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Indirect pulp capping in young patients: immunohistological study of pulp–dentin complex

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Abstract

Indirect capping is a complex therapy exclusively needed in deep cavities that provides, using biomaterials, a disinfection of the dentinary sore and seals the dentinary tubules, protects the pulp of physical mechanisms and chemical agents and stimulates the mechanisms that produce new dentin. Following this idea, we studied the histological changes in the dental pulp tissue and also the specific immunohistochemical response in various structures when an indirect capping technique was used. We used special histological techniques followed by classical staining or by immunohistochemical reaction in order to assess the odontoblastic, and the vascular reaction. The immunohistochemical study allows us to evaluate the changes in the pulp–dentin complex, as the result of the changes in the dentinal tubules permeability and the biological reactions at this level.

Keywords: indirect capping, pulp–dentin complex, biomaterials, immunohistochemistry, cytochrome c, α-SMA.

Introduction

Morphofunctional interrelations between pulp and dentin are established in the moment of the tooth genesis and they are maintained during its entire life, and that makes the researchers consider it an entity named the pulp–dentin complex. The dental pulp, even considered an immature connective tissue, is one of the most specialized tissues in the organism and it assure the dentin synthesis, and mineralization and its lifespan starting with the odontogenesis, before the eruption of the tooth and continuing after the tooth eruption. The process of mineralization slows during the functional period of the tooth. When a noxious factor affects the most specialized cell in the dental pulp, the odontoblast, the dental pulp tissue reacts with a series of local changes that are directing to protect its vitality, meaning reactionary dentin deposits [1, 2].

The protective action of the dental pulp is possible due to its complex structure of cells (mesenchymal, undifferentiated cells – named Hohl cells, fibroblasts, histiocytes, odontoblasts), connective fibers (various collagen, mainly type I, but also type III, IV and VIII and oxytalan fibers – a variety of elastic fibers), ground substance, a rich blood and lymphatic vessels supply, somatic an vegetative nervous fibers.

During pathological conditions, such as a carious lesion or a coronal filling, all this structural elements suffer a certain level of injury [3, 4].

In our study, we emphasize the general alterations in the pulp tissue organization but also the specific changes of the main vital structures – vessels and nerve fibers – when using indirect capping techniques.

In order to label the odontoblast cells, we used an anti-S-100 protein antibody. Anti-vimentin antibody has

been used to label pulp cells with mesenchymal origin and anti-α-smooth muscle actin (α-SMA) in order to label the smooth muscle fibers from the vessel walls.

We gave a special attention to the immunolabeling of apoptotic phenomena using the anti-cytochrome c antibody, according the findings that its release from the mitochondria into the cytosol is a required step for apoptosis activation [5, 6].

Materials and Methods

Materials

We treated by indirect capping technique 24 first and second premolars, both maxillary and mandibulary from 14 healthy patients, aged 9–16-year-old. The teeth were to be extracted for orthodontic reasons. On these teeth, we prepared deep cavities following the general preparation rules for cavities, then we applied calcium hydroxide materials (Dycal, Cacidor), zinc oxide eugenol, glass ionomers (Fuji II LC, Vitremer) and dentinary adhesives (Scotchbond, Prime & Bond, Gluma Bond, Excite).

Methods

All teeth were washed in saline solution and fixed in 4% paraformaldehyde for 10 days. The whole tooth was decalcified in 4 M formic acid solution for 10–20 days. Immediately after, the piece was sectioned in sagittal plane and decalcified for another week. We determined the end point of decalcification using mechanical methods. After decalcification, the tissue was hydrated and processed for paraffin embedding. Paraffin embedding was made following the standard procedure. Five μm serial paraffin sections were mounted on poly-lysinated slides. We made usual Hematoxylin–Eosin and trichromic staining.

Immunohistochemistry

Serial sections were dewaxed and incubated for 30 minutes with 0.3% hydrogen peroxide in methanol in order to block the endogenous peroxidase. Blocking of non-specific binding was made with non-immune serum 1:75 diluted in PBS for 20 minutes at room temperature. Sections were incubated with the primary antibodies listed in Table 1 in a moist chamber for 12 hours at -4^{0}C. Table 1 contains the description of primary antibodies used in our experiment.

