats. Class II major histocompatibility complex (MHC)-positive cells dramatically changed their locations and populations in correlation with the degeneration/regeneration of the odontoblast layer following tooth injuries [18,19]. Therefore, *in vivo* approaches are suitable to analyze the interplay of these cell populations and track the chronological changes in the injured dental pulp.

Direct pulp capping (DPC) is usually carried out when pulp is exposed by caries or in mechanical, chemical, or physical trauma to avoid pulp death [20,21]. Clinically, when a pulpal exposure leads to long-term irritation and inflammation at the exposure site, the outcome of hard-tissue formation is not predictable [13,22]. To date, several kinds of materials have been used for DPC treatment, including calcium hydroxide (CH), calcium silicate-based cements, and experimental antibiotic-containing pastes. Nevertheless, there is no consensus among practitioners on which material should be selected for DPC treatments to improve the prognosis of the injured dental pulp [23–25]. The combination of antibacterial drugs such as ciprofloxacin, metronidazole, and minocycline has been reported to be effective against the bacterial flora of humans in carious dentin, dental plaque, and necrotic pulp [26–28]. Previous studies using a mouse model for the evaluation of therapeutic reagents on intentionally delayed replanted teeth demonstrated the positive effects of this triple antibiotic combination (TAC) as a solution on the pulpal healing process. Thus, different concentrations of the triple antibiotic solution (TAS) elicited various healing patterns ranging from sustained inflammatory responses to tertiary dentin formation in the pulp chamber [29]. Moreover, the application of TAS may suppress osteoblast differentiation by the migration of dendritic cells to the injury site and via the activation of stem/ progenitor cells, resulting in the acceleration of odontoblast-like cell differentiation [30]. This finding is supported by a previous study using a rat cavity preparation model, where the recruitment of dendritic cells preceded the differentiation of odontoblast-like cells and matrix deposition following the application of alpha tricalcium phosphate (αTCP) cementum containing the TAC [31]. Although several case reports have shown the effectiveness of the TAC in ointment with macrogol (M) and propylene glycol (P), referred to as triple antibiotic paste (TAP) in regenerative treatments, responses of the infected and/or injured dental pulp to TAP remain to be fully clarified at the cellular and gene level. Hence, this study aimed to elucidate responses of the oral-microflora-exposed dental pulp to capping with TAP in mouse molars, compared with those to CH cement.

2. Materials and methods

2.1. Dental cavity preparation and direct pulp capping

All animal experiments complied with the guidelines by the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of the Environment, and the Science Council of Japan and were carried out in accordance with the Act on Welfare and Management of Animals. All animal experiments were conducted in compliance with a protocol that was reviewed by the Institutional Animal Care and Use Committee and approved by the President of Niigata University (#132–3). Forty-seven male Crlj:CD1 mice (6 weeks old) were purchased from Charles River Laboratories Japan (Yokohama, Japan). All animals received an intraperitoneal injection of chloral hydrate (maximum dose of 350 mg/kg) prior to the operation. For histological analyses (n = 19), an injury model for pulp exposure and direct capping treatment was performed according to the previous study of Sato et al. [31] with some modifications. A class I cavity was prepared on the maxillary first molars to expose the dental pulp by use of an air turbine with a tungsten carbide bur (diameter 0.5 mm) under water-cooling. The cavities were maintained without any further treatment such as air drying, etching, or filling for 24 h. Subsequently, the exposed pulp was capped with TAP [a combination of ciprofloxacin, metronidazole, and minocycline in ointment with macrogol (M) mixed with propylene glycol (P)], in the TAP group, or was capped with CH cement (Life, Kerr, Portland, OR, USA) in the CH group, in addition to a mixture of macrogol and propylene glycol (MP, control group), followed by a self-cured glass ionomer cement filling (Fuji II, GC Corporation, Tokyo, Japan). The maxillary left and right molars were treated with different capping materials to avoid increasing the number of experimental animals. For measuring the variations of gene expression after capping treatments (n = 28), the previous experimental design was slightly modified. Following dental pulp

exposure, the teeth were immediately capped with the TAC in propylene glycol (TAC-P), or CH cement, or propylene glycol alone (control group) and restored by a glass ionomer cement filling (Fuji II). The maxillary left and right molars were treated with the same capping agent. The protocol for the preparation of TAP is described elsewhere [32].

2.2. Tissue preparation

Materials were collected from groups of 3—6 treated teeth at Week 1 [n = 4 (TAP), 4 (CH), and 3 (MP)], 2 [n = 6 (TAP), 5 (CH), and 3 (MP)], and 3 [n = 6 (TAP), 4 (CH), and 3 (MP)] after capping treatment. At each stage, the animals were transcardially perfused with physiological saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH = 7.4) under deep anesthesia induced by an intraperitoneal injection of chloral hydrate. The maxillae were removed *en bloc* and immersed in the same fixative for an additional 12 h at 4 °C. After decalcification in Morse's solution (10% sodium citrate and 22.5% formic acid) for 4—6 days at 4 °C, the specimens were processed for embedding in paraffin and cut sagittally at a thickness of 4 μm. Sections were processed for hematoxylin-eosin (HE) staining, azocarmine and aniline blue (AZAN) staining, and immunohistochemistry.

