Responses of infected dental pulp to αTCP-containing antimicrobials in rat molars*

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Summary. α-tricalcium phosphate (αTCP) with the addition of antimicrobials such as ciprofloxacin, metronidazole, and cefaclor (3Mix) has been applied to sterilize the infected dentin and pulp in vivo. Both clinical and animal experiments have shown that 3Mix is effective for sterilizing infected tissues. However, the responses of the infected dental pulp to 3Mix remain to be fully determined at the cellular level. This study aims to clarify the responses of neural elements and immune cells to antimicrobials during the healing process of infected pulp using immunohistochemistry for protein gene product (PGP) 9.5 and class II major histocompatibility complex molecules using both light and electron microscopy. An artificial pulp exposure was prepared on the maxillary molar of 14-week-old rats and maintained without any treatment for 12 - 24 h. Subsequently, the exposed pulp was covered with αTCP or αTCP containing 3Mix, followed with glass ionomer cement. A pulp abscess lacking both dendritic cells and PGP 9.5-reactive nerve fibers was induced after pulp capping with αTCP; in contrast, numerous dendritic cells accumulated along the pulp-dentin border followed by the differentiation of odontoblast-like cells and matrix deposition after the application of αTCP containing 3Mix. PGP 9.5-reactive nerve fibers were also densely distributed and surrounded the accumulated dendritic cells in the medial dental pulp beneath αTCP containing 3Mix. The findings indicate that the application of αTCP containing 3Mix to the infected pulp induces an intense accumulation of dendritic cells, suggesting that these cells play crucial roles in the differentiation of odontoblast-like cells under pathological conditions.

Introduction

In carious or traumatic pulpal exposures, dental pulp is easily infected and the bacteria may survive within the dentinal tubules or pulp tissue even after treatment. In addition, it has been reported that bacteria of carious dentin can invade the pulp tissue through dentinal tubules without pulpal exposure by the carious process (Hoshino et al., 1992). Bacterial flora of humans in carious dentin (Hoshino, 1985), dental plaque (Hoshino et al., 1989), and necrotic pulp (Sato et al., 1993b) have been reported to consist mainly of obligate anaerobes. A mixture of ciprofloxacin, metronidazole, and minocycline or cefaclor

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formation that might enhance pulpal protection (Schroder, 1985). During the reparative process of exposed pulp, primary odontoblasts lost as a result of extensive damage can give rise to dentin bridge formation (Sato et al., 1992; Sato et al., 1993a; Hoshino et al., 1996). Furthermore, a-tricalcium phosphate (aTCP), with the addition of 3Mix — referred to as aTCP containing 3Mix — has been shown to be effective against bacteria in dentinal lesions (Sato et al., 1992; Sato et al., 1993a; Hoshino et al., 1996). Thus, aTCP containing 3Mix has been used to sterilize infected dentin and pulp in vivo.

In animal studies, the effects of antibacterial drugs on bacterially contaminated dental pulp were investigated using monkeys (Yoshida et al., 1995). Hard tissue barrier formation was delayed by aTCP containing 3Mix as compared with calcium hydroxide although aTCP containing 3Mix effectively disinfected pulpal lesions without destroying any of the sound pulp tissue. Calcium hydroxide-based materials have been extensively used for direct pulp capping because of their potential to induce hard tissue repair, which can give rise to dentin bridge formation that might enhance pulpal protection (Schroder, 1985). During the reparative process of exposed pulp, primary odontoblasts lost as a result of extensive damage are replaced with newly differentiated odontoblast-like cells (Schroder, 1985; Goldberg and Smith, 2004). This process follows the sequential steps of proliferation, migration, and differentiation of progenitor cells before matrix secretion at the exposure site. However, the responses of infected pulp to antimicrobials, especially cellular responses, remain to be fully determined.

