The influence of haemostatic agents on healing of healthy human dental pulp tissue capped with calcium hydroxide

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Abstract

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Aim To investigate the hypothesis that different haemostatic agents could impair the histological response of human pulps capped with calcium hydroxide.

Methodology Forty-five third molars scheduled for extraction were selected. Class I cavities with pulp exposures were prepared. Three agents were used to control bleeding: 0.9% saline solution (control, $n = 14$), 5.25% sodium hypochlorite ($n = 16$) and 2% chlorhexidine digluconate ($n = 15$). The pulps were dressed with hard-setting calcium hydroxide cement. After 7, 30 or 90 days, teeth were extracted, formalin-fixed and prepared for histochemical techniques. The biological response was categorized using the following criteria: inflammatory response, soft tissue organization, reactionary dentine and reparative dentine. Data were submitted to statistical analysis, using nonparametric Kruskal–Wallis one-way analysis of variance on ranks. Differences amongst groups were detected using Dunn's method.

Results The statistical analysis disclosed that whilst inflammatory response decreased over time, reactionary dentine deposition and reparative dentine formation increased in the latter periods of evaluation ($P < 0.05$). The three agents had similar performances for all criteria evaluated. The conventional pulp response to calcium hydroxide was observed over time, and complete pulp healing was observed in 88% of the specimens after 90 days.

Conclusion The three haemostatic agents did not impair the healing process following pulp exposure and capping with calcium hydroxide at different time intervals investigated.

Keywords: biocompatibility, calcium hydroxide, haemostatic agents, pulp healing.

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Introduction

The maintenance of pulp vitality has been a challenge for restorative dentistry (Smith *et al.* 2002), yet it is essential for the preservation of the pulp–dentine complex. In the last few decades, vital pulp therapies have been developed; however, the long-term clinical evaluation of teeth with capped pulps has demonstrated a significant reduction in the maintenance of vitality (Barthel *et al.* 2000).

Several factors influence the outcome of pulp capping, amongst them the effective control of bleeding, which is necessary to improve the favourable prognosis of conservative vital therapy (Stanley 1989). An ideal haemostatic agent should be bacteriostatic and/or a bactericide, when used in pulp exposures created by caries removal. If the control of bleeding fails, the development of a blood clot between the capping material and pulp tissue would prevent intimate contact, favouring the persistence of a chronic inflammatory

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response whilst impairing the healing process (Schroder 1973, 1985, Stanley 1989, Cox *et al.* 1998, 1999, Costa *et al.* 2001).

Few studies have investigated the influence of haemostatic agents on the healing process. One of the most common agents to control pulp bleeding is saline solution. However, this substance might not be effective in some situations (Stanley 1989). The application of sodium hypochlorite (NaOCl) has been considered successful in adhesive pulp capping and has shown biocompatibility when used as a haemostatic agent (Cox *et al.* 1998, Costa *et al.* 2001, Hafez *et al.* 2002). However, a severe cytotoxic effect has been observed in cell cultures with sodium hypochlorite, even in low concentrations (Heling *et al.* 2001). The biocompatibility of chlorhexidine digluconate has not been determined completely (Thomas *et al.* 1995). Disastrous results were obtained in adhesive capping in monkey pulps using 2% chlorhexidine as the haemostatic agent (Pameijer & Stanley 1998). Conversely, Horsted-Bindslev *et al.* (2003) observed only mild inflammatory reactions after application of 0.2% chlorhexidine digluconate in human pulps.

The null hypothesis of this study was that different haemostatic agents do not impair the healing process of human pulps capped with calcium hydroxide. Thus, the study evaluated the interference of three haemostatic solutions on the repair process (0.9% saline solution, 5.25% sodium hypochlorite or 2% chlorhexidine digluconate) used prior to calcium hydroxide application.

Materials and methods

Experimental design and direct pulp-capping procedures

The research protocol was approved by the Ethics Committee, Federal University of Pelotas (no. 048/2002). All patients provided written consent to participate in the study. Forty-five human maxillary third molars scheduled for extraction for orthodontic reasons were selected. Teeth were from 14 males (54%) and 12 females (46%), with a mean age of 23 (±2.59) years. Local anaesthesia was administrated (Mepivicaine 2% with 1 : 100,000 epinephrine – DFL Indústria e Comércio Ltda, Rio de Janeiro, RJ, Brazil). The operative and restorative procedures were carried out under rubber dam isolation. Prophylaxis was performed with rubber cups and pumice.

