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Histological evaluation of the rat dental pulp after indirect capping with sildenafil or L-NAME incorporated into a bioadhesive thermoresponsive system

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ABSTRACT. We evaluated the histological dental pulp state *in vivo* after indirect pulp capping using sildenafil or L^G-nitro-L-arginine (L-NAME), incorporated into a new bioadhesive thermoresponsive system (BTS). Male Wistar rats were subjected to an upper and lower first molar class I cavity preparation followed by indirect pulp capping with sildenafil or L-NAME. Calcium hydroxide (CaOH₂) was used as a control. The teeth and surrounding bone were properly dissected and processed for Nissl's staining. Pulp state was evaluated considering the morphological aspects of the inflammatory response, type of inflammatory infiltrate, organization of the odontoblast layer, blood vessel condition, and presence of abscesses or necrosis. The results were expressed as average of observations. The most intense inflammatory response was observed 3 days after the cavity preparation. No identified changes were detected in the dental pulp response of the molars treated with L-NAME compared with those treated with CaOH₂. A dual effect was observed in the teeth treated with sildenafil. While low sildenafil concentration (0.015% w w⁻¹) promoted effects comparable to CaOH₂, at a higher concentration (0.15% w w⁻¹), sildenafil caused a severe inflammatory response and pulp necrosis. This pioneering suggest that NO pathway activity may be a determinant in the process of dental pulp healing.

Keywords: indirect dental pulp capping, bioadhesive thermosensitive system (BTS), sildenafil citrate, L-NAME, rats.

Avaliação histológica da polpa dental do rato após capeamento pulpar indireto usando sildenafil ou L-NAME incorporados a um sistema bioadesivo termorresponsivo

RESUMO. Avaliou-se o estado histológico da polpa dental *in vivo* após capeamento pulpar indireto usando sildenafil ou LG-nitro-L-arginine (L-NAME), incorporados num novo sistema bioadesivo termorresponsivo (BTS). Ratos Wistar machos foram sujeitos ao preparo cavitário, classe I nos primeiros molares superiores e inferiores, seguido de capeamento pulpar indireto com sildenafil ou L-NAME incorporados em BTS. Hidróxido de cálcio (CaOH₂) foi usado como controle positivo. Os dentes e tecidos adjacentes foram adequadamente dissecados e processados para a coloração de Nissl. O estado da polpa foi avaliado quanto aos aspectos morfológicos e tipo do infiltrado inflamatório, organização da camada de odontoblastos, condições dos vasos sanguíneos e presença de abscesso ou necrose. A resposta inflamatória mais intensa foi observada três dias após o preparo cavitário. Nenhuma alteração foi detectada na polpa dental dos molares tratados com L-NAME comparados com aqueles tratados com CaOH₂. Um efeito dual foi observado nos dentes tratados com sildenafil. Enquanto baixas concentrações de sildenafil (0.015% v v⁻¹) promoveram efeitos comparáveis com o CaOH₂, altas concentrações (0.15% v v⁻¹) causaram uma severa resposta inflamatória e necrose pulpar. Este estudo pioneiro sugere que a atividade da via do NO pode ser determinante no processo de cicatrização.

Palavras-chave: capeamento pulpar indireto, sistema bioadesivo termorresponsivo, citrato de sildenafil, L-NAME, ratos.

Introduction

The dentist continually refers to the maintenance of dental integrity and functionality. Restorative procedures involve pulp protection to decrease the aggression produced by chemical, biological, mechanical, and thermal agents (Smith, 2002). After deep cavity preparations to remove the soft demineralized or infected dentin, the pulp must be

indirectly capped using materials that can stimulate biological process that lead to the deposition of tertiary dentin and pulp healing (Tziafas, Koliniotou-Koumpia, Tziafa, & Papadimitriou, 2007).