2.3. Immunohistochemistry and TUNEL assay

Immunohistochemistry was conducted using a mouse anti-rat anti-nestin monoclonal antibody diluted 1:100 (Millipore, Temecula, CA; catalog number: MAB353) and a rat anti-mouse Ki-67 monoclonal antibody diluted 1:100 (Dako Japan, Tokyo, Japan; catalog number: M7249). The Envision + Horseradish Peroxidase System (Dako Japan, catalog number: K5027) and the avidin-biotin peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) method using biotinylated anti-rat immunoglobulin G (Vector Laboratories; catalog number: BA-4000) were used for nestin and Ki-67 immunohistochemistry, respectively. For the final visualization of the sections, 0.05 mol/L Tris—HCl buffer (pH = 7.6) containing 0.04% 3-3′-diaminobenzidine tetrahydrochloride and 0.0002% H_2O_2 was used. The immunostained sections were counterstained with hematoxylin. Apoptosis was quantified by terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL) with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore; catalog number: S7100). Negative controls were performed by replacing the primary antibodies or terminal deoxynucleotidyl transferase enzyme with phosphate-buffered saline (PBS).

2.4. Quantitative real-time PCR analysis

Total RNA was isolated from the dental pulp tissue of treated teeth at each observation stage (Weeks 1 and 2) using the Trizol system (Invitrogen, Life Technologies, Carlsbad, CA). Complementary DNA (cDNA) was synthesized from the RNA with the Prime-Script RT reagent Kit (Takara Bio Inc., Shiga, Japan; Cat. # RR037A). Quantitative analyses of gene expression for *β-actin*, *Nestin*, *Nanog* and *Cd11c* were performed by quantitative real-time polymerase chain reaction (qRT-PCR) using TB Green Premix Ex Taq (Takara Bio Inc., Shiga, Japan; Cat. # RR420A) on a Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan) according to the following amplification conditions: 30 s at 95 °C; 40 or 50 cycles of 95 °C for 5 s and 60 °C for 30 s; dissociation for 15 s at 95 °C; and 30 s at 60 °C. Gene expression levels were calculated relative to the levels of *β-actin* mRNA using the comparative Ct (2−ΔΔCt) method. The oligonucleotide primers specific for the target sequences are listed in Supplementary Table S1.

2.5. Cell counting

The numbers of Ki-67- positive cells in the coronal and root pulp of each sample (3.4×10^4 grid was selected) and the percentage of TUNEL-positive areas was calculated separately. All data were presented as the mean and standard deviation of each group. Furthermore, the number of cells in the coronal and root pulp at Week 1 and 2 after pulp capping treatments and between groups were compared using one-way analysis of variance (ANOVA) multiple comparisons adjusted by the Bonferroni test using statistical software (SPSS 21.0 J for Windows; SPSS Japan, Tokyo, Japan).

2.6. Statistical analysis of nestin-positive perimeters, newly formed hard-tissue areas and the occurrence of tertiary dentin or bone-like tissue areas in the pulpal tissue after capping treatment

The percentage of nestin-positive perimeters in the total perimeter of the pulp-dentin border was calculated on Weeks 1, 2, and 3 using Image J software (Image J 1.45s, National Institutes of Health). Similarly, the percentage of newly formed hard tissue and the percentage of occurrence of tertiary dentin or bone-like tissue areas in the total area of the pulp chamber were quantified on Weeks 1, 2, and 3 using WinRoof image processing software (WinRoof Version 7.4; Mitani Corporation, Tokyo, Japan). All data were presented as the mean and standard deviation of each group and analyzed by the Student *t* test using statistical software (SPSS 21.0 J for Windows).

3. Results

3.1. Histological and nestin immunohistochemical evaluation of the dental pulp in the negative control group

One week after capping treatment, pulpal tissue in the MP group degenerated in the exposed areas and expanded apically (Fig. 1A). Increased collagen fibers were observed at the boundary between the intact and degenerated areas (Inset in A). Coronal pulp lost positive immunoreactivity for nestin, although its expression remained in the cervical and root areas in most of the samples (Fig. 1D). Two weeks after the operation, the dental pulp showed extensive areas of hemorrhage, while some fibroblasts remained in the coronal pulp (Fig. 2A and B). A total lack of nestin-immunopositive expression (Fig. 2C) was observed at this stage and Week 3 (Fig. 3C), where an accumulation of bacteria was also observed in the pulpal tissue of some samples (Fig. 3A and B; arrowheads).