The dentin-pulp complex is capable of repair after tooth injuries such as caries, attrition, abrasion, and dental procedures including cavity preparation, resulting in tertiary dentin formation (Nanci, 2008). In rat molars, the procedures of cavity preparation and tooth replantation induce destructive changes in odontoblasts at the affected site as well as an acute inflammatory reaction (Ohshima, 1990; Nakakura-Ohshima et al., 2003; Ohshima et al., 2003). In these experimental models, pulpal mesenchymal cells take the place of the degenerated odontoblasts to differentiate into odontoblast-like cells, resulting in the formation of tertiary dentin. Our recent studies using rat models have demonstrated that the temporal appearance of dendritic cells at the pulp-dentin border is a decisive phenomenon to induce the differentiation of odontoblast-like cells after tooth injuries such as cavity preparation and tooth replantation (Ohshima et al., 1995; Shimizu et al., 2000; Nakakura-Ohshima et al., 2003; Ohshima et al., 2003). However, there is no available data on how dendritic cells are involved with the odontoblast differentiation in the regenerative process following direct capping with aTCP containing 3Mix on the infected pulp. Some investigations have suggested that neural elements — including calcitonin gene-related peptide (CGRP) — influence the degree of tissue damage and pulp healing after tooth injury; much more extensive reinnervation causes the regeneration of odontoblast-like cells (Kvinnsland et al., 1991; Byers et al., 1992) since that protein is capable of enhancing wound healing and promoting fibroblast proliferation (Taylor and Byers, 1990; Byers and Taylor, 1993; Trantor et al., 1995). This study aims to clarify the responses of neural elements and immune cells to antimicrobials in the regenerative process following direct capping with aTCP containing 3Mix on the infected pulp. It employed immunohistochemistry for protein gene product (PGP) 9.5, which is a general neuronal marker, and class II major histocompatibility complex (MHC) molecules, which are expressed in the dendritic cells and macrophages, using both light and electron microscopy.

Materials and Methods

Procedure of cavity preparation and pulp capping

All experiments were reviewed by the Committee on the Guidelines for Animal Experimentation of Niigata University and performed according to the recommendations or under the conditions proposed by the Review Committee. Sixteen Wistar rats, 100 days old, were used in this study. Under anesthesia by an intraperitoneal injection of chloral hydrate (the maximum dose of 350 mg/kg), a round-shaped cavity was prepared by an air turbine with a tungsten carbide bur (diameter: 0.6 mm) under water-cooling to expose the pulpal horn on the medial surface of the upper-left first molar. The cavity was left open to the oral environment for 12 - 24 h without any treatment. Before any placement of the capping agent into the cavities, all gross plaque or debris found there were washed away with sterile saline. The exposed pulp tissue was then capped with aTCP (New Apatite Liner type I; Dentsply-Sankin, Tokyo) containing 3% (w/w) metronidazole + 1% (w/w) ciprofloxacin + 1% (w/w) cefaclor (3Mix), or aTCP without any antibiotics. The remaining cavity was filled with light-cured glass ionomer lining cement (Vitabond; 3M, MN, USA). The upper-right first molar of the same animal was used as a control. The region selected for observation was the periphery of the pulp tissue in the mesial coronal portion.
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(EDTA-2Na) solution for 6 weeks at 4°C, the samples were processed for cryosectioning for which the tissue blocks were equilibrated in a 30% sucrose solution for cryoprotection. The specimens were cut sagittally at a thickness of 25-50 μm with a freezing microtome (FX-801; Yamato Kohki, Tokyo), collected into cold phosphate-buffered saline (PBS), and treated as free-floating sections.

Histological procedures

Materials were collected from groups of one to five animals each at intervals of 1, 3, 5, 7, and 14 days after pulp capping. At each stage, the animals were anesthetized by an intraperitoneal injection of chloral hydrate (350 mg/kg) and transcardially perfused with physiological saline followed by 4% paraformaldehyde in a 0.1M phosphate buffer (pH 7.4). The maxillae including both the control and prepared teeth were removed en bloc and immersed in the same fixative for an additional 12 h. Following decalcification in a 5% ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) solution for 6 weeks at 4°C, the samples were processed for cryosectioning for which the tissue blocks were equilibrated in a 30% sucrose solution for cryoprotection. The specimens were cut sagittally at a thickness of 25-50 μm with a freezing microtome (FX-801; Yamato Kohki, Tokyo), collected into cold phosphate-buffered saline (PBS), and treated as free-floating sections.