The pulp must heal from inflammatory reactions caused by the cavity preparation procedures and presence of caries lesions. If left unattended, then this will eventually progress to pulp necrosis (Yu &

Abbot, 2007). The dental pulp inflammatory reaction has been characterized by blood flow modifications, immune cell actuation, and neural reactivity (Law, Baumgardner, Meller, & Gebhart, 1999; Da Silva, Issa, & Del Bel, 2008). Experimental studies indicated that nitric oxide (NO) participates as a regulator of vascular homeostasis (Lohinai, Balla, Marczis, Vass, & Kovách, 1995; Berggreen & Heyeraas, 2003; Law et al., 1999), mediator of proinflammatory activity (Da Silva et al., 2008), and modulator of the afferent sensitivity of the dental pulp (McCormack & Davies, 1996). To date, the presence of NO has been detected in the normal and inflamed dental pulp in humans and many animal species (Kerezoudis, Olgart, & Fried, 1993; Lohinai, Székely, Benedek, & Csillag, 1997; Felaco et al., 2000; Di Nardo Di Maio et al., 2004). Nitric oxide has been shown to be part of the first line of hard dental tissue defense against invading oral microorganisms (Silva Mendez, Allaker, Hardie, & Benjamin, 1999). Nitric oxide has also been recognized as an indicator of cell differentiation during the formation of reparative dentine (Yasuhara et al., 2007). Furthermore, NO expression in odontoblasts during tertiary dentinogenesis is synchronized with other odontoblast differentiation markers, such as osteocalcin and alkaline phosphatase (Mei et al., 2007). This evidence indicates that the NO system is a potential molecular target in dental pulp healing.

Nitric oxide is produced by a group of isoenzymes collectively named NO synthase (NOS; Bredt & Snyder, 1994). When activated, NOS generates 3'5'-guanosine monophosphate (GMP), which is rapidly degraded by phosphodiesterase (PDE). Pharmacological inhibition of PDE-5 increases the intracellular concentration of cyclic GMP (cGMP) that, in turn, may cause vascular smooth muscle relaxation and concomitantly increase blood flow. Systemic or local inhibition of NOS by L^G-nitro-L-arginine (L-NAME) has been shown to increase vascular pulp resistance (Lohinai et al., 1995) and reduce the diameter of the pulpal arteriole (Kispélyi et al., 2005).

Sildenafil citrate is a PDE-5 inhibitor used in the management of erectile dysfunction and pulmonary arterial hypertension (Montani et al., 2009; Dorsey, Keel, Klavens, & Hellstrom, 2010). It also induces angiogenesis after experimental stroke (Li et al., 2007). Based on the pharmacological actions of sildenafil and L-NAME, these compounds may influence the dental pulp response after injury or cavity preparation. However, the clinical efficacy of dental pulp treatments intrinsically depends on the

release of the drug and mechanical properties of the formulation. We recently developed a semisolid formulation that consists of a bioadhesive thermoresponsive system (BTS) incorporated with sildenafil for endodontic application (Fabri, Cupertino, Hidalgo, Oliveira, & Bruschi, 2011). The rheological, mechanical, and bioadhesive properties of this system were characterized and shown to be beneficial both for the insertion of the formulation into the endodontic space and its subsequent retention.

Therefore, the aim of the present study was to evaluate the *in vivo* histological dental pulp state after indirect pulp capping using sildenafil or L-NAME, both incorporated into a new BTS.

Material and methods

Materials

Poloxamer 407 (P407) was a kind gift from BASF (São Paulo, SP, Brazil), and Carbopol 934P (C934P) was purchased from B.F. Goodrich (Brecksville, OH, USA). Triethanolamine (TEA) was purchased from Galena (Campinas, São Paulo State, Brazil) and used as a neutralizing agent. Sildenafil citrate and L-NAME were purchased from Pfizer (Suzhou, China) and Sigma (St. Louis, MO, USA). Calcium hydroxide (CaOH₂) was obtained from Biodinâmica (São Paulo, São Paulo State, Brazil). All other chemicals were purchased from Merck (Darmstadt, Germany) or Synth (Diadema, São Paulo State, Brazil).

Preparation of Formulations

C934P (0.10, 0.25, and 0.50%, w w⁻¹) was initially dissolved in distilled water using a mechanical stirrer. Following complete dissolution, P407 (15 and 20%, w w⁻¹) was added to this preparation, and the mixture was stored at 4°C for 12h to ensure complete wetting. This BTS was then stirred to ensure complete mixing of the two components, neutralized with TEA, and stored at 4°C for 24h (Bruschi et al., 2007).

Sildenafil (0.015 and 0.15%) and L-NAME (10⁻⁴ mol L⁻¹) were added to the BTS by manual stirring (Fabri et al., 2011). The samples were then transferred into amber ointment jars, evacuated to remove incorporated air, and stored at 4°C for at least 24h prior to use.

