Acta Veterinaria (Beograd), Vol. 57, No. 5-6, 573-584, 2007.

DOI: 10.2298/AVB0706573D UDK: 619:611-018

HISTOLOGICAL EVALUATION OF ODONTOBLAST-LIKE CELLS RESPONSE AFTER CAPPING APPLICATION OF CALCIUM HYDROXIDE AND HYDROXILAPATITE IN DOG'S PULP

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(Received 12. May 2007)

This study was conducted to observe the response of dog's dental pulp to hydrpxylapatite (Hap) and calcium hydroxide when used as pulp capping materials. After the pulps of 22 teeth were exposed, they were capped with either Hap or calcium hydroxide. Histological analysis was performed 8 weeks after treatment. Results showed that pulp exposure caused irreversible injury of odontoblasts, which were subsequently replaced by similar, odontoblast-like cells. These cells were able for reparative dentin bridge formation in 6 cases treated with calcium hydroxide and almost all the cases treated with hydroxylapatite. The presence of odontoblast-like cells seemed to be crucial for reparative dentinogenesis. The capping material was of less importance. In conclusion neither calcium hydroxide nor hydrxylapatite had completely satisfied all the requirements of an ideal capping material, although Hap can be recognized as a superior alternative to calcium hydroxide.

Key words: odontoblast-like cells, dental pulp, histology, Hap, calcium hydroxide

INTRODUCTION

Dental pulp is a vital tissue whose response to damage is associated with deposition of a hard tissue barrier called dentin bridge (Schroeder 1985). Histologically, this barrier has characteristics of reparative dentin. Reparative dentin formation is misunderstood, as this form of dentin can be the product of either preexistnig odontoblasts stimulated to deposit additional dentin or new cells differentiated from the pulp following odontoblast death i.e. cells that are odontoblast-like and produce a substance not strictly identical to normal dentin (Gronthos, 2002). Following pulp exposure, primary odontoblasts are often irreversibly injured. Odontoblasts are postmitotoc terminally differentied cells and cannot proliferate to replace subjacent irreversibly injured odontoblasts. Consequently, the origin of odontoblast-like cells, which produce the dentine bridge following pulp exposure, has proved to be controversial. Autoradiographic studies have indicated that new odontoblast-like cells may be derived from other

pulp cell populations by a process of differentiation (Fitzgerald *et al*., 1990). However, the mechanisms by which pulp cells differentiate into odontoblast-like cells and secrete dentine bridges in the absence of the basement membrane or dental epithelium are not fully understood (Tzifas, 1994).

The most likely progenitor cell population for odontoblast-like cells was assumed to be the fibroblasts located in the pulp core (Fitzgerald *et al*., 1990). Whilst cultured fibroblast cell lines have the ability to migrate in response to cytokine signalling molecules, cultures of pulp fibroblasts did not appear to differentiate into cells with odontoblast-like phenotypes or secretory characteristics (Hanks *et al*., 1998). Nevertheless, reparative dentinogenesis can be observed in tooth pulp tissue in vitro, indicating that new odontoblast-like cells can originate from other pulp cell populations (About *et al*., 2000). Feith *et al*. (1970) demonstrated that undifferentiated progenitor cells of the pulp parenchyma divide and migrate toward the site of pulp exposure, where the dentin bridge is secreted. This cellular differentiation pattern provided some support for the theory that odontoblast-like progenitor cells were resident undifferentiated mesenchymal cells (D'Souza *et al*., 1995). However, recent attention has focused on cells associated with pulp vasculature, most probably pericytes (Carlile *et al*., 2000), or pericytes progenitor cells such are myofibroblast cells (Alliot-Licht *et al*., 2001). Pericytes are solitary vascular smooth muscle cells associated with capillaries. It is widely accepted that pericytes represent pluripotent progenitor cells in the adult and their differentiation and migration have been reported to occur during tissue repair. In human dental pulp pericyte migration and differentiation have been observed (Carlile *et al*., 2000). However, the transition of these cells to a fibroblast phenotype may explain the difficulty in distinguishing these cells from the endogenous fibroblasts of the pulp.