Table 1 – ***Antibodies used in the immunohistochemical study***

Antibody	Dilution	Source
Anti-S-100[1]	1:200	Dako, code A5114
Anti-α-SMA[2]	1:100	Dako, code M0851
Anti-cytochrome c[1]	1:50	Santa Cruz Biotechnology Inc., code H-104

[1]Rabbit polyclonal antibody; [2]Mouse monoclonal antibody.

After excess reagent removing, sections were further incubated with biotinylated secondary antibody (goat anti-rabbit for polyclonal antibodies and horse anti-mouse secondary antibody for monoclonal primary antibodies) diluted 1:200 for 30 minutes. After washing with PBS, further incubation was carried out with Avidin–Biotin-peroxidase complex (Vector Laboratory) 1:100 for 60 minutes. Peroxidase activity was revealed with 3,3'-diaminobenzidine (Sigma Chemical Co.). The sections were counterstained with Mayer's Hematoxylin and mounted in Eukit.

Controls. In control sections, the primary antibody was replaced by non-immune serum. Known positive tissue controls were used for S-100 and α-SMA.

Results

According to the data registered during the clinical examination and the histological asses, we emphasize the morphopathological and clinical aspects of each tooth from this study in different periods of time after we applied the different materials and techniques of indirect capping.

During this study, we noticed some agreements but also disagreements between the clinical subjective and objective signs on one hand and the morphological aspects of the dentin and dental pulp on the other.

The indirect capping was made with zinc eugenate, calcium hydroxide and glass ionomers materials and they did not clinically induce any severe inflammatory reaction, no matter how much time passed since we made the treatment. The good clinical results (with no signs of inflammation or dentin sensitivity) that we obtain following the indirect capping technique by using zinc eugenate, calcium hydroxide and glass ionomers materials are in a complete correspondence with normal morphological aspects of the pulp and dentin, and these aspects can be seen on usual histological sections and during the immunohistochemical study.

For these groups of teeth, the pulp tissue presents a complete odontoblastic layer, with a normal Weil zone, a zone rich in fibroblasts and a central pulp with normal characteristics. The odontoblasts were regularly arranged in 3–4 layers, next to the cavity (Figure 1).

We did not notice any significant odontoblast loss. This is why we used them as control groups, being able to evaluate the histological process of the samples and to identify the efficiency of the fixation, decalcification and the marking agent that we used.

The odontoblasts situated in a proper alkaline environment immediately begin to synthesize a dentinary matrix that will be materialized due to the calcium ions influx from the obturation level. The dental pulp ensures the increased necessities of oxygen and of mineral substances belonging to active odontoblasts, by increasing the vascularization at this level (Figure 2).

For the teeth treated with dentinal adhesives, a pre-inflammatory hyperemia was clinically diagnosed in the first weeks after the treatment and this hyperemia had signs of pulpal inflammation and dentinal hypersensitivity. From a histological point of view, these cases were not accompanied by destructions of the odontoblastic layer, but only a few slightly swollen cells with a vascular picture marking an intense hyperemia (Figure 3).

The important plasmocyte infiltration makes the Weil zone no different from the rest of the pulp, so the layering of the normal pulp became scarce.

The teeth treated with dentinal adhesives manifest an increased clinical inflammatory phenomenon. After three

months, we found in the pulp a disorganized odontoblast layer and an advanced state of suffering for the cells. They presented cytoplasmatic vacuolisations, lipidic loadings of the cytoplasm and small nuclei. The vessels were dilated and congested, with a remarkable agglomeration of white sanguine elements forming obturating plugs (Figure 4).

The nervous threads are totally destroyed at the level of inflammatory center. The collagen matrix is totally compromised and suppurating micro-foci are present in the entire pulp. Above the dental pulp, we notice a predentinal area, a regular dentin layer and a zone that presents disorganized dentinal tubules and fragmented odontoblasts process.