Class I cavities were prepared, using a round diamond bur no. 1014 (KG Sorensen Ind. & Com. Ltda, São Paulo, SP, Brazil) to remove enamel and a carbide bur no. 330 (KG Sorensen) for dentine removal. Burs were used in high-speed handpieces, under copious air/distilled water cooling, and were replaced after each cavity preparation. The cavity floor was deepened until pulp exposure. The active point of the bur determined the size of the exposure (0.08 mm). The procedures were performed to avoid pulp tissue damage by bur intrusion, which could impair healing and also affect the histological analysis.

The cavities were randomly divided into three groups, based on different agents used to treat the pulp exposure: 0.9% saline solution (control); 2% chlorhexidine digluconate or 5.25% sodium hypochlorite (Table 1). A randomization table was used to define the agent, so that each patient had one of the treatments distributed by chance, and necessary adjustments were made to assure an equal treatment distribution. In those cases where the patient provided more than one tooth, different agents were applied in the same individual. Soon after pulp exposure, a sterilized cotton pellet soaked in the appropriate solution, based on the group, was applied with light pressure for 30 s. The cavity was then gently dried with absorbent paper and sterilized cotton pellets.

Hard-setting calcium hydroxide cement – Dycal Advanced Formula II® (Dentsply, Petrópolis, RJ, Brazil) was used as the capping material. Following the capping procedure, the cavity preparation was conditioned with 35% phosphoric acid for 30 s in enamel and 15 s in dentine. Two coats of a one-bottle adhesive system (Single Bond; 3M ESPE, St Paul, MN, USA) were applied and photo-activated for 20 s. The cavities were incrementally filled with a microhybrid composite resin

Table 1 Representation of the study design

	Number of specimen (*n*)/days			
Group	7 days	30 days	90 days	Total specimens (*n*)
Saline solution	4	5	5	14
Sodium hypochlorite	5	5	6	16
Chlorhexidine digluconate	5	5	5	15
Total specimens (*n*)	14	15	16	45

Saline solution (control group): 0.9% sodium chloride – Áster Medical Products, SP, Brazil (batch 0944/08/T); 5.25% sodium hypochlorite: Uso Indicado – pharmacy of handling (certificate of quality ISO 9002); 2% chlorhexidine digluconate: FGM Dentistry Products, SC, Brazil (batch 30381).

(Filtek Z250; 3M ESPE). Each increment was photoactivated for 20 s. A XL 3000 (3M ESPE) light curing unit was used during the study, with an energy >450 mW cm^{-2}. After rubber dam removal, the occlusion was checked to remove premature occlusal contacts, which could lead to additional mechanical injury and interfere with the healing process. One experienced operator performed all the operative and restorative procedures.

Histopathological examination

According to International Standardization Organization (ISO) 7405 guidelines (ISO-7405 1997), the teeth were extracted after 7, 30 or 90 days (Table 1). The apices were sectioned with a diamond bur, under air-water cooling. The teeth were fixed in 10% formalin solution for 48 h and then demineralized in 20% formic acid. Specimens were paraffin-embedded and serially sectioned (4 μm). Serial sections were stained by haematoxylin and eosin (HE) to observe the healing process or the Brown & Hopps (1973) technique to detect the presence of bacteria. Histological sections were evaluated using light microscopy based on the criteria shown in Table 2.

Statistical evaluation

Each criterion for each specimen was determined and the results were submitted to statistical analysis, using the software SigmaStat for windows 3.0 (SPSS Inc., Chicago, IL, USA). The confidence level was set at 95% ($P < 0.05$) and the data were evaluated using non-parametric Kruskal–Wallis one-way analysis of variance on ranks. Differences amongst groups were detected using Dunn's method.

Results

Morphological analysis

Seven-day follow up

• *Group I* ($n = 4$) – *control, 0.9% saline solution* (Fig. 1a): no inflammatory infiltrate was observed. Frequently, when evaluating the tissue organization, areas of haemorrhage could be seen. Some samples showed tissue loss at the exposure site with a thin underlying necrotic layer. In other specimens, the tissue loss was larger. Neither reactionary dentine nor dentine barrier formation was detected at the exposure site.