3.2. Histological and nestin immunohistochemical evaluation of the dental pulp in the CH cement group

In the CH group, the dental pulp showed degenerative features near the site of injury, with a mild expansion toward the apical region one week after the operation (Fig. 1B). Increased collagen fibers were observed at the boundary between the intact and degenerated areas (Inset in B). Nestin-immunopositive expression was partially detected in the coronal odontoblasts, but intensely expressed in the root pulp, together with other pulpal cells (Fig. 1E). Two weeks after the operation, the odontoblasts lost their pseudostratified features (Fig. 2D), and several bundles of collagen fibers began to occupy the pulp chamber, as demonstrated by AZAN staining (Fig. 2E). Nestin-immunopositive expression was lost in the odontoblast layer but detected in other pulpal cells forming filamentous-like structures (Fig. 2F). At Week 3, hard-tissue deposition was observed in the dental pulp of most samples (Fig. 3D). Two healing patterns, tertiary dentin and/or bone-like tissue were
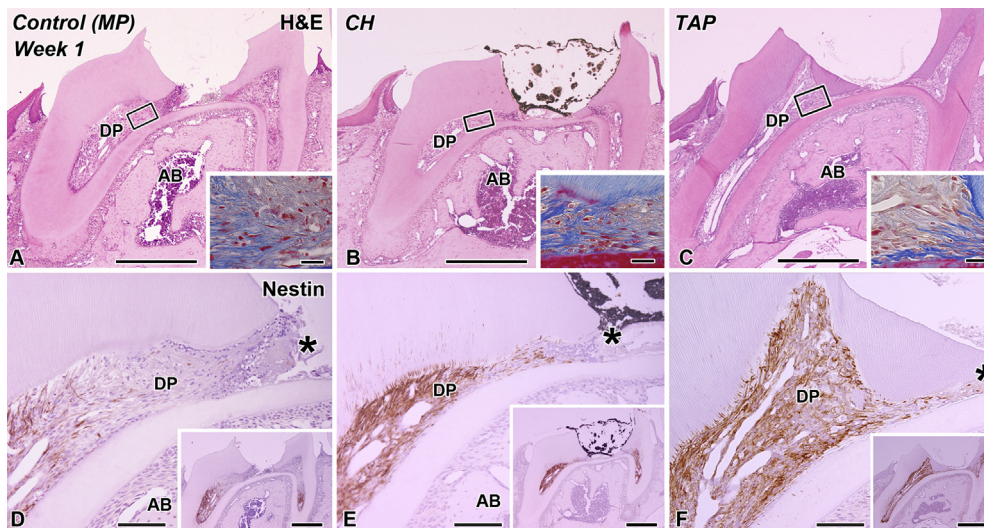
Fig. 1. Histological and nestin immunohistochemical changes in the dental pulp one week after treatment. Hematoxylin eosin (HE) (A–C) staining, AZAN staining (upper insets in A-C), and nestin immunohistochemistry (D–F) at Week 1 after direct capping treatment with MP (A, D), CH (B, E), and TAP (C, F) (A–C) The pulpal tissue degenerates in the exposed areas, while increased collagen fibers are observed at the boundary between the intact and degenerated areas (Inset in A-C). The degenerated area heavily expands apically in the control and mildly in the CH group (D) Coronal pulp lacks positive immunoreactivity for nestin, although its expression remains in the cervical and root area in most of the samples (E) Nestin immunoexpression is partially detected in the coronal odontoblasts, but intensely expressed in the root pulp, together with other pulpal cells (F) Survival and/or newly differentiated odontoblasts in the pulpal tissue are recognized in the nestin immunostained section, except for the exposure site. The root pulp shows a positive staining in all the groups (lower insets from D-F). DP, dental pulp; AB, alveolar bone; *, Site of exposure. Scale bars = (A–C) 500 μm (D–F) 100 μm (Upper insets: 25 μm, lower insets: 500 μm).
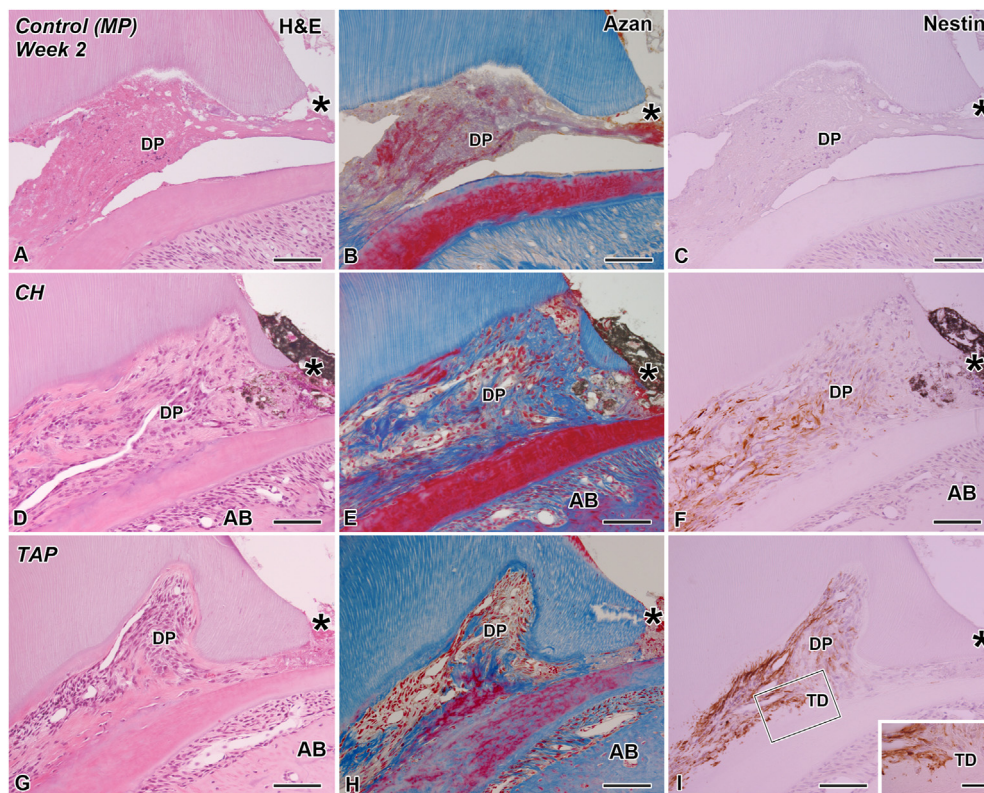