The present experiment was undertaken with aim to study pulpal cell response and the onset of reparative dentin formation after capping by application of calcium hydroxide and hydroxylapatite on mechanically exposed pulp.

MATERIAL AND METHODS

Experimental animals

Three healthy 12 months old beagle dogs were used in the experiment. All experimental procedures were carried out in accordance with the European Communities Directive (86/609/EEC). The experiment was approved by the Ethical Committee of the Faculty of Stomatology, Belgrade University.

Material used

The following materials were used:

– Life^{R} Kerr Co., Orange County, CA, USA (commercial calcium hydroxide);

– Apatec^{R} Stomygen (commercial hydroxylapatite)

Experimental procedure

The dogs were anaesthetized after premedication with atropine sulphate (0.5 mg/kg bodyweight), with an intramuscular injection of Conbern® (0.005 mg/kg) Bayne, Germany and intravenous injection of Nembutal, Abbott Labs, Chicago USA (25 mg/kg)

Intact teeth – first molars, canines and third incisors from both jaws, with a healthy periodontium, were used. The teeth were isolated with cotton rolls, polished and washed with 0.5% chlorhexidine. Buccal class V cavities were prepared by inverted cone carbide bur mean size 31 with an air turbine with sterile saline spray. The pulps were exposed with round bur, producing a wound the size of the cutting edge of the bur. The exposed pulps were capped with calcium hydroxide and commercial hydroxylapatite. The guidelines of the International Standards Organization (ISO 7405) require the established capping materials to be used as the control group for measuring pulp responses. $Ca(OH)_2$ is commonly used as the control group because it is generally the most well established material (Schroeder, 1985; Cox and Bergenholz, 1986; D'Souza *et al*. 1995).

The material was placed at the exposure site and the cavities were immediately restored with amalgam. The observation period lasted 8 weeks. After that, the animals were sacrificed with an overdose of Nembutal®, the jaws were dissected and the teeth were fixed and processed for light microscopy analysis.

Histological procedure

Twenty-two teeth (divided equally in both groups) were fixed in 10% buffered formalin (pH 7.2) and processed for light microscope analysis. The teeth were demineralised for 40-50 days in 5% trichloracetic acid and embedded in paraffin. Serial sections 6 μm thick were cut transversally through the exposure site and stained with haematoxylin-eosin as well as with Gram. The amalgam was gently removed before sectioning.

Histological analysis was performed according to the next criteria:

a. hard tissue bridge formation (continuity, morphological aspect, thickness, localization);

b. inflammatory reaction - chronic or acute and extension of the reaction.

The intensities of these histological measures were classified into four grades: none, slight, moderate and severe, according to modified reported criteria (Stanley, 1968; Mjor and Tronstad, 1972):

– none – the pulp contained only a few, or an absence of inflammatory cells;

– slight – the pulp had localised inflammatory lesions composed of polymorphonuclears or lymphocytes at the site of exposure;

– moderate – the pulp had inflammatory lesions involving more that one third of the pulp;

– severe – the pulp was largely necrotic following inflammatory cells activity.

c. capillary dilatation and proliferation;

d. scar tissue formation;

e. atrophic degeneration of pulp tissue;

f. presence of operative debris, giant cells and particles of capping material;

g. the presence of microorganisms;
h. adhesion of capping material to dentin.

RESULTS

Odontoblast-like cells and dentin bridge formation

The results of the analysis revealed histological changes in the superficial reaction zone close to the capping material-pulp interface. These changes varied from minimal to very severe. Pulp capping resulted in mineralised bridge formation, while the pulp tissue was consistently found to be of normal structure, without any signs of inflammation or tissue degeneration, in 6 specimens in the group treated with calcium hydroxide. Hard tissue barrier consisted of mineralised matrix which was related to cuboidal or columnar cells and was separated from surrounding dentin with a clearly visible demarcation line (Figure 1). These cells had a distinctive elongated columnar morphology, with a clear nuclear, cytoplasmic and secretory polarity, resembling that of odontoblst-like cells. These newly differentiated cells have a similar phenotype to odontoblast cells, but with tubular continuity with dentine bridge secretion. They were intimately positioned at the dentine bridge border, in a polarized alignment relative to the direction of the dentine bridge formation. All the bridges were of the tubular type and they exhibited continuity with the lateral dentine, had sinuous dentine tubules, fewer in number and with a markely smaller diameter than those further away.