Table 2 Scores attributed for the levels of the histological criteria evaluated (ISO 7405 and adapted from Akimoto *et al.* 1998)

	Criteria for the morphological analysis			
Scores	Inflammatory response	Soft tissue organization	Reactionary dentine	Reparative dentine
1	None: the pulp contained few inflammatory cells or an absence of inflammatory cells associated with cut tubules of the cavity floor	Normal: there was no injury, disruption or loss of cell survival	None: no evidence of additional dentine deposition at the injury site	None: no dentine barrier formation
2	Mild: the pulp had localized inflammatory cell lesions predominated by polymorphonuclear leucocytes or mononuclear lymphocytes	Mild: there was a superficial loss of cell survival at the site of injury	Mild: there was a mild increase in dentine deposition, constituted by a thin layer produced by the original odontoblasts	Mild: there was some dentine deposition by the odontoblast-like cells at the exposure site, in focal areas
3	Moderate: the pulp had polymorphonuclear leucocytes lesions involving more than one-third of the coronal pulp	Extense: there was an extensive loss of cell survival involving more superficial cells	Intense: there was deposition of a thick and uniform layer of reactionary dentine	Intense: there was uniform dentine formation by the odontoblast-like cells at the exposure site
4	Severe: the pulp tissue was largely necrotic, following chronic inflammatory cell injury	–	–	–

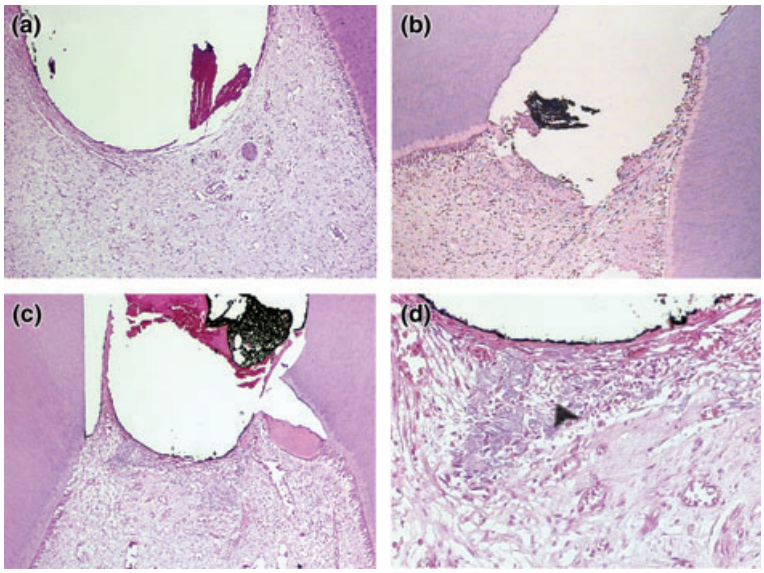

Figure 1 Seven-day follow-up. (a) 0.9% SS group. Loss of pulp tissue is observed, which is covered by a thin necrotic layer. Neither inflammatory infiltrate nor reactionary and reparative dentine was observed. (b) 5.25% SH group. A small loss of soft tissue, restricted to the exposure site, and mild chronic inflammatory infiltrate were observed adjacent to this area. There was no evidence of any reactionary dentine or dentine barrier formation. (c) 2% CD group. Exposure area demonstrated small pulp tissue retraction and presence of inflammatory cells. (d) Magnification of c (×100). The arrow-head highlights the focus of the inflammatory infiltrate. *SS*, Saline solution; *SH*, sodium hypochlorite; *CD*, chlorhexidine digluconate. Haematoxylin–eosin stain, original magnification ×25 (a, b and c) and ×100 (d).

• *Group II* ($n = 5$) – *5.25% sodium hypochlorite* (Fig. 1b): Generally, mild chronic inflammatory infiltrate was observed adjacent to the pulp exposure. Haemorrhage was also noted in the pulp tissue. Almost the entire pulp showed normal morphology, exhibiting loss of soft tissue organization restricted to the exposed pulp area. There was no evidence of any reactionary dentine or dentine barrier formation.

• *Group III* ($n = 5$): *2% chlorhexidine digluconate* [Fig. 1(c, d)]: Frequently, the samples treated with this solution demonstrated similar inflammatory pulp response and soft tissue organization to 5.25% sodium hypochlorite. Similarly, no reactionary dentine or dentine barrier was observed.