Fig. 2. Histological and nestin immunohistochemical changes in the dental pulp two weeks after treatment. Hematoxylin eosin (HE) staining (A, D and G), AZAN staining (B, E, and H), and nestin immunohistochemistry (C, F, and I) at Week 2 after direct capping treatment with MP (A–C), CH (D–F), and TAP (G–I) (A and B) The dental pulp shows extensive areas of hemorrhage, while some fibroblasts remain in the coronal pulp (C) A total lack of nestin immunoexpression is observed at this stage (D) The odontoblasts lose their pseudostratified features (E) Several bundles of collagen fibers begin to occupy the pulp chamber (F) Nestin immunoexpression is lost in the odontoblast layer but detected in other pulpal cells forming filamentous-like structures (G) Hard-tissue formation is first observed in the coronal pulp, especially in the pulpal floor near the exposure site (H) Collagen fibers are clearly organized around the deposition of hard tissue (I) Positive-nestin immunostaining confirms the presence of newly differentiated odontoblasts and the initiation of tertiary dentin formation near the exposure site. DP, dental pulp; AB, alveolar bone; TD, tertiary dentin; *, Site of exposure. Scale bars = (A–I) 100 μm (Inset in I) 25 μm.
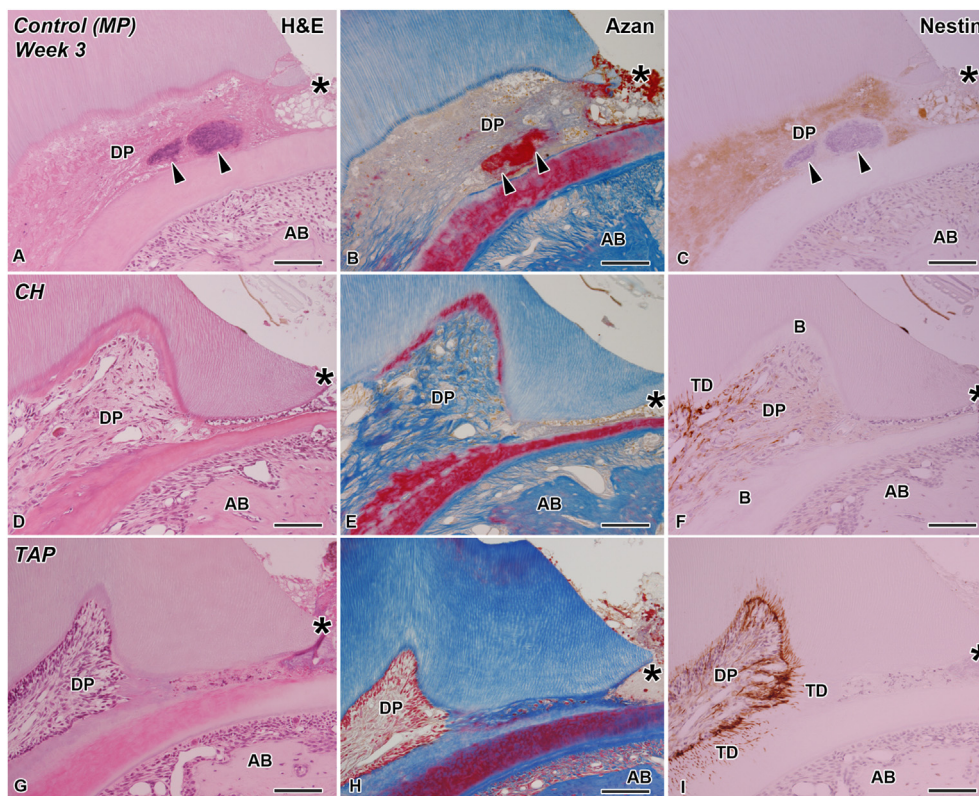
Fig. 3. Histological and nestin immunohistochemical changes in the dental pulp three weeks after treatment. Hematoxylin eosin (HE) (A, D and G), AZAN staining (B, E, and H), and nestin immunohistochemistry (C, F, and I) at Week 3 after direct capping treatment with MP (A–C), CH (D–F), and TAP (G–I) (A) Accumulation of bacteria (arrowheads) is observed in the pulpal tissue of some samples (B) The coronal pulp lacks collagen fibers (C) The pulpal tissue shows a negative staining for nestin immunohistochemistry (D) Hard-tissue deposition is observed in the dental pulp of most samples (E) Collagen fibers continue to be deposited in the dental pulp along with the ongoing healing process (F) Nestin immunostaining shows two healing patterns, tertiary dentin and/or bone-like tissue in the coronal pulp (G and H) Hard tissue is consistently deposited near the exposure site, forming a bridge-like structure in the repaired dental pulp (I) Nestin-expressing differentiated odontoblasts align in the border of the newly formed tertiary dentin barrier. DP, dental pulp; AB, alveolar bone; B, bone-like tissue; TD, tertiary dentin; *, Site of exposure. Scale bars = (A–I) 100 μm.

randomly deposited in the coronal pulp of the CH group as shown by nestin immunostaining (Fig. 3F). However, the deposition of collagen fibers continued at this stage, indicating that the healing process was still ongoing (Fig. 3E).

3.3. Histological and nestin immunohistochemical evaluation of the dental pulp in the TAP group

Following capping treatment with TAP, the injured dental pulp retained most of its normal features (Fig. 1C) after one week, including the presence of blood vessels and a regular organization of collagen fibers at the boundary between the intact and degenerated areas, as showed by AZAN staining (Inset in Fig. 1C). Nestin immunostaining demonstrated the presence of survival and/or newly differentiated odontoblasts in the pulpal tissue, except for at the site of injury (Fig. 1F). As a result, hard-tissue formation is first observed in the coronal pulp (Fig. 2G) at Week 2, especially in the pulpal floor near the injury site. Collagen fibers were clearly organized around the deposition of hard tissue (Fig. 2H), while a positive-nestin immunostaining confirmed the presence of newly differentiated odontoblasts and the initiation of tertiary dentin formation (Fig. 2I). Finally, at Week 3, hard tissue was consistently deposited near the exposure site forming a bridge-like structure in the injured dental pulp (Fig. 3G and H). Differentiated odontoblasts aligned in the border of the newly formed tertiary dentin barrier, as demonstrated by intense immunoreaction for nestin (Fig. 3I).