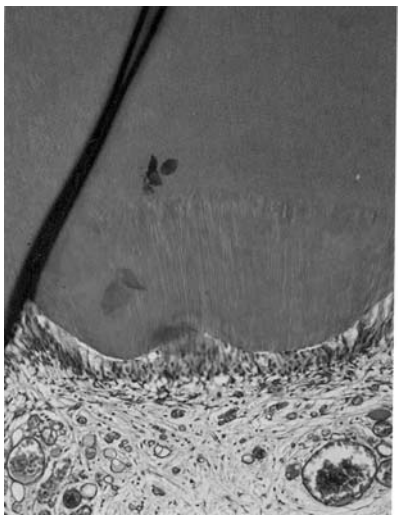


Figure 1. H.E.40x. Microphotograph of reparative dentin formation in the group treated with calcium hydroxide. Dentin bridge is separated from surrounding dentin by a clearly seen demarcation line. Odontoblasts-like cells and underlying pulp tissue is of normal structure

The healing process in the group treated with hydroxylapatite occurred in a way similar to the group treated with calcium hydroxide, although tissue response was more adequate (Figure 2). Dentin bridge formation was observed in almost all the analysed specimens (in only two cases dentin bridge formation failed). The presence of odontoblast-like cells was found to be the most important factor influencing dentine bridge formation. Small densities of odontoblasts were associated with decreased dentine bridge formation. Bacterial microleakage appeared to have an effect on the density of odontoblast-like cells, as well as did the selection of pulp capping materials.

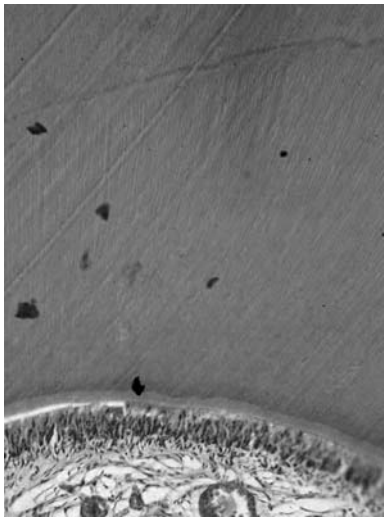


Fig.2. H.E. 40x. Microphotograph of reparative dentin formation in the group treated with hydroxylapatite. The lenght of dentine bridge formation is highly variable, but in most of the samples treated with calcium hydroxide it is significantly increased

The area of dentine bridge formation was highly variable. In many cases hard tissue bridges showed slight irregularities with diverse morphology. In all the cases dental pulp had an odontoblast-like cells layer whose cell number was lowest next to the hard tissue bridges.

Inflammatory reaction, bacteria microleakage and vascular reaction

Chronic inflammatory reaction occurred in 8 specimens of the group treated with calcium hydroxide and 5 specimens of the group treated with Hap, with variable intensity and extension. Most of the analysed teeth had a slight or moderate inflammation. The rest of the samples were characterised by an absence of inflammatory cells.

The Gram stain method showed a small number of gram-positive cocci in the dentinal tubules beneath the capping material in two cases, in the group

treated with $Ca(OH)_2$ (Figure 3) bacteria were not found in the dental pulp in not even one specimen. It could be difficult to explain whether these bacteria invaded the dentin during the capping procedure, or whether they came into dentinal tubules through the cavity margins later on, during the healing process, due to microleakage of the restorative material. Neutrophilic infiltrates or

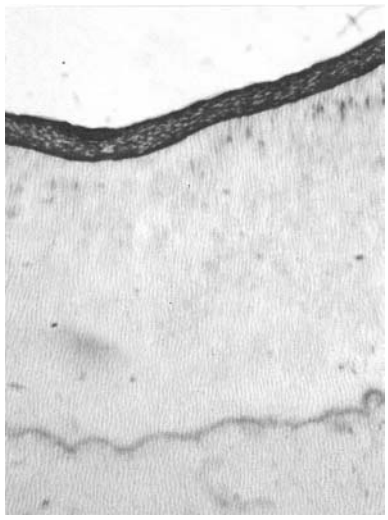


Figure 3. Gram, 40x. Microphotograph of the dental pulp capped with calcium hydroxide. Scar tissue formation with severe pulp degeneration can be observed. Small number of bacteria is located in dentinal tubules just beneath the placed material.