Animals

Twenty-three male Wistar rats (*Rattus norvegicus*), 90 days old and weighing 240-320 g, were housed two per cage in a temperature-controlled room (23 ± 1°C) that was maintained on a 12h/12h light/dark

cycle. All of the animals had *ad libitum* access to food and water. The procedures were conducted in accordance with the Brazilian Society of Neuroscience and Behavior Guidelines for the Care and Use of Laboratory Animals, which comply with international laws, and approved by the local committee on animal ethics (CEAE 034/2007). All efforts were made to minimize animal suffering.

Experimental Procedure

Each animal was anesthetized with an intramuscular injection of a mixture of 7 mg kg⁻¹ xylazine (Rompun, Bayer, São Paulo, São Paulo State, Brazil) and 65 mg kg⁻¹ ketamine (Dopalen, Sespo Ind. Com. Ltda, São Paulo, Brazil) and administered in a volume of 1 mL kg⁻¹ body weight. Class I cavities were prepared on the upper and lower first molars, without pulp exposure, using a high-speed contra-angle at 120,000 rpm. Round diamond burs (0.6 mm diameter, 0.05 ISO; Maillefer, France) were used, cooled with copious amounts of sterile water. The depth of the cavities was standardized at approximately 0.6 mm, corresponding to the active part of the bur. Each cavity was then air dried, filled with the different treatments, and temporarily restored with glass-ionomer cement (Vidrion, SS White, São Paulo, São Paulo State, Brazil). The animals did not receive any postoperative medication.

Experiment I: Time-course of dental pulp response to the cavity preparation

This experiment determined the time-course of the dental pulp response to the cavity preparation to determine the optimal interval to conduct the pharmacological treatments (Experiment II).

The rats (n = 13) had their upper and lower first molars prepared as described above, with no medication, and were randomly divided into the three experimental groups according to the time that elapsed after the surgical procedures: 1, 3, and 7 days.

Experiment II: Effects of pharmacological treatments on indirect pulp capping

This experiment was conducted to evaluate the effects of the different pharmacological treatments (i.e., sildenafil and L-NAME) incorporated into the BTS on indirect pulp capping. The rats (n = 10) had their upper and lower first molars prepared, which were randomly treated alternately, such that the treatment of the upper first molars (right and left) differed from the lower first molars (right and left) and all of the teeth received the pharmacological

treatments. The cavities were prepared and filled with the following treatments:

- i) CaOH₂
- ii) BTS
- iii) BTS + 0.015% sildenafil
- iv) BTS + 0.15% sildenafil
- v) BTS + 10⁻⁴ mol L⁻¹ L-NAME

The pulps capped with CaOH₂ powder mixed with saline were used as a standard control. Sildenafil and L-NAME incorporated into the BTS were directly applied to the cavity in a volume of 20 µl. The concentration of L-NAME was based on a published study (Kispélyi et al., 2005). The concentration of sildenafil was calculated as a concentration equimolar to the L-NAME dose. The animals were sacrificed 3 days after the surgical procedures.

Histology

The animals were anesthetized with an intraperitoneal overdose of sodium pentobarbital (Thiopentax, Cristália, SP, Brazil) and transcardially perfused with saline followed by 4% paraformaldehyde in 0.01 M phosphate buffer (4% PFA), pH 7.4. The teeth and surrounding bone were then resected and fixed in 4% PFA overnight. The samples were decalcified with 10% EDTA in 0.1 M phosphate buffer for 14 days. After decalcification, the samples were dehydrated with a graded series of ethanol, embedded in paraffin, and sectioned along their coronal axis at 7 µm in a rotating microtome (Leica RM2445, Nussloch, Germany). The sections were placed on gelatin-coated slides. The slides that contained a set of four sections were stained for hematoxylin-eosin (H&E) and analyzed under a light microscope (Olympus BX50, Tokyo, Japan).

The H&E staining was used to investigate the dental pulp response, considering the morphological aspects of the inflammatory response, such as the intensity (i.e., mild, moderate, or severe) and type of inflammatory infiltrate (polymorphonuclear [PMN] or mononuclear [MN]), organization of the odontoblast layer, blood vessel condition, and presence of abscesses or necrosis (Six, Decup, Lasfargues, Salih, & Goldberg, 2002; Vier-Pelisser, Figueiredo, Cherubini, Braga Filho, & Figueiredo, 2007; Parolia et al., 2010).

All of the slides were coded before the histological analysis to avoid bias.