In our immunohistochemical study, we used *anti-S-100 protein* in order to mark odontoblasts cells.

We observe that constantly during the study, the S-100 protein marked positively the nerves of the pulp. This is why it was considered a marker for internal control of positive reaction for protein S-100, obtained for different components of the pulp–dentin complex (Figure 5).

The S-100 antibody marked constantly and highly positive both the entire body of the cell and the cytoplasm of the odontoblasts but also sometime their nucleus. These positive answers to protein S-100 antibody were noticed in cases with *indirect capping with calcium hydroxide based materials, zinc oxide eugenol and glass ionomers* that determined favorable reactions of the pulp and odontoblasts around the cavity.

The walls of the blood vessels were marked with anti-α-SMA (Figure 6). We noticed a rich vascular supply made mainly of capillary, but also veins and arteriole in the central pulp. Pulp vessels showed characteristic feature for an active process of new dentin genesis. The vessels dilatation is present on the entire dental pulp tissue.

Indirect capping using *dentinal adhesives* determined, in many cases, severe or moderate inflammatory reactions, proving the existence of a certain degree of pulpal toxicity of these materials.

Severe inflammatory responses obtained in indirect capping by hybridization made us pursue a more careful investigation of the cellular outcome in the pulp–dentin complex. In order to assess the response of various cells populations to the chemical stress, we used the *cytochrome c* as an apoptotic marker.

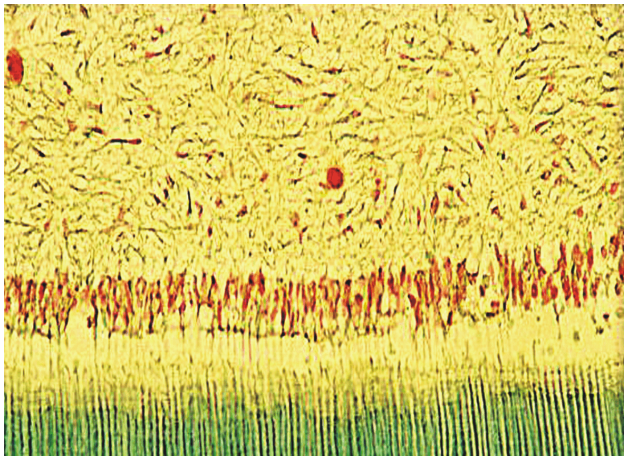

Figure 1 – ***Pulp–dentin complex: general representation. We notice the dentin, predentin, and odontoblast layer at the periphery of the pulp; in Weil zone, fewer cells and in the central pulp a high cellular density. Trichromic staining, ob. ×20.***

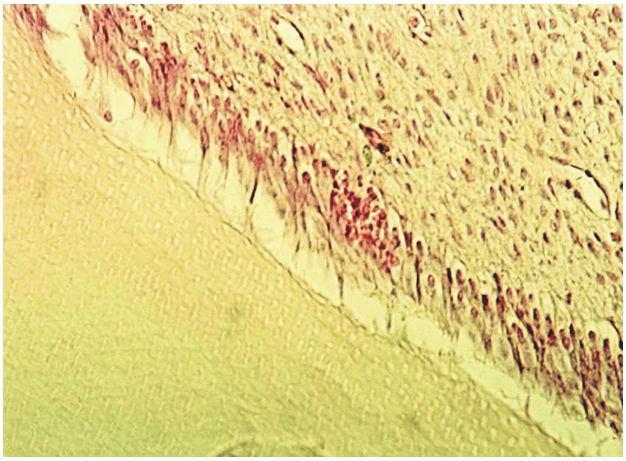

Figure 2 – ***Pulp–dentin complex. Note the richness in cells and vessel in the superficial dental pulp layer. Among the odontoblasts, many dilated capillaries. HE staining, ob. ×20.***