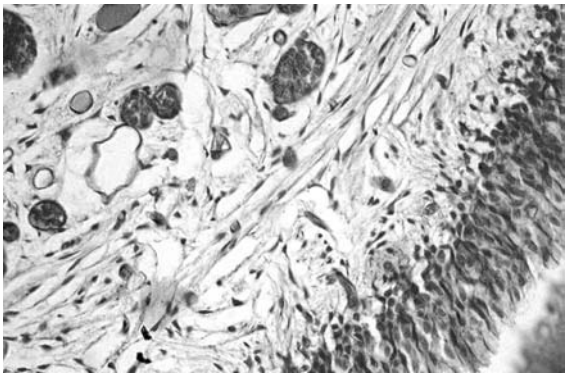
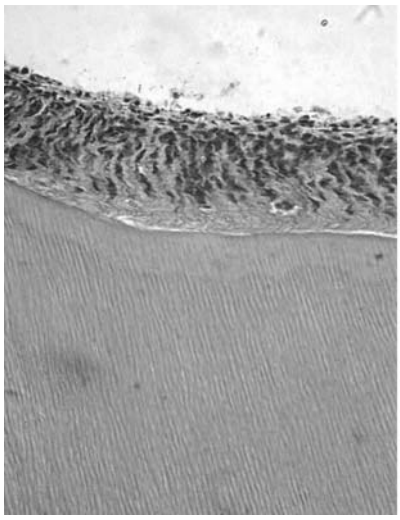


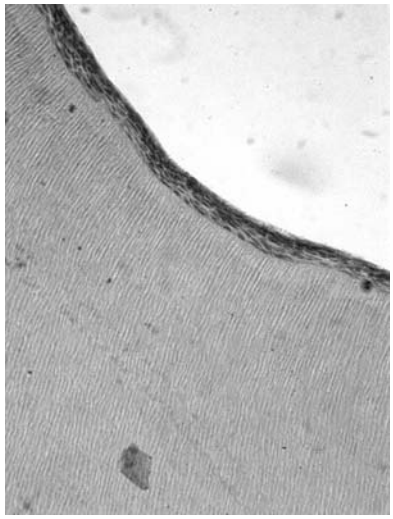
Figure 4. H.E. 100x. Microphotograph of the dental pulp capped with hydroxylapatite. Blood vessels are dilated with the signs of the stasis. This finding is probably related to the chemical irritation of the capping material and mechanical trauma during cavity preparation, rather than the presence of bacterial infection. The cells of the inflammatory infiltrate are not seen. The integrity of odontoblast-like cells is completely mantained

microabscesses were not found. Blood vessels were significantly dilated with signs of stasis in all analysed specimens in both groups (Figure 4). This finding was probably related to the chemical properties of the capping material and mechanical trauma during cavity preparation, rather than the presence of bacterial infection. The category of pulp inflammation appeared to have little effect on dentine bridge formation following pulp exposure.

Scar tissue formation and atrophic degeneration

In five specimens in the group treated with $Ca(OH)_2$, and two specimens treated with Hap reparative processes in the dentin were complicated with severe degeneration of odontoblasts, necrosis and replacement with fibroblasts. These cells secreted collagen (scar tissue), which could stay unmineralised or became mineralised (in only one case). Although mineralised scar tissue did not posses histological characteristics of normal dentin, it had the ability to close the pulp chamber. In spite of that, this could not be considered as an acceptable therapeutic result, because of the severe atrophic degeneration of the deeper layers of the dental pulp, which was observed in all the cases in which the healing process was characterised by scar tissue formation (Figures 5 and 6). It would be difficult to presume which mechanism or mechanisms were involved in such an inadequate tissue response.