Immunohistochemical analysis

Free floating sections were processed for the avidin-biotin peroxidase complex (ABC) method using antibodies to class II MHC molecules (OX6-monoconal antibody, diluted 1:5000; Serotec Ltd., Oxford, UK) and PGP 9.5,
Fig. 2. Light (a–d) and electron micrographs (e) of toluidine blue staining (a), and OX6- (c–e) and PGP 9.5- (b) immunoreactivities obtained from a semithin section (a, d), cryosections (b, e), and an ultrathin section (e) in αTCP-treated teeth after 3 days (a) and 2 weeks (c–e) (A: abscess, C: prepared cavity, D: dentin, DP: dental pulp). a: A large abscess lesion including numerous neutrophils occupies the entire mesial coronal pulp. b, c: The abscess lesion lacks OX6-immunopositive cells and PGP 9.5-immunoreactive nerves, which are densely localized beneath the abscess lesion and not observed along the pulp-dentin border beneath the affected dentin or the exposed area. d, e: The accumulation of neutrophils is observed in direct contact with αTCP (*) at the exposed area. Bars = 100 μm (a, b, c), 50 μm (d), 5 μm (e).

Fig. 3. Light (a–g) and electron micrographs (h) of toluidine blue staining (b), and OX6- (a, c, e–h) and PGP 9.5- (d) immunoreactivities from a semithin section (b, g), cryosections (a, c–f), and an ultrathin section (g) in αTCP containing 3Mix-treated teeth after 1 (a), 3 (b), 5 days (c), and 1 week (d–h) (C: prepared cavity, D: dentin, DP: dental pulp, PD: predentin, *: differentiating mesenchymal cells). a: OX6-immunopositive cells are increased in number compared with the control. b: The affected dental pulp is occupied with granulation tissue lacking the abscess lesion. c: OX6-immunopositive cells are densely distributed in the mesial coronal pulp.
Fig. 3. **d, e:** Arborized PGP 9.5-immunoreactive nerves innervate the mesial coronal pulp where OX6-immunopositive cells are densely distributed. (**f–h**) Figure **f** is a higher magnification of the boxed area in **e**. OX6-positive cells are arranged along the pulp-dentin border beneath drilled dentin and the exposed area beneath αTCP containing 3Mix to extend their cellular processes into the dentinal tubules (arrows). **Inset:** Higher magnification of the boxed area in **h**. Tubulovestibular structures (arrowheads) are observed in the cytoplasm of OX6-positive cells. Bars = 100 μm (**a–e**), 50 μm (**f**), 25 μm (**g**), 5 μm (**h**).
Fig. 4. Light (a–c) and electron micrographs (d–f) of OX6- (b–f) and PGP 9.5- (a) immunoreactivities obtained from a semithin section (e), cryosections (a, b), and an ultrathin section (d–f) in αTCP containing 3Mix-treated teeth after 2 weeks (C: prepared cavity, D: dentin, DP: dental pulp, OB: odontoblast-like cells, PD: predentin). a: PGP 9.5-immunoreactive nerves are densely distributed in the coronal pulp. b: OX6-immunopositive cells are densely distributed in the coronal pulp. c–f on the next page.
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Bonferroni's test (one-way analysis of variance; ANOVA) using statistical software (SPSS 16.0J for Windows; SPSS Japan, Tokyo).

Results

Controls

The dental pulp was composed of three layers: a differentiated odontoblast layer, a subodontoblastic layer including a cell-rich zone, and the center of the pulp tissue. Many OX6-immunopositive cells were widely distributed throughout the dental pulp, predominantly in the periphery of the pulp tissue (Fig. 1a). Most of the OX6-immunopositive cells in the subodontoblastic region exhibited dendritic profiles; some of them extended their processes into the odontoblast layer (Fig. 1b). Dense innervation of PGP 9.5-immunoreactive nerves was found in the dental pulp (Fig. 1c). The positive nerve bundles entered the pulp through both the apical foramina and medullary tubes to extend throughout the entire pulp. They arborized extensively in the coronal pulp to form the subodontoblastic nerve plexus of Raschkow beneath the odontoblast layer. A major population of nerve fibers from this plexus penetrated into the predentin beyond the odontoblast layer (Fig. 1d).

Pulp capping with αTCP without any antibiotics

The odontoblasts under the prepared cavity suffered severe damage; many odontoblasts at the affected site showed an impaired cell layer. A large abscess lesion including numerous neutrophils occupied the entire mesial coronal pulp at Day 3 (Fig. 2a) and remained at the same size until 2 weeks after pulp capping. The abscess lesion lacked OX6-immunopositive cells and PGP 9.5-immunoreactive nerves, which were densely localized beneath the abscess lesion and not observed along the

Statistical analysis of OX6-immunoreactivity

We compared the OX6-immunoreactivity at the mesial dental pulp beneath the prepared cavity in control and αTCP containing 3Mix-treated teeth (control, week 1, and week 2 groups). Quantitative analysis was performed in 3 samples for each group. We selected the grid (280 x 188 µm²), and the immunoreactive area fractions (%) of each specimen were calculated using image-analysis software (WinRoof 5.6; Mitani Corp Ltd, Fukui). All data were presented as the means and standard deviations (SD) of each group. Comparisons among different groups (control, week 1, and week 2 groups) were performed by
pulp-dentin border beneath the affected dentin or the exposed area (Fig. 2b, c). The accumulation of neutrophils was observed in direct contact with αTCP at the exposed area under both light and electron microscopes (Fig. 2d, e).