Thirty-day follow up

• *Group I* ($n = 5$) – *control, 0.9% saline solution* [Fig. 2(a, b)]: Variable histological findings were observed in the control group when the inflammatory cell response and reactionary dentine deposition/dentine barrier formation criteria were analysed. Some samples exhibited no inflammatory cells or osteodentine barrier. Other specimens showed a moderate inflammatory response associated with superficial necrosis at the exposure site, either without dentine barrier formation or with some reparative dentine deposition. A small loss of pulp tissue was noted in all samples.

• *Group II* ($n = 5$) – *5.25% sodium hypochlorite* [Fig. 2(c, d)]: No inflammatory cell infiltrate was observed. Frequently, a dentine barrier was formed by thin irregular nontubular dentine with cellular inclusions. However, one case showed reparative tubular dentine with a well-established odontoblastic layer under it. Generally, a small, thin rim of new reactionary dentine was observed.

• *Group III* ($n = 5$): *2% chlorhexidine digluconate* [Fig. 2(e, f)]: Inflammatory cell response was usually absent, although two cases demonstrated scattered mononuclear cells throughout the pulp tissue. A thicker dentine barrier than that formed with 0.9% saline solution or 5.25% sodium hypochlorite was observed. Additionally, initial tubular dentine barrier was observed under nontubular dentine in some cases. A thin layer of reactionary dentine was frequently present along adjacent walls of cavity preparation. Little pulp tissue was lost at the exposure site.

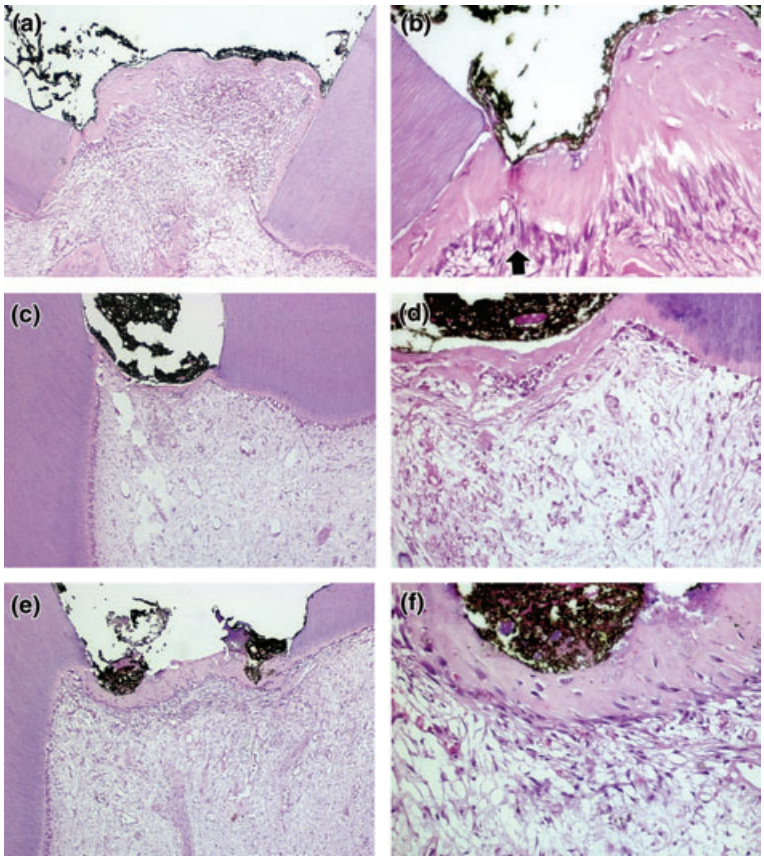

Figure 2 Thirty-day follow up. (a) 0.9% SS group. Dentine barrier formation can be observed at the exposure interface, which showed foci of haemorrhage. (b) In evidence (from a), the odontoblast-like cells aligned under reparative dentine (arrow). (c) 5.25% SH group. A thin, nontubular dentinal barrier was formed. Small loss of tissue was present at the exposure site. (d) Specimen treated with 5.25% SH group exhibiting dentine barrier continuous with the reactionary dentine. (e) 2% CD group. Thick osteodentine barrier was present. No inflammatory cells were observed. (f) In higher magnification (from e), dentine barrier. *SS*, saline solution; *SH*, sodium hypochlorite; *CD*, chlorhexidine digluconate. Haematoxylin–eosin stain, original magnification ×25 (a, c and e) and ×100 (b, d and f).