Figures 5 (H.E. 100x) and 6(H.E.40x). Microphotograph of dental pulp treated with calcium hydroxide. Severe degeneration of odontoblasts, their necrosis and replacement with fibroblas (Fig. 5). These cells secrete collagen which form scar tissue, and could close the pulp chamber. Severe atrophic degeneration of dental pulp is also observed (Fig. 6.)

The presence of capping particles in pulp tissue

In both groups black particles of capping material were observed dispersed in the pulp tissue in a small number of specimens. This finding included a complete destruction of the pulp and failure of dentin bridging. It could be presumed that capping material was accidentally placed in the pulp tissue. In few cases treated with hydroxylapatite, true denticles have been observed in the pulp chamber. It would be difficult to explain why a material with strong osteoinductive properties, when placed in the pulp tissue should induce denticle formation.

Adhesion of capping material to dentine

The hydrpxyapatite provided excellent mechanical properties as capping material and contributed to better adhesion to dentine. It had satisfactory tensile strength that has been shown to eliminate completely a contraction gap between the capping material and dentine. This was not the case with groups treated with calcium hydroxide, in which a visible gap between the dentin and capping material was present in all analysed samples (Figure 7). This finding was related with sealing properties of the used material and its ability to prevent bacterial invasion.

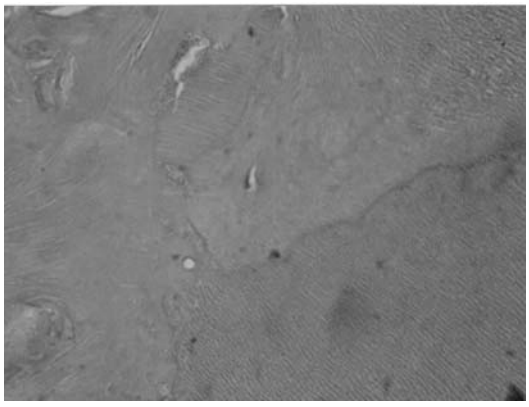


Figure 7. (H.E. 100x). Microphotograph of the dentin-hydroxylapatite interface. Intimate contact without any gaps can be observed. This finding is important from the mechanical and biological point of view. Intimate contact between dentin and hydroxylapatite prevents bacterial invasion and facilitates reparative dentinogenesis

DISCUSSION

The present study has not directly addressed the mechanisms by which odontoblast-like cell differentiation and reparative dentinogenesis take place leading to dentine bridge formation. However, this study has highlighted some aspects of the regenerative potential of the dental pulp. Murray *et al*. (2003) have reported the beginning of odontoblast-like cell differentiation and secretory

activity between 7 and 27 days. Similar time scales were observed in the pulp tissue after capping with isolated dentine matrix components, and growth factors particularly of TGF-family (Smith *et al*. 1990), (Tziafas, 1994).

This present study investigated noninfected mechanical pulpal exposures; the only source of infection was by bacterial microleakage through the cavity margins. In situations of accidental trauma and caries pulp exposure, it is likely that the pulp tissue will be infected with bacteria prior to pulp capping, and this will probably cause more severe degenerative reactions and impaired reparative responses. In the controlled conditions in this present study, it was not possible to mimic caries or accidental pulp exposure. However, it was possible to correlate the effects of the variables of pulp-capping treatments directly to the kinetics of odontoblast-like cell formation and their dentine bridge secretory activity following mechanical pulp exposure.

Calcium hydroxide is the most commonly used pulp-capping material, and it has been claimed to promote dentine bridge formation (Schroeder, 1972; 1985). However, $Ca(OH)_2$ did not appear to influence odontoblast-like density in comparison with mineral trioxide aggregate (MTA) pulp capping (Murray *et al*. 2003). This finding is in agreement with studies that have demonstrated the natural ability of the pulp to form dentine bridges under most types of pulp-capping materials, as long as a bacteriometic seal is provided (Cox *et al.* 1987). Also, in agreement with previous studies, it was observed that bacterial microleakage reduced the odontoblast-like secretion of dentine bridges. Although there are many properties that are necessary for dental materials to function adequately, from our findings, it is postulated that one of the most important properties of a pulp-capping material is its capacity to prevent bacterial microleakage.