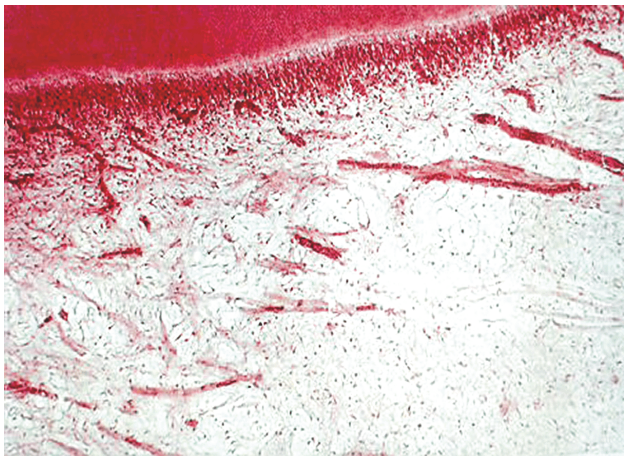

Figure 3 – ***Pulp-dentin complex. We notice the dentin, predentin, odontoblastic layer and pulpal tissue rich cells. An intense vascular hyperemia, intercellular edema and scarce collagen fibers. HE staining, ob. ×10.***

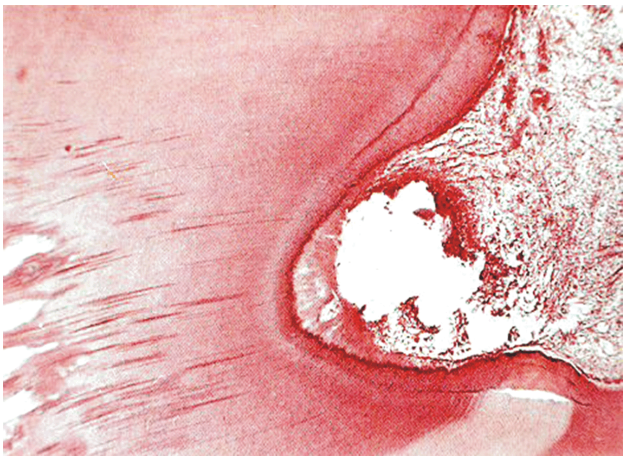

Figure 4 – ***Localized suppurated pulpitis with almost complete disorganization of the cell layers. HE staining, ob. ×10.***

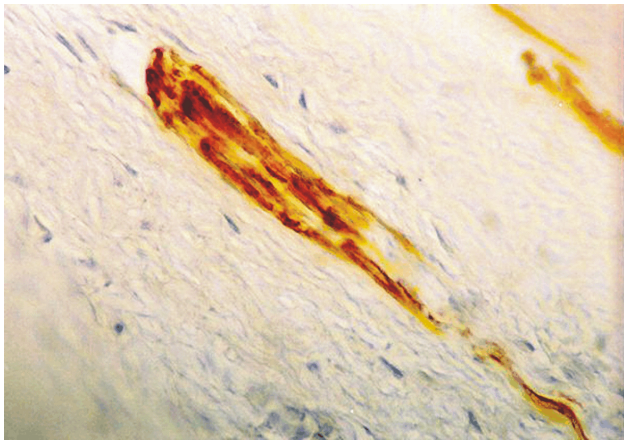

Figure 5 – ***An intense positive reaction for S-100 antibody in central pulp nervous fibers, ob. ×40.***

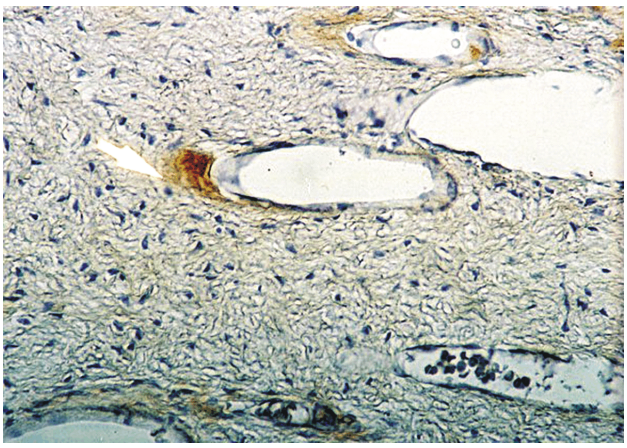

Figure 6 – ***Positive reaction for α-SMA in vascular cells but absent in lymphatic vessel, ob. ×20.***

Positive reactions for cytocrome c were found in the odontoblast layer as well as in some pulpal cells, especially myocytes that were marked with α-SMA in serial sections. We found a more intense expression of cytochrome c in the cells of the blood vessels walls than cytoplasm of the odontoblasts (Figure 7).