3.4. The progression of the healing process and hard-tissue deposition after pulp capping treatment

The percentage of nestin-positive perimeters or regenerated surfaces of the dental pulp was analyzed at each observation point. TAP favored the survival and/or accelerated the differentiation process of odontoblast-like cells from Week 1–3 followed by the CH group, especially at Weeks 2 and 3, where a significant difference was clearly noted between TAP group and MP control group (Fig. 4A). Similarly, the analysis of the percentage of newly formed hard-tissue areas showed that in the TAP group, the occurrence rate of hard-tissue deposition following pulp capping was significantly higher than that in the CH group at Week 2, and this tendency continued at Week 3 (Fig. 4B). In addition, the percentage of occurrence of tertiary dentin or bone-like tissue areas was calculated based on the nestin-positive (tertiary dentin) or nestin-negative (bone-like tissue) hard-tissue areas in the total pulp area. TAP induced the higher occurrence rate of tertiary dentin at Week 2, compared with the MP group (p < 0.05) and the CH group. At Week 3 following capping treatment, TAP kept the favorable tendency toward tertiary dentin formation, whereas increased deposition of bone-like tissue was observed at this stage in the group treated with CH cement (Fig. 4C).

3.5. Evaluation of cell proliferation in the dental pulp of experimental and control groups

The analysis of Ki-67-positive cells showed an overall favorable tendency toward the TAP group compared with the CH group at all
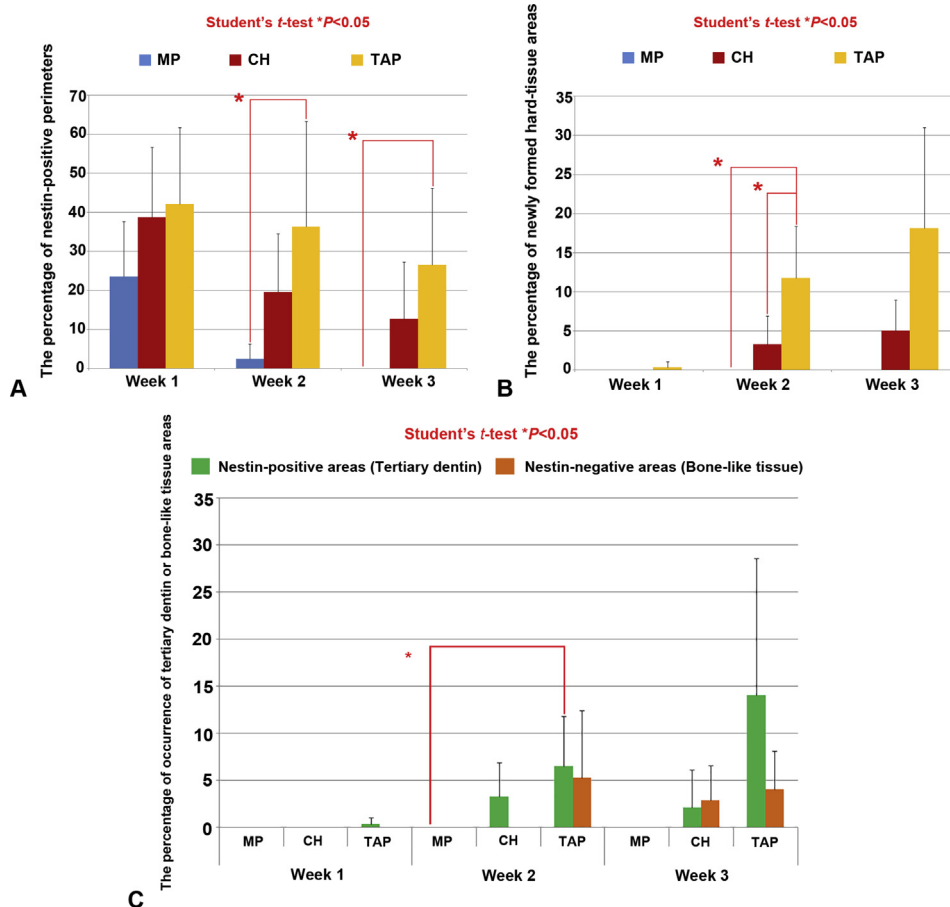
Fig. 4. Quantitative analyses of the progression of the healing process and hard-tissue deposition after pulp capping treatments. (A) The percentage of nestin-positive perimeters or regenerated surfaces of the dental pulp. The use of TAP favored the survival and/or accelerated the differentiation process of odontoblasts, especially at Weeks 2 and 3, when a significant difference was clearly noted between the TAP group and the MP control group (B) The analysis of the percentage of newly formed hard-tissue areas. The occurrence rate of hard-tissue deposition following pulp capping was significantly higher in the TAP group than in the CH group at Week 2, and this tendency continued at Week 3 (C) The percentage of occurrence of tertiary dentin or bone-like tissue areas. TAP induced the higher occurrence rate of tertiary dentin at Week 2, compared with the MP group ($p < 0.05$) and the CH group, keeping the favorable tendency toward tertiary dentin formation at Week 3, whereas increased deposition of bone-like tissue was observed at this stage in the CH group.

observation stages. TAP accelerated cell proliferation in the coronal and root pulp at Week 2 after capping treatment, whereas a significant difference between TAP and the other groups still was seen in the root pulp three weeks after treatment. In contrast, the number of proliferative cells dramatically decreased in both coronal and root dental pulp from Week 1—3 in the MP negative control group (Fig. 5A and B).

3.6. Evaluation of the apoptotic activity in the dental pulp of experimental and control groups

The apoptotic activity was analyzed by measuring the percentage of TUNEL-positive areas in the dental pulp of the experimental and control groups. After pulp-capping treatments, the highest occurrence rate of cell death was found in the control group followed by the CH group at Weeks 2 and 3, where a significant difference was noted between the MP and TAP groups (Fig. 5C).