Whilst it has been established that odontoblast-like cells require adhesion to an appropriate surface before cell differentiation and dentine bridge formation can begin (Veis, 1985), little attention has been directed towards the possible role of the cells of the subjacent reorganizing tissue. The role of these cells remains unclear, although they may well have a supporting function in relation to odontoblast-like cell activity. Approximately two cells per unit area of the subjacent reorganizing tissue, appear to be necessary to support odontoblast-like cell density per unit area beneath the dentine bridge formation (Murray *et al*. 2003).

Whether or not capping material is an irritant to dental pulp has been controversial. Studies have reported that pulpal inflammation is a consequence of irritation from the capping material itself, rather than bacterial invasion by microleakage (Brannstorm and Nyborg, 1972; White *et al*., 1994; Ebihara and Kato, 1996). Severe inflammation was not seen with any of the used materials. It has been reported that a characteristic reaction, such as a necrotic layer on the surface of the exposed pulp with application of pure calcium hydroxide, may be observed (Watts *et al*., 1994). However, in the present study Life®, did not induce the typical necrotic layer seen with pure calcium hydroxide. This observation is in agreement with previous reports (Sonoda, 1997).

Once death of odontoblasts occurred, for whatever reason, the reaction of pulp can be compared to that of skin connective tissue. As there was inflammation, leading to proliferative and secretory responses. New cells differentiate from undamaged pulpal cells and lay down collagen and form scar tissue (Gronthos, *et al*. 2002). Scar tissue can become mineralised.

The basic reparative response of dentin-pulp complex can be impaired by a number of local factors. First is the fact that it was assumed, on basis of normal embryologic development, that an epithelial factor was needed for the differentiation of odontoblasts (Smith and Lesot H, 2001). This fact, combined with the recognition that mature odontoblasts cannot divide, led to the conclusion that cells able to form hard tissue were generally unavailable in dental pulp. Second, the restorative materials do not precisely mimic the epithelium's ability to cover and seal. It is now recognized that microleakage can occur around some restorative materials, so that low-grade persistent infection modifies and delays the pulpal response.

CONCLUSION

An ideal direct pulp capping material would maintain the vitality and function of the dental pulp, form a dentine bridge, have appropriate mechanical properties and adhesion to dentine in order to prevent microleakage. Neither calcium hydroxide nor hydrxylapatite had completely satisfied these requirements, although Hap can be recognized as a superior alternative to calcium hydroxide.

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Acta Veterinaria (Beograd), Vol. 57. No. 5-6, 573-584, 2007.

HISTOLOŠKA PROCENA ODGOVORA ODONTOBLASTIMA-SLIČNIH ĆELIJA NAKON DIREKTNOG PREKRIVANJA PULPE PASA KALCIJUM HIDROKSIDOM I HIDROKSILAPATITOM

DANILOVIĆ VESNA, KRŠLJAK ELENA i LAČKOVIĆ VESNA

SADRŽAJ

Cilj ovog rada je bio ispitivanje odgovora zubne pulpe pasa nakon njenog direktnog prekrivanja hidroksilapatitom (Hap) ili kalcijum hidroksidom. U eksperimentu su korišćena 22 zuba kod kojih je nakon arteficijelnog otvaranja, zubna pulpa prekrivana Hap-om ili kalcijum-hidroksidom. Osam nedelja nakon prekrivanja, urađena je histološka analiza. Dobijeni rezultati su ukazali da je tokom arteficijelnog otvaranja pulpe došlo do ireverzibilnog oštećenja odontoblasta i njihove zamene ćelijama sličnim odontoblastima. Odontoblastima-slične ćelije stvorile su mostić reparativnog dentina u 6 uzoraka iz grupe tretirane kalcijum hidroksidom i skoro svim uzorcima tretiranih Hap-om. Uočeno je da je prisustvo odontoblastima-sličnih ćelija presudno za uspeh reparativne dentinogeneze, dok je vrsta korišćenog materijala za direktno prekrivanje, bila od manje važnosti. Pri tome ni hidroksilapatit ni kalcijum hidroksid ne zadovoljavaju sve zahteve koji se postavljaju pred idealne materijale za direktno punjenje. Hap ipak predstavlja superiorniju alternativu u odnosu na kalcijum hidroksid.