Results

Experiment I

One day after the cavity preparation, the histological analysis of the dental pulp showed mild PMN inflammatory infiltrate and a column-shaped

odontoblastic layer that surrounded the usual connective tissue elements of the pulp (Figure 1). Local disruption and disorganization of the odontoblast layer was detected in 50% of the specimens on the third day (Table 1), together with inflammatory infiltrate that was even more prominent subjacent to the cavity preparation (Figure 1). At 7 days, the odontoblast layer appeared to be organized and nearly repaired (Figure 1). In all of the intervals, the blood vessels had a normal aspect, with no evidence of abscesses or necrosis.

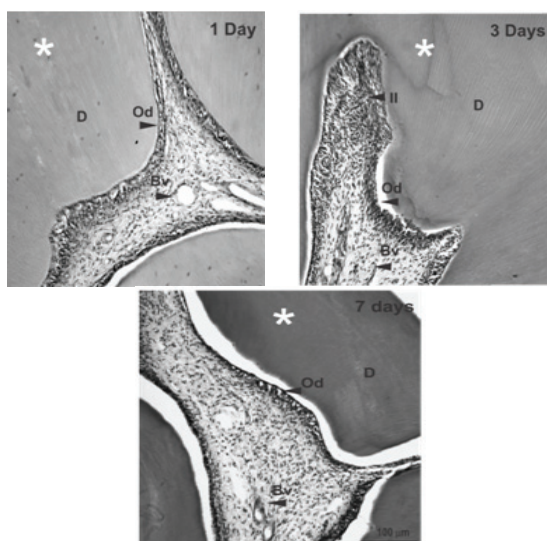


Figure 1. Representative photomicrographs of the rat first molar dental pulp 1 day, 3 days, and 7 days after the cavity preparation and immediate glass-ionomer cement restoration. At 3 days, an area of intense inflammatory infiltrate, concentrated subjacent to the cavity preparation, and disorganization of the odontoblast layer are evident (arrow). At 7 days, inflammation was mild, and the odontoblast layer was organized, similar to the first day. (*) Location of the cavity preparation; (Od) odontoblast layer; (D) dentin; (II) inflammatory infiltrate; (Bv) blood vessel. Hematoxylin-Eosin staining.

Table 1. Results of histological analyses of the rat first molar dental pulp 1, 3, and 7 days after indirect pulp capping. The cavities were prepared and immediately restored with glass-ionomer cement.

	Day 1	Day 3	Day 7
Inflammatory infiltrate	PMN	PMN	PMN
Cells Intensity	Mild	Moderate	Mild
Odontoblast layer	Organized	Organized (50%) Desorganized (50%)	Organized
Blood vessels	Present	Present	Present
Necrosis	Absent	Absent	Absent

Experiment II

As seen in Figure 2 and Table 2, teeth filled with CaOH_2 showed moderate PMN inflammatory infiltrate and an organized odontoblast layer. Teeth

filled with BTS showed inflammatory infiltrate that varied from moderate (25%) to severe (50%), especially in proximity to the cavity preparation. The blood vessels were ingurgited, with no sign of necrosis (Figure 2). The results obtained with sildenafil were dual. At the low concentration (0.015%), sildenafil did not change the histological parameters compared with CaOH_2 (Table 2, Figure 2). However, at the higher concentration (0.15%), sildenafil induced severe pulp inflammation in all of the teeth evaluated. The odontoblast layer appeared disorganized or nonexistent, with evidence of necrosis and abscesses (Figure 2). The results obtained with 10^{-4} mol L-NAME were similar to the results obtained with CaOH_2 , showing an organized odontoblast layer and moderate inflammatory infiltrate (Table 2, Figure 2).