Ninety-day follow up

• *Group I* ($n = 5$) – *control, 0.9% saline solution* [Fig. 3(a, b)]: Inflammatory response was always accompanied by a loss of pulp tissue limited to the exposure area. Variable degrees of dentine barrier formation were observed. Fibrodentine/osteodentine was mainly present and two specimens exhibited a tubular dentine barrier with odontoblast-like cells aligned beneath. Reactionary dentine was observed in all specimens, as a thick and uniform deposition in the adjacent walls of cavity exposure.

• *Group II* ($n = 6$) – *5.25% sodium hypochlorite* [Fig. 3(c, d)]: Inflammatory cell response was not present in any specimen. Dentine barrier formation was always observed as a continuous hard tissue composed of an osteodentine outer layer and an inner layer of tubular dentine, with odontoblast-like cells aligned at the pulp tissue interface. A small loss of pulp tissue was noted, just near to the exposure site. In relation to reactionary dentine formation, a thick layer was observed in all cases.

• *Group III* ($n = 5$): *2% chlorhexidine digluconate* (Fig. 3e). Only one case, in which the dentine barrier was not formed, demonstrated the presence of a few scattered inflammatory cell responses at the exposure interface. In the remaining specimens, at this interval, thick dentine barriers were found with odontoblast-like cells subjacent to them. Soft tissue formation and reactionary dentine findings were closely related to those observed for sodium hypochlorite.

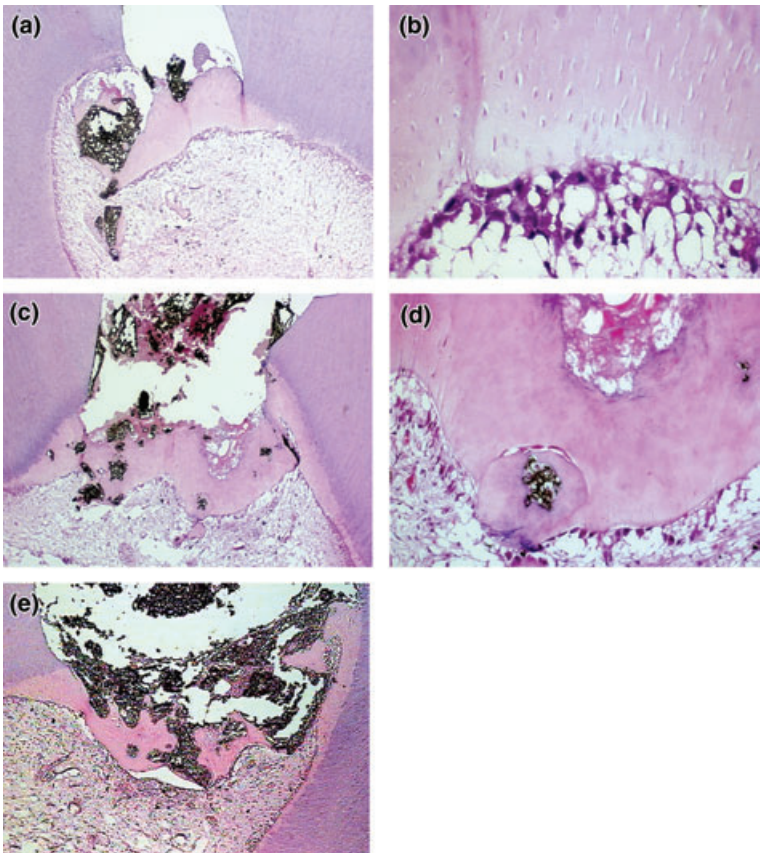

Figure 3 Ninety-day follow up. (a) 0.9% SS group. At the exposure site, dentine barrier with calcium hydroxide inclusions was noted. (b) In evidence (from a), the alignment of odontoblast-like cells under tubular dentine barrier. (c) 5.25% SH group. Dentine barrier formation was observed composed of an outer layer of osteodentine and an inner layer of tubular dentine, with odontoblast-like cells aligned beneath. A thick layer of reactionary dentine was also noticed. (d) 5.25% SH group, specimen highlighting the tubular organization of dentine barrier, which was underlined by odontoblast-like cells. Calcium hydroxide inclusions were also present. (e) 2% CD group. Exposure site showing dentine barrier and calcium hydroxide cement. *SS*, saline solution; *SH*, sodium hypochlorite; *CD*, chlorhexidine digluconate. Haematoxylin–eosin stain, original magnification ×25 (a, c and e) and ×100 (d) and ×200 (b).