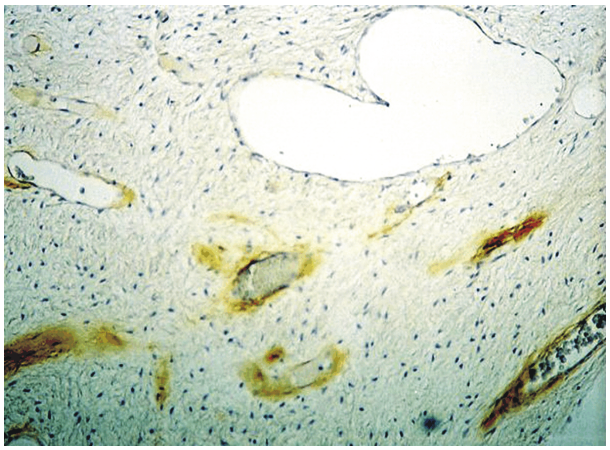

Figure 7 – ***Scarce positive reaction for cytochrome c in some cells of the vascular wall and in few mesenchymal cells, ob. ×20.***

Myocytes were not constantly marked for cytochrome c in all vessels, but more frequently, the immunoreactions were present in the capillary endothelium.

Some mesenchymal cells (namely fibroblasts) of the pulp present also in inconstant reaction for cytochrome c (Figure 7). Nervous fibbers of the dental pulp, positive for S-100 antibody as we notice in serial sections, display a scarce and inconstant positivity for cytochrome c.

Discussion

We assess a correlation between the clinical response and the morphologic one to the various substances used in indirect capping. We used the antibody against S-100 protein, known as a marker for cells originated from the neural crests, for glial cells from the central nervous system [7] and for Schwann cells from the peripheral nervous system [8], in order to label the odontoblast layer. Origin of the odontoblast cells in the neural crests was sustained by the fact that these cells are specific labeled with S-100 antibody and HNK (Leu7) [9, 10]. Recently, for S-100 protein is mentioned its involvement in different biological functions, like cell proliferation, apoptosis, cell motility, exocytosis, cell cytoskeleton organization [11]. In our study, the positivity of S-100 protein was high at the level of the nerves in the pulp, being used as an internal control marker.

Following the application of indirect capping with biomaterials (based on calcium hydroxide, zinc oxide eugenol and glass ionomers), we noticed that the odontoblasts were marked constantly and high positively with S-100 antibody. This is why we considerate that stimulated odontoblasts in a proper alkaline environment immediately start to synthesize a dentinary matrix, which will be mineralized due to the calcium ions at the obturation level.

The increase of the positive reaction intensity to S-100 protein for both the cell body and the cytoplasmatic processes of the odontoblasts involved in the new dentin regeneration, supports more our idea of the favorable role played by biomaterials over the dental pulp [12].

Histological comparisons of $Ca(OH)_2$ and resin pulp capping in a recent report by study tend to support resin adhesives as an alternative to calcium hydroxide [13, 14].

Treatment for this group of teeth should be oriented toward preserving pulpal vitality in order to return these injured teeth to acceptable normal function, appearance, and repair for better prognosis and prolonged tooth retention [1, 3, 15].

Dental pulp ensures also the higher oxygen necessities and mineral substances of the active odontoblasts by increasing the blood flow at this level. This is how we explain *the dilated blood vessels* in certain teeth from our study, in the absence of inflammatory response.

The walls of the blood vessels have been marked with α-SMA antibody, which has a positive reaction for actin of the cells laying the vessels walls [16, 17].