3.7. Expression levels of selected genes during the healing process of the dental pulp following capping treatment

The mRNA expression of selected genes during the pulpal healing process was analyzed by qRT-PCR, using specific primers for cDNA encoding *Nestin*, *Cd11c*, and *Nanog* at Weeks 1 and 2 after

the operation. There were not statistically differences except for the TAC-P and MP groups regarding *Cd11c* at Week 1. The mRNA expression of the odontoblast differentiation marker *Nestin* was higher in the TAC-P group than in the CH and MP groups at Week 1, followed by a steady decrease of its expression in all groups at Week 2 (Fig. 6A). In addition, the mRNA expression of dendritic cell marker, *Cd11c*, sharply increased from Week 1—2 in the TAC-P group, followed by the CH group. In contrast, the MP group markedly decreased its expression two weeks after treatment (Fig. 6B). Furthermore, the TAC-P group showed the highest mRNA expression of stem cell marker *Nanog* among the three groups at Week 1, whereas its expression decreased by Week 2, unlike the MP group, which displayed an opposite behavior. Interestingly, no variation was observed in the CH group at this stage (Fig. 6C).

4. Discussion

The present study elucidated the dynamics of pulpal healing following DPC with TAP compared with a CH-based material. In addition, we established an animal model using mice for evaluation of the pulpal responses to dental materials at the cellular and gene level by immunohistochemistry and qRT-PCR analysis. The use of TAP as a capping agent for the treatment of oral-microflora-exposed dental pulps consistently promoted cell proliferation and
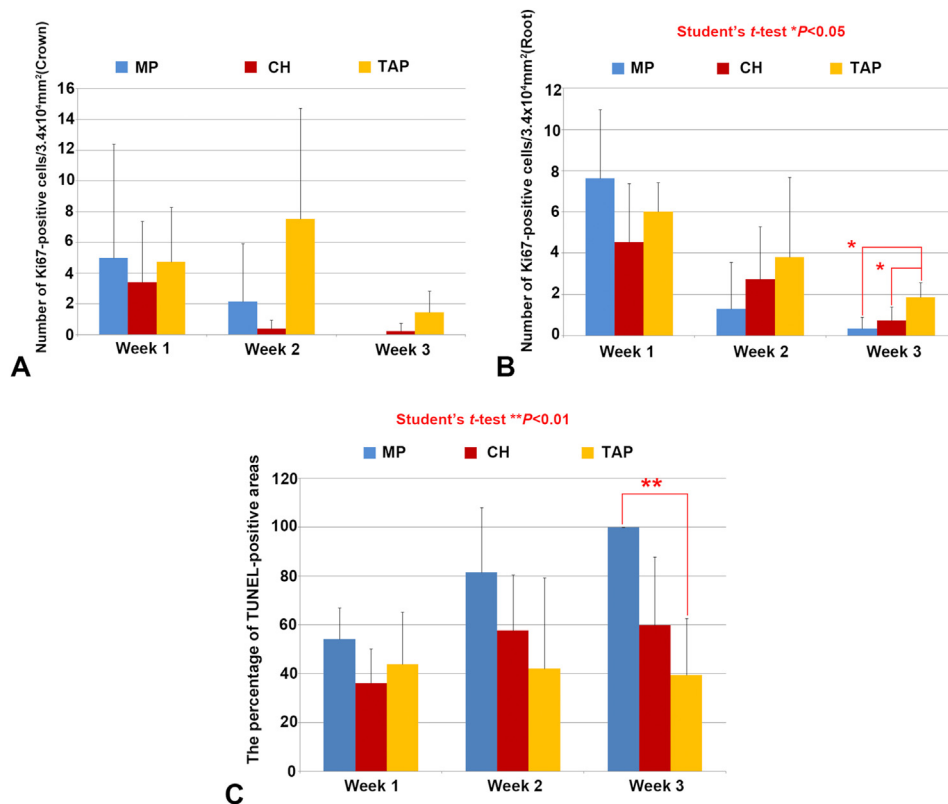
Fig. 5. Quantitative evaluation of cell proliferation and apoptotic activity in the dental pulp of experimental and control groups. (A) TAP accelerated cell proliferation in the coronal pulp at Week 2, whereas in the MP group, the number of proliferative cells dramatically decreased from Week 1–3, followed by that in the CH group (B) A significant difference between TAP and the other groups was seen in the root pulp at Week 3 after treatment. Similarly, the number of proliferative cells in the root dental pulp sharply decreased from Week 1–3 in the MP negative control group followed by the CH group (C) The percentage of TUNEL-positive areas in the dental pulp. The control group shows the highest occurrence rate of cell death, followed by the CH group at Weeks 2 and 3, when a significant difference was noted between the TAP and MP groups.

tended to decrease apoptotic activity in the pulpal tissue during its healing process, favoring the differentiation of odontoblast-like cells and the deposition of tertiary dentin. In contrast, the mixed healing pattern consisting of bone-like tissue and tertiary dentin, and total necrosis were seen in the CH cement and MP groups, respectively. The injury model used in our study induced the contamination of oral microflora into the dental pulp, leading to a severe inflammatory condition following application of the TAP or CH as capping agents. The effects of TAP on the injured tissue brought about better results as to the survival of odontoblasts and the acceleration of the odontoblast-like cell differentiation. Consequently, a rapid deposition of hard tissue took place at the injury site three weeks after operation. The histological analysis showed the occurrence of tubular and atubular dentin-like matrix with some cellular inclusions and newly-formed hard tissue compatible with the features of reparative dentin. Furthermore, this study showed that application of TAP might directly activate stem/progenitor cells and dendritic cells that reside in the afflicted pulpal tissue, favoring their participation during the chronological changes of the repair process. The differentiation of odontoblast-like cells following tooth injuries and the subsequent deposition of reparative dentin at the exposure site imply a complex scenario involving the participation of progenitors, supportive and immune cells that remain to be fully clarified [33,34]. In our study, the dental pulp was exposed to the oral microflora for 24 h to contaminate the tissue before capping treatment. Preliminary microbiological studies performed using the samples from our study have shown that the part of the oral microflora population changes from baseline levels following pulp exposure. For instance, certain