Bacterial contamination

There was no bacterial staining in any section in all periods of time as observed using Brown & Hopps (1973) technique. The efficiency of the staining technique was tested using carious teeth as a positive control. All teeth in the positive control were stained using this method.

Statistical results

Significantly higher inflammatory response was detected for specimens at 7 days than for specimens at 90 days ($P < 0.01$). The inflammatory response was not influenced by different agents ($P > 0.05$). The three agents tested presented similar behaviours regarding soft tissue organization. The postoperative time had a significant influence ($P < 0.05$) on reactionary dentine formation: 90 days > 30 days > 7 days. Also, sodium hypochlorite exhibited more reactionary dentine at 90 days than the three agents at 7 days ($P < 0.05$). No significant difference was observed amongst agents for reparative dentine formation. However, 90-day specimens exhibited greater reparative dentine formation than 7-day specimens ($P < 0.001$). The 30-day specimens were similar to 90-day specimens.

Discussion

In this study, the agents used to control bleeding were biocompatible. Despite there being no significant differences observed between them, during the morphological descriptive analysis at the 7 days follow-up period, it was possible to observe the inert response to saline solution, when compared with sodium hypochlorite or chlorhexidine digluconate. No inflammatory response was observed for saline, whilst the two other solutions exhibited a mild inflammatory infiltrate. Sodium hypochlorite has tissue dissolution capacity, interfering with the cytoplasmic membrane integrity by causing irreversible enzymatic inhibition, biosynthetic alterations in cell metabolism and phospholipids destruction in lipid peroxidation (Estrela *et al.* 2003). This is the reason for its bactericidal effect as well as the explanation for adjacent pulp cell destruction. Chlorhexidine digluconate provides the capacity to precipitate cellular

membrane proteins, altering the cellular osmotic balance (Estrela *et al.* 2003), causing cellular lyses. In a recent study, Hernandez *et al.* (2005) observed that fresh mineral trioxide aggregate (MTA) mixed with chlorhexidine produced increased cytotoxicity when compared with fresh MTA and sterile water. However, this finding was not observed in set MTA with chlorhexidine (material which was allowed to dry for 24 h). Despite the initial mild inflammatory response observed in the present study, chlorhexidine and sodium hypochlorite solutions did not impair the inherent healing capacity of pulp tissue, which was confirmed by dentine barrier formation and absence of inflammation at the 90-day follow up.

At 30 days, variable results were observed mainly in relation to reparative dentine deposition. In some specimens, dentine deposition was formed predominately by fibrodentine or osteodentine, whilst in others there was complete dentine barrier formation. In a few other samples this barrier was absent. These findings could be related to the high metabolic activity and the transitory characteristics of this period, showing different speeds in the deposition of dentine matrix in different specimens.

After 90 days, the three agents had similar responses. Independent of the used substance, in this period there was a tendency for more organized dentine barriers with the experimental agents, presenting slightly increased tubular dentine deposition, indicating an advanced stage in the repair process. However, significant enhanced reactionary dentine deposition was observed with ageing (90 days > 30 days > 7 days). At 90 days, sodium hypochlorite presented significantly more reactionary dentine deposition than all agents at 7 days. Sodium hypochlorite has the capacity to solubilize the mineralized dentine matrix (Rosenfeld *et al.* 1978, Zhao *et al.* 2000) releasing growth factors, especially TGF-β (Tziafas *et al.* 2000, Smith 2003). It has been demonstrated that TGF-β1 and TGF-β3 isoforms have the ability to stimulate extracellular matrix secretion by odontoblast cells, thus, stimulating reactionary dentine deposition (Smith 2003).

The results obtained in this study confirmed the null hypothesis tested that the three agents did not cause interference in healing process after capping with calcium hydroxide. Nevertheless, these results were obtained in healthy pulps and the correlation with the response in inflamed pulps should be made with caution, because the presence of bacteria or their metabolites provokes pulp degeneration and expressive molecular alterations (Huang *et al.* 2005). Therefore, additional studies should investigate the response of these agents in pulp tissue that has previously been injured during the caries process.

Conclusions

This histological study showed that 5.25% sodium hypochlorite and 2% chlorhexidine digluconate have similar behaviours when compared with the control (0.9% saline solution), and that no agent had impaired the pulp healing process following capping with calcium hydroxide.

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