Indirect capping with dentinal adhesives frequently determines intense or moderate inflammatory pulpal reactions, so we think they have a high level of pulpal toxicity. The consequent phenomena in the pulp–dentin complex have been followed using immunohistochemical labeling against cytochrome c, considered an early apoptotic marker [18, 19].

Studies have proved the normal position of cytochrome c, a transporter of electrons, between mitochondrial membranes

of the cell [5, 6]. From this level, cytochrome c is released into the cytosol due to the changes of the permeability in the mitochondrial membrane under the influence of some external factors that stimulate the activity of the complex transition orifice of permeability. Once moved in the cytoplasm, the cytochrome c starts the caspase cascade, which leads finally to the cell death [5, 6, 20].

Examining the bad results obtained at capping, using hybridization with dentinary adhesives, it might be suggested that cytotoxic molecules of the dentinary adhesives act over the cells in the vessel walls and promote the release of cytochrome c, finally leading to the death of those cells.

The blood vessels are branching at the odontoblast layer in a rich capillary network, where the bulk of the metabolic exchanges take place. We notice that the cells from the walls of the blood vessels are most sensitive for the toxicity of the substances used. Consecutively, we can affirm that the odontoblast death is mainly the result of the indirect lack of oxygen and nutritional substances following the blood vessels disruption, more than a consequence of direct action of cytotoxic molecules from the dentinary adhesives.

The intense reaction to cytochrome c on pulp fibroblast explains the reduced quantity of reparatory dentin. The pulpal elements, once destroyed, could not be differentiated in odontoblasts anymore, reducing the capacity of osteo-dentin production. The lack of oxygen and the low nutritional contribution affected all them.

If the quantity of diffused cytotoxic substances is too high, as it happened when dentinary adhesives were used, the edema and the rise of blood thickness can compromise the remove of these materials. The danger occurs when these noxious substances get into the systemic blood circulation or endanger the maintenance of the tooth vitality.

These results occur, probably, because the local barriers of the pulp are broken.

The fact that the nervous fibers do not show signs of apoptosis phenomena, might represent a morphologic base of the recent studies which sustain that under the influence of some irritating factors, the activity of the nerves increases to get the pain and control the blood circulation.

If the fibers are over stimulated, the active peptide secretion will start, determining a vasodilatation of the blood vessels and an increase of capillary permeability. The pressure of the intercellular fluids rises. This leads to the collapse of the blood vessels, necrosis of the entire pulp tissue, also destroying the nervous fibers [21].

The fact that the marked structures for the S-100 protein do not have a positive reaction for the cytochrome c too, sustains the theory that S-100 protein could be an anti-apoptotic factor [7, 8].

When we analyze the deposition of secretory dentin, as well as the multiple ways of protection of the dental pulp, we conclude that we have to keep finding new materials and treatment methods that can value all the biological resources of these tissues.

It is very important to keep in mind that there are many facts that can have an influence on the pulp reorganization process [22], and their careful consideration is necessary for the optimum utilization of the regenerative ability of the dentin to corroborate the biological state of the tooth with the clinical situation of the dental diseases [23].

Conclusions

Due to their particular structure and organization in the dental pulp, the blood vessels are the first components of the pulp tissue responsible of the unbalance determined by indirect capping treatment. They constantly manifest apoptotic phenomena, emphasized by the cytochrome c expression when the tooth is injured by the dentinary adhesives toxicity. The immunohistochemical results corroborated with moderate and severe inflammatory reactions demonstrate that dentinary adhesives show high toxic potential, so we are allowed to not consider them as biomaterials. We can conclude that associating an intermediary biomaterial that protects and stimulates the vital functions of the pulp, the dentinar adhesive can successfully rice to the physiological repair of the pulp–dentin complex.

Contribution Note

All authors have equal contributions to the study and the publication.

References

[1] Abu-Tahun I, Torabinejad M, *Management of teeth with vital pulps and open apices*, Endod Topics, 2010, 23(1):79–104.

[2] Hilton TJ, *Keys to clinical success with pulp capping: a review of the literature*, Oper Dent, 2009, 34(5):615–625.