bacterial species found in the plaque on teeth moved into the dental pulp and proliferated 24 h after pulp exposure, suggesting the onset of a possible infection in the pulp tissue (Sato T et al. unpublished data). As a result, a strong inflammatory reaction was consistently observed in the MP group, that showed the highest percentage of TUNEL-positive areas, followed by the CH cement group at each observation stage. In contrast, the application of TAP improved the resolution of the inflammation and promoted the increase of proliferative cells in the coronal and root pulp at Week 2 after the operation, suggesting that TAP may enhance the function of immune cells. Previous studies have noted that DCs participate in the initial defense reaction, serving as a biological sensor for the external stimuli arriving through the exposed dentinal tubules [35]. DCs arrange at the dentin-pulp interface to catch foreign antigens, and subsequently, other immune cells such as T cells, neutrophils, and macrophages accumulate in the pulp according to the extension of the lesion and the development of the inflammatory process [36–38]. Although we did not confirm the infection of the exposed pulpal tissue, nor did we show immunohistochemical evidence for DCs following direct pulp capping, the analysis of the pulpal healing process at the gene level indicated that the application of TAC-P tended to induce the highest mRNA expression of the DCs marker *Cd11c* 2 weeks after treatment, correlating with the peak of proliferative cells shown at this stage by Ki-67 immunohistochemistry. Given that cell proliferation precedes odontoblast-like cells differentiation, we hypothesized that TAP may contribute to a sterile environment that allows active pulpal cell proliferation and simultaneous activation of DCs. In contrast, an intense inflammatory reaction occurring in the samples of control group leads to an
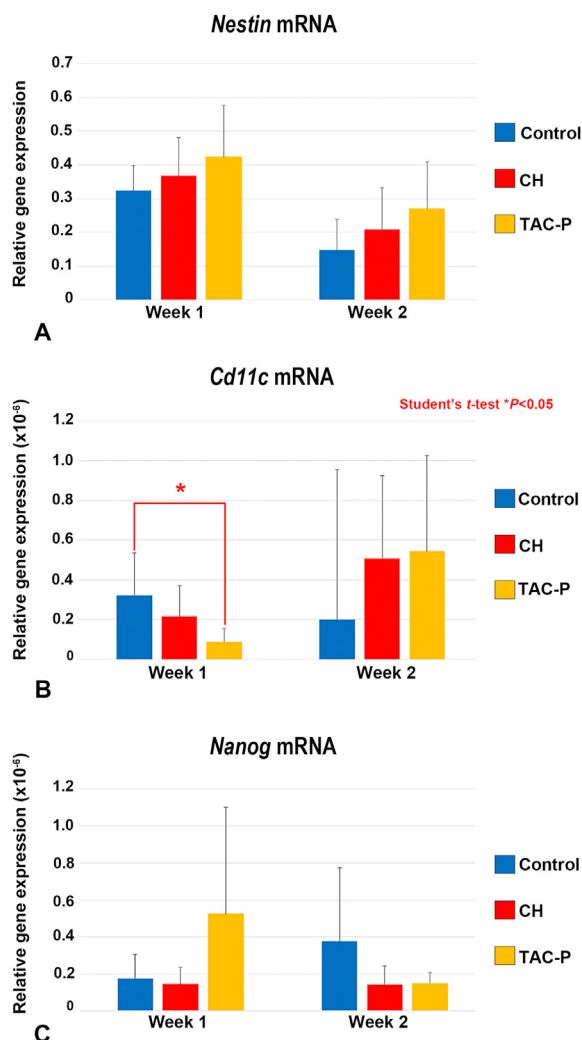
Nestin mRNA

A

Cd11c mRNA

Student's *t*-test **P*<0.05

B

Nanog mRNA

C

Fig. 6. Expression profile of odontoblast differentiation process—related genes. (A) Comparative mRNA expression levels for *Nestin* (B) *Cd11c*, and (C) *Nanog* among the TAC-P, CH and control groups at Weeks 1 and 2 after direct capping treatment. Gene expression levels were calculated relative to the levels of *β-actin* mRNA using the comparative Ct (2−ΔΔCt) method.

increased activity of antigen-presenting and other immune pulpal cells, as demonstrated by the statistical difference in the expression levels for *Cd11c* between the control and TAP group at week 1. Subsequently, DCs may aggregate along the pulp-dentin border near the injury site to contribute to the differentiation process of odontoblast-like cells under pathological conditions. This hypothesis is supported by a previous study in which high expression levels of *Cd11c* mRNA were first observed in the dental pulp of intentionally delayed replanted teeth treated with TAS on days 1, 5, and 7 after their repositioning into the alveolar socket [30]. Nevertheless, further studies are necessary to confirm whether an infection is established in the dental pulp and to test the effectiveness of TAP on the activation of immune cells including DCs, their recruitment towards the site of injury, and odontoblast-like cell differentiation.