**Pulp capping with αTCP containing 3Mix**

Many odontoblasts at the affected site showed an impaired cell layer irrespective of the addition of 3Mix. The affected dental pulp was occupied with granulation tissue lacking the abscess lesion at days 1-3, where OX6-immunopositive cells were increased in number (Fig. 3a, b). Numerous OX6-immunopositive cells were densely distributed throughout the mesial coronal pulp at 5 days and 1 week after surgery (Fig. 3c, e). Interestingly, they were arranged along the pulp-dentin border beneath the drilled dentin and the exposed area beneath αTCP containing 3Mix; they extended their cellular processes into the dentinal tubules (Fig. 3e, f). Transmission electron microscopy demonstrated intense immunoreactions of class II MHC antigen being confined to the cell membrane as well as OX6-immunopositive cells at the pulp-dentin border possessing tubulovestibular structures in their cytoplasm. Large mesenchymal cells with clear nucleoli appeared next to the OX6-immunopositive cells localized along the pulp-dentin border and contained developed cell organelles such as rough endoplasmic reticulum, Golgi apparatus, and mitochondria (Fig. 3g, h). Arborized PGP 9.5-immunoreactive nerves innervated the coronal pulp where OX6-immunopositive cells were densely distributed (Fig. 3d, e).

OX6-immunopositive cells remained densely distributed in the coronal pulp 2 weeks after surgery (Fig. 4b). PGP 9.5-immunoreactive nerves showed features similar to the previous stage: they were densely distributed in the coronal pulp (Fig. 4a). The newly-formed dentin matrix was observed in the exposed area although the matrix occasionally included the dentin tips that had been contaminated in the prepared cavity during the surgery. Newly-differentiated odontoblast-like cells were arranged subjacent to the deposited dentin matrix, and OX6-immunopositive cells retreated beneath the odontoblast-like cells (Fig. 4c, f). Multinuclear giant cells appeared within the pulp granulation tissue protruding through the opening of the experimental cavity, where the existence of odontoblast-like cells and a matrix deposition was also observed (Fig. 4c, d). These giant cells developed ruffled membranes and contained numerous vacuoles including electron dense materials in their cytoplasm as confirmed by electron microscopy (Fig. 4e).

Statistical analysis of OX6-immunoreactivity

Statistical analysis of OX6-immunoreactivity showed that the fraction of the immunopositive area in the experimental groups was significantly larger than that in the control (Fig. 5).

**DISCUSSION**

The present immunocytotoxic study has clearly demonstrated cell dynamics in the pulpal repair process following pulp capping with αTCP containing 3Mix on the infected pulp tissue using both light and electron microscopy. Pulp capping with αTCP without any antibiotics induced a large abscess lesion including numerous neutrophils in the infected pulp during the experimental periods in rat models. The healing response to direct pulp capping with αTCP on the non-infected pulp of monkeys was characterized by the formation of an atubular matrix by cuboidal cells without any necrotic layer (Ikami et al., 1990; Yoshiba et al., 1994, 1995). Thus, pulp capping with αTCP alone on the infected pulp appears to be contraindicated in clinical dentistry. On the other hand, pulp capping with αTCP containing 3Mix elicits no abscess lesion, resulting in the pulpal repair observed in the present study. The previous study using monkey models also demonstrated that teeth capped with αTCP containing 3Mix effectively disinfected
bacterially contaminated pulp tissue without destroying the surrounding pulp tissue—although this study failed to induce hard tissue formation 4 weeks after surgery (Yoshiba et al., 1995). This inconsistency regarding hard tissue formation following direct capping with αTCP containing 3Mix may be attributed to the differences between animal species such as rats and monkeys and/or experimental procedures. The collagenous matrix underlying the αTCP layer begins 14–21 days after pulp amputation in monkey models (Ikami et al., 1990) whereas the newly formed matrix underlying mineral trioxide aggregate (MTA) appears 5 days after pulp amputation in rat models (Kuratate et al., 2008). Cavity preparation in rats induces the arrangement of newly-differentiated odontoblasts along the pulp-dentin border 2 days after surgery, and a newly-deposited dentin matrix appears on Day 3 (Harada et al., 2008). These findings indicate that the pulpal healing process following tooth injuries in rats is two or three times faster than that in monkeys, where it may take more than 4 weeks to complete pulpal healing following pulp capping.