[3] Bentley K, Janyavula S, Cakir D, Beck P, Ramp L, Burgess J, *Mechanical and physical properties of vital pulp therapy materials*, J Dent Res, 2012, 91:Abstract No. 258.

[4] Schmalz G, Galler KM, *Tissue injury and pulp regeneration*, J Dent Res, 2011, 90(7):828–829.

[5] Liu X, Kim CN, Yang J, Jemmerson R, Wang X, *Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c*, Cell, 1996, 86(1):147–157.

[6] Skulachev VP, *Cytochrome c in the apoptotic and antioxidant cascades*, FEBS Lett, 1998, 423(3):275–280.

[7] Hachisuka H, Mori O, Sakamoto F, Sasai Y, Nomura H, *Immunohistological demonstration of S-100 protein in the cutaneous nervous system*, Anat Rec, 1984, 210(4):639–646.

[8] Stefansson K, Wollmann RL, Moore BW, *Distribution of S-100 protein outside the central nervous system*, Brain Res, 1982, 234(2):309–317.

[9] Le Douarin N, *The neural crest*, Cambridge University Press, Cambridge, 1982.

[10] Lumsden AGS, *Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ*, Development, 1988, 103(Suppl):155–169.

[11] Bronckart Y, Decaestecker C, Nagy N, Harper L, Schäfer BW, Salmon I, Pochet R, Kiss R, Heizman CW, *Development and progression of malignancy in human colon tissues are correlated with expression of specific Ca(2+)-binding S100 proteins*, Histol Histopathol, 2001, 16(3):707–712.

[12] Tziafas D, Belibasakis G, Veis A, Papadimitriou S, *Dentin regeneration in vital pulp therapy: design principles*, Adv Dent Res, 2001, 15:96–100.

[13] Briso AL, Rahal V, Mestrener SR, Dezan Junior E, *Biological response of pulps submitted to different capping materials*, Braz Oral Res, 2006, 20(3):219–225.

[14] Büyükgüral B, Cehreli ZC, *Effect of different adhesive protocols vs calcium hydroxide on primary tooth pulp with different remaining dentin thicknesses: 24-month results*, Clin Oral Investig, 2008, 12(1):91–96.

[15] Maltz M, Oliveira EF, Fontanella V, Carminatti G, *Deep caries lesions after incomplete dentine caries removal: 40-month follow-up study*, Caries Res, 2007, 41(6):493–496.

[16] Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C, *Alpha-smooth muscle actin expression upregulates fibroblast contractile activity*, Mol Biol Cell, 2001, 12(9):2730–2741.
[17] Sappino AP, Schürch W, Gabbiani G, *Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations*, Lab Invest, 1990, 63(2):144–161.
[18] Jacotot E, Ferri KF, Kroemer G, *Apoptosis and cell cycle: distinct checkpoints with overlapping upstream control*, Pathol Biol (Paris), 2000, 48(3):271–279.
[19] Willingham MC, *Cytochemical methods for the detection of apoptosis*, J Histochem Cytochem, 1999, 47(9):1101–1110.
[20] Skulachev VP, *Why are mitochondria involved in apoptosis? Permeability transition pores and apoptosis as selective mechanisms to eliminate superoxide-producing mitochondria and cell*, FEBS Lett, 1996, 397(1):7–10.
[21] About I, Mitsiadis TA, *Molecular aspects of tooth pathogenesis and repair: in vivo and in vitro models*, Adv Dent Res, 2001, 15:59–62.
[22] Jontell M, Okiji T, Dahlgren U, Bergenholtz G, *Immune defense mechanisms of the dental pulp*, Crit Rev Oral Biol Med, 1998, 9(2):179–200.
[23] Duque C, Hebling J, Smith AJ, Giro EM, Oliveira MF, de Souza Costa CA, *Reactionary dentinogenesis after applying restorative materials and bioactive dentin matrix molecules as liners in deep cavities prepared in nonhuman primate teeth*, J Oral Rehab, 2006, 33():452–461.

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