The selection of an adequate dental material for vital pulp therapies is critical to elicit a positive response following severe injuries [23—25,39]. In this context, the use of *in vivo* strategies is necessary to track the chronological changes and to recognize the different healing patterns formed in the injured pulp as results of the direct application of capping agents. To our understanding, the present results constitute the first report of the pulpal responses to

TAP as a capping agent at the cellular and gene level, using a murine injury model. Hard-tissue formation was clearly observed from Week 2 after treatment in both the TAP and CH groups, whereas a bridge-like structure was predominantly observed in the TAP group at Week 3. In contrast, total necrosis was seen in the MP group even at this stage. Using nestin as a marker for newly differentiated odontoblast-like cells [40], we were able to distinguish the nature of the hard tissue formed in the dental pulp. The presence of a strong immunoreaction for nestin in the periphery of the bridge-like structure and other hard-tissue areas near the exposure site confirmed that TAP induced reparative dentin formation, whereas CH cement induced a mixture of bone-like tissue and reparative dentin in the dental pulp, as shown by nestin-negative and -positive staining in the surrounding pulpal cells. These findings were also reported in previous *in vivo* studies using TAS. Intentionally delayed replanted molars of mice showed different healing patterns after the treatment with TAS, ranging from total necrosis to reparative dentin according to the concentration of each antibacterial drug [29]. Another study using the same model showed that TAS promoted the regeneration of the dental pulp and maintained the proper size of the pulp chamber [30]. Moreover, a clinical trial conducted in primary teeth of patients of 3—6 years of age showed that direct capping treatment using TAP reached an overall outcome rate of 62.5% after 12 months, preceded by its combination with Simvastatin (also known as 3Mixtatin) and mineral trioxide aggregate (MTA) [41]. On the other hand, CH is still considered the gold standard material for direct capping treatments. The properties of CH-based cements have been extensively reported *in vivo* and *in vitro* elsewhere. Although CH possesses antibacterial properties due to its high pH (approximately 12) and promotes dentine bridge formation at the exposure site, there are some disadvantages that cause fluctuations on its successful outcome rate; such as the occurrence of high irritation in the pulpal tissue and tunnel defect formation in the dentine bridge [23]. The previous *in vitro* study also demonstrated that CH could regulate odontogenic/osteogenic differentiation by stimulating the interaction of EphrinB1 ligand with its receptor EphB2 [42]. This finding supports our current data, where bone-like tissue and reparative dentin were found in the pulp of teeth treated with CH-based cement. Alternatively, new bioactive materials such as MTA or tricalcium silicate cements (Biodentine) have shown promising results *in vivo* and *in vitro*, including in several randomized clinical trials in immature and mature teeth [43—46]. According to a recent systematic review and meta-analysis on the use of dressing materials for pulpotomy, there is not enough evidence to conclude the superiority between MTA and other pulpotomy-dressing agents such as CH, TAP, calcium—enriched mixture, or platelet-rich fibrin, as they showed similar success rate in the treatment of immature permanent teeth [47]. Similarly, another systematic review concluded that there was no evidence of the superiority of any material currently used for DPC in vital primary teeth [24]. Although we still lack conclusive long-term clinical studies, the use of new dental materials with biological properties have gained popularity toward a new era of regenerative dentistry.

In recent years, TAP has been widely used in regenerative endodontic procedures (REP) for the treatment of the infected root canals due to its superior antibacterial effects. Even at low concentrations, the application of TAP demonstrated to eradicate *Enterococcus faecalis* without side effects on the viability of stem cells of the apical papilla (SCAP) [48—50]. However, the use of TAP at high concentrations negatively affected the survival of SCAP, whereas lower concentrations of the drugs as well as CH contributed to their survival and proliferation [51]. This finding was reinforced by another study in which lower concentration of TAC showed no cytotoxic effects on DPSCs [52]. On the other

hand, the main disadvantage of using TAP in REP refers to the risk of tooth discoloration, as showed by three recent systematic reviews [53—55]. The concern about discoloration has provided alternatives to TAP, such as the double antibiotic paste (DAP), based on metronidazole and ciprofloxacin; or the modified TAP (mTAP), where minocycline is replaced by a third antibiotic. Both alternatives have shown to be effective against the microflora of the root canal, although they showed cytotoxic effects *in vitro* [56—58].

Previous data showed that the disinfection with TAP favors the influx of SCAP inside the root canal following the evoked-bleeding step in REP [59]. In our study, the high mRNA expression of the stem cell marker *Nanog* at Week 1 after treatment suggests that the application of TAC-P favored the activation of stem cells/progenitors residing in the injured dental pulp, resulting in a positive trend in the experimental group. This finding is supported by a previous *in vivo* report in which prolonged immersion time of extracted teeth in TAS was conducive to the upregulation of stem cell markers *Oct 3/4 A* and *B* in the dental pulp at Day 1 following the repositioning into their alveolar sockets [30]. In addition, it was found that minocycline, effectively induced a set of genes, including *Yap 1*, *Sox 10*, *Birc5*, *Pcna*, *Synapsin*, and *Notch 1* (cleaved), and decreased the activation of *Caspase 3* [60]. These genes are known to affect cell proliferation, apoptosis, synaptic transmission, and nerve transmission, which also may have a potential relationship with the activation of the internal biological mechanisms leading to the healing process of the injured pulpal tissue. Nevertheless, direct evidence of progenitor cell activation is necessary to clarify the relationship between topical antibiotics and reparative dentin formation following severe injuries.

5. Conclusion

In summary, the use of TAP as a pulp-capping agent, compared with CH cement, accelerated the healing process of oral-microflora-exposed dental pulp in mouse molars. Further studies evaluating the effectivity of the TAP and its comparison with other antibiotic pastes such as DAP or mTAP and other advanced bioactive materials are necessary to clarify the concerns of DPSCs and DCs during the repair processes of the dental pulp following tooth injuries and pulp exposure in order to be considered as a potential biomaterial in the clinical setting.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.reth.2020.10.001.

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