The temporal appearance of class II MHC-positive cells, which are categorized as dendritic cells on the basis of their ultrastructural features—including cell organelles such as tubulovesicular structures and a lack of phagosomes (Ohshima et al., 1994), was observed along the pulp-dentin border in the pulp repair process after direct capping with αTCP containing 3Mix in the infected pulp in the present study. In contrast, class II MHC-positive cells were localized beneath the abscess lesion instead of arranging along the pulp-dentin border as in the case of pulp capping with αTCP alone. These findings suggest that the accumulation of dendritic cells at the pulp-dentin border may be a prerequisite phenomenon for the differentiation of odontoblast-like cells; this is consistently observed in the pulpal repair process following tooth injuries irrespective of the types of injuries such as cavity preparation or tooth replantation reported in previous studies (Ohshima et al., 1995; Shimizu et al., 2000; Nakakura-Ohshima et al., 2003; Ohshima et al., 2003) or the direct pulp capping reported in this study. A crucial role for class II MHC-positive cells in the process of odontoblast differentiation is supported by our recent results showing that the secretion of the granulocyte-macrophage colony-stimulating factor (GM-CSF) and osteopontin by immunocompetent cells such as macrophages and dendritic cells play respective roles in the maturation of dendritic cells and the differentiation of odontoblasts in the regenerative pulp tissue following tooth transplantation (Saito et al., 2011). Regarding the relationship between class II MHC-positive cells and neutrophils, their differing functions have been demonstrated after chromium, thulium, erbium:yttrium-aluminum-garnet (CrTmEr:YAG) laser irradiation: class II MHC-positive cells are temporarily arranged along the pulp-dentin border and extend their cytoplasmic processes into the dentinal tubules at the initial stage but disappear from there at postoperative 24 h; subsequently, the abscess lesion including numerous neutrophils appears until Day 3 following the bacterial infections via the dentinal tubules at 24 h (Suzuki et al., 2004). In general, the class II MHC-positive cells participate in the initial immune response to serve as antigen-presenting cells (Steinman, 1991). The increase of neutrophils with phagocytotic activity may be attributed to bacterial infection after laser ablation in the previous study (Suzuki et al., 2004) and pulp exposure to the oral environment followed by pulp capping with αTCP in this study. Thus, class II MHC-positive cells may play dual roles in both the initial immune response and the differentiation of odontoblast-like cells under pathological conditions.

It is noteworthy that the appearance in this study of multinucleated giant cells seen in the repair process after pulp capping with αTCP containing 3Mix using rat models coincides with the results in the previous study using direct capping with αTCP in monkey models (Ikami et al., 1990), suggesting that they are engaged in degrading αTCP in the repair process. Interestingly, multinuclear giant cells with numerous ruffled membranes appeared within the pulpal granulation tissue protruding through the opening of the experimental cavity, implying that they migrate out from the dental pulp together with mesenchymal cells to differentiate further into odontoblast-like cells. The similarity in distribution patterns between class II MHC-positive cells and neural elements in this study may indicate an intimate functional correlation between them. Previous immunohistochemical studies have demonstrated a close spatial relationship between these two components in human (Jontell et al., 1996) and rat dental pulp (Okiji et al., 1997), suggesting the involvement of neural elements in the functional capacities of class II MHC-positive cells.

In conclusion, this study showed that numerous dendritic cells accumulated along the pulp-dentin border, followed by the differentiation of odontoblast-like cells and a matrix deposition after the application of αTCP containing 3Mix in rat models. PGP 9.5-reactive nerve fibers were also densely distributed and surrounded the accumulated dendritic cells in the medial dental pulp beneath αTCP containing 3Mix. The findings indicate that the application of αTCP containing 3Mix to the infected pulp induces the temporal appearance of dendritic cells, indicating that these cells may play crucial
roles in the differentiation of odontoblast-like cells under pathological conditions.

References

Banchs F, Trope M: Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? J Endod 30: 196-200 (2004).

Byers MR, Kvinessland I, Bothwell M: Analysis of low affinity nerve growth factor receptor during pulpal healing and regeneration of myelinated and unmyelinated axons in replanted teeth. J Comp Neurol 326: 470-484 (1992).

Byers MR, Taylor PE: Effect of sensory denervation on the response of rat molar pulp to exposure injury. J Dent Res 72: 613-618 (1993).

Goldberg M, Smith AJ: Cells and extracellular matrices of dentin and pulp: a biological basis for repair and tissue engineering. Crit Rev Oral Biol Med 15: 13-27 (2004).

Harada M, Kenmotsu S, Nakasone N, Nakakura-Ohshima K, Ohshima H: Cell dynamics in the pulpal healing process following cavity preparation in rat molars. Histochem Cell Biol 130: 773-783 (2008).

Hori R, Kohno S, Hoshino E: Bactericidal eradication from carious lesions of prepared abutments by an antibacterial temporary cement. J Prosthet Dent 77: 348-352 (1997).

Hoshino E: Predominant obligate anaerobes in human carious dentin. J Dent Res 64: 1195-1198 (1985).

Hoshino E, Sato M, Sasano T, Kota K: Characterization of bacterial deposits formed in vivo on hydrogen-ion-sensitive field-effect transistor electrodes and enamel surfaces. Jpn J Oral Biol 31: 102-106 (1989).

Hoshino E, Ando N, Sato M, Kota K: Bacterial invasion of non-exposed dental pulp. Int Endod J 25: 2-5 (1992).

Hoshino E, Kurihara-Ando N, Sato I, Uematsu H, Sato M, Kota K, Iwaku M: In-vitro antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. Int Endod J 29: 125-130 (1996).

Ikami K, Iwaku M, Ozawa H: An ultrastructural study of the process of hard tissue formation in amputated dental pulp dressed with alpha-tricalcium phosphate. Arch Histol Cytol 53: 227-243 (1990).

Jontell M, Okiji T, Dahlgren U, Bergenholtz G: Interaction between perivascular dendritic cells, neuropeptides and endothelial cells in the dental pulp. In: Dentin pulp complex: Proceeding of the International Conference on Dentin/Pulp Complex 1995. (Shimono M, Maeda T, Suda H, Takahashi K eds) Quintessence, Tokyo, 1996 (p. 182-187).

Kuratate M, Yoshiha K, Shigetani Y, Yoshiha N, Ohshima H, Okiji T: Immunohistochemical analysis of nestin, osteopontin, and proliferating cells in the reparative process of exposed dental pulp capped with mineral trioxide aggregate. J Endod 34: 970-974 (2008).

Kvinessland I, Heyeraas KJ, Byers MR: Regeneration of calcitonin gene-related peptide immunoreactive nerves in replanted rat molars and their supporting tissues. Arch Oral Biol 36: 815-826 (1991).

Longman LP, Preston AJ, Martin MV, Wilson NH: Endodontics in the adult patient: the role of antibiotics. J Dent 28: 539-548 (2000).

Mohammadi Z, Abbott PV: On the local applications of antibiotics and antibiotic-based agents in endodontics and dental traumatology. Int Endod J 42: 555-567 (2009).

Nakakura-Ohshima K, Watanabe J, Kenmotsu S, Ohshima H: Possible role of immunocompetent cells and the expression of heat shock protein-25 in the process of pulpal regeneration after tooth injury in rat molars. J Electron Microsc (Tokyo) 52: 581-591 (2003).

Nanci A: Ten Cate’s oral histology : development, structure, and function. Mosby, Inc., and affiliate of Elsevier Inc., St. Louis, MO, 2008 (p. 1-411).

Ohshima H: Ultrastructural changes in odontoblasts and pulp capillaries following cavity preparation in rat molars. Arch Histol Cytol 53: 423-438 (1990).

Ohshima H, Kawahara I, Maeda T, Takano Y: The relationship between odontoblasts and immunocompetent cells during dentinogenesis in rat incisors: an immunohistochemical study using OX6-monomonal antibody. Arch Histol Cytol 57: 435-447 (1994).

Ohshima H, Sato O, Kawahara I, Maeda T, Takano Y: Responses of immunocompetent cells to cavity preparation in rat molars: an immunohistochemical study using OX6-monomonal antibiotic. Connect Tissue Res 32: 303-311 (1995).

Ohshima H, Nakakura-Ohshima K, Takeuchi K, Hoshino M, Takano Y, Maeda T: Pulpal regeneration after cavity preparation, with special reference to close spatio-relationships between odontoblasts and immunocompetent cells. Microsc Res Tech 60: 483-490 (2003).

Okiji T, Jontell M, Belichenko P, Dahlgren U, Bergenholtz G, Dahlstrom A: Structural and functional association between substance P- and calcitonin gene-related peptide-immunoreactive nerves and accessory cells in the rat dental pulp. J Dent Res 76:1818-1824 (1997).
Infected pulpal responses to antimicrobials

Ozan U, Er K: Endodontic treatment of a large cyst-like periradicular lesion using a combination of antibiotic drugs: a case report. *J Endod* 31: 898-900 (2005).

Pinheiro SL, Simionato MR, Imparato JC, Oda M: Antibacterial activity of glass-ionomer cement containing antibiotics on caries lesion microorganisms. *Am J Dent* 18: 261-266 (2005).

Saito K, Nakatomi M, Ida-Yonemochi H, Kenmotsu SI, Ohshima H: The expression of GM-CSF and osteopontin in immunocompetent cells precedes the odontoblast differentiation following allogenic tooth transplantation in mice. *J Histochem Cytochem* 59: 518-529 (2011).

Sato I, Ando-Kurihara N, Kota K, Iwaku M, Hoshino E: Sterilization of infected root-canal dentine by topical application of a mixture of ciprofloxacin, metronidazole and minocycline in situ. *Int Endod J* 29: 118-124 (1996).

Sato T, Hoshino E, Uematsu H, Kota K, Iwaku M, Noda T: Bactericidal efficacy of a mixture of ciprofloxacin, metronidazole, minocycline and rifampicin against bacteria of carious and endodontic lesions of human deciduous teeth in vitro. *Microb Ecol Health Dis* 5: 171-177 (1992).

Sato T, Hoshino E, Uematsu H, Noda T: In vitro antimicrobial susceptibility to combinations of drugs on bacteria from carious and endodontic lesions of human deciduous teeth. *Oral Microbiol Immunol* 8: 172-176 (1993a).

Sato T, Hoshino E, Uematsu H, Noda T: Predominant obligate anaerobes in necrotic pulps of human deciduous teeth. *Microb Ecol Health Dis* 6: 269-275 (1993b).

Schroder U: Effects of calcium hydroxide-containing pulp-capping agents on pulp cell migration, proliferation, and differentiation. *J Dent Res* 64 Spec No: 541-548 (1985).

Shimizu A, Nakakura-Ohshima K, Noda T, Maeda T, Ohshima H: Responses of immunocompetent cells in the dental pulp to replantation during the regeneration process in rat molars. *Cell Tissue Res* 302: 221-233 (2000).

Steinman RM: The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 9: 271-296 (1991).

Suzuki T, Nomura S, Maeda T, Ohshima H: An immunocytochemical study of pulpal responses to cavity preparation by laser ablation in rat molars by using antibodies to heat shock protein (Hsp) 25 and class II MHC antigen. *Cell Tissue Res* 315: 311-319 (2004).

Takahighe T, Cruz EV, Asgor Moral A, Hoshino E: Endodontic treatment of primary teeth using a combination of antibacterial drugs. *Int Endod J* 37: 132-138 (2004).

Taylor PE, Byers MR: An immunocytochemical study of the morphological reaction of nerves containing calcitonin gene-related peptide to microabscess formation and healing in rat molars. *Arch Oral Biol* 35: 629-638 (1990).

Trantor IR, Messer HH, Birner R: The effects of neuropeptides (calcitonin gene-related peptide and substance P) on cultured human pulp cells. *J Dent Res* 74: 1066-1071 (1995).

Trope M: Regenerative potential of dental pulp. *J Endod* 34:S13-17 (2008).

Yoshiba K, Yoshiba N, Iwaku M: Histological observations of hard tissue barrier formation in amputated dental pulp capped with alpha-tricalcium phosphate containing calcium hydroxide. *Endod Dent Traumatol* 10: 113-120 (1994).

Yoshiba K, Yoshina N, Iwaku M: Effects of antibacterial capping agents on dental pulps of monkeys mechanically exposed to oral microflora. *J Endod* 21: 16-20 (1995).