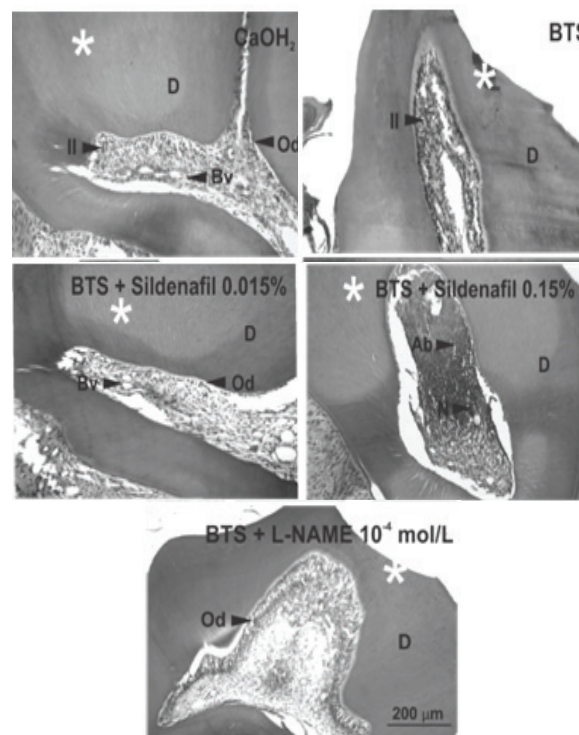


Figure 2. Representative photomicrographs of the rat first molar dental pulp 3 days after the experimental procedure. The cavities were prepared, filled with CaOH_2 , bioadhesive thermoresponsive system (BTS), BTS + 0.015% sildenafil, BTS + 0.15% sildenafil, and BTS + 10^{-4} mol L⁻¹ L-NAME and immediately restored with glass-ionomer cement. Notice that CaOH_2 , BTS + 0.015% sildenafil, and BTS + L-NAME produced similar effects on dental pulp. Severe PMN inflammatory infiltrate, areas of abscesses and necrosis, and a disorganized odontoblast layer were observed in dental pulp treated with BTS + 0.15% sildenafil. (*) Location of cavity preparation; (Od) odontoblast layer; (D) dentin; (II) inflammatory infiltrate; (N) necrosis; (Ab) abscess; (Bv) blood vessel. Hematoxylin-Eosin staining.

Table 2. Results of histological analyses of the rat first molar dental pulp 1, 3, and 7 days after indirect pulp capping. The cavities were prepared, filled with different pharmacological treatments, and immediately restored with glass-ionomer cement.

	CaOH ₂	BTS	Sildenafil 0.015%	Sildenafil 0.15%	L-NAME 10 ⁻⁴ mol L ⁻¹
Inflammatory infiltrate	PMN	PMN	PMN	PMN	PMN
Cells Intensity	Mild	Moderate (25%) Severe (50%)	Mild	Severe	Moderate
Odontoblast layer	Organized	Organized (50%) Desorganized (50%)	Organized	Desorganized	Organized
Blood vessels	Present	Present	Present	Present	Present
Necrosis	Absent	Present	Absent	Present	Absent

Discussion

The comprehension of the molecular mechanisms involved in the pulp response and introduction of new capping materials for the delivery of exogenous signaling molecules to be used in dental pulp protection are important for dentistry. This was a pioneering study that used a BTS that contained compounds that interfere with NO neurotransmission in indirect pulp protection in rats. In the present study, upper and lower first molars in rats were processed for a class I cavity preparation followed by indirect pulp capping with sildenafil or L-NAME incorporated into the BTS. Indirect capping with CaOH₂ was used as a standard control. The most severe inflammatory pulp response was detected 3 days after the cavity preparation. No identified changes were detected in the dental pulp response of the molars treated with L-NAME compared with those treated with CaOH₂. A dual effect was observed in the teeth treated with sildenafil. The low concentration of sildenafil (0.015% w w⁻¹) promoted effects comparable to CaOH₂. The higher concentration of sildenafil (0.15% w w⁻¹) caused a severe inflammatory response and pulp necrosis.

The dentin-pulp complex is capable of repair after tooth injury, such as caries, attrition, abrasion, and dental procedures, including cavity preparation, resulting in tertiary dentin formation. The cavity preparation induces destructive changes in odontoblasts at the affected site followed by pulp mesenchymal cell migration to the damaged odontoblast layer and differentiation into new odontoblasts (Izumi, Eida, Matsumoto, & Inoue, 2007). The effects on the odontoblast layer are initiated approximately 6h after the cavity preparation and ends approximately 4 days after the injury, culminating in the reestablishment of local homeostasis (D'Souza et al., 1999). In the present study, an inflammatory response was detected 3 days after the cavity preparation. At this time point, the odontoblast layer was disorganized. At 7 days, the inflammatory response was mild, and the odontoblast layer appeared almost repaired. These results are consistent with a previous study, in which

cell proliferation markers, such as bromodeoxyuridine and metallothionein, were detected in odontoblasts, pulp cells, and endothelial cells in the pulp core 3 days after the cavity preparation (Izumi et al., 2007). These findings indicate that the cavity preparation results in the proliferation and differentiation of newly differentiating odontoblasts and angiogenesis during the dental pulp healing process.

Regenerative procedures can be defined as biologically based procedures designed to replace damaged structures, including dentin and root structures, and cells of the pulp-dentin complex (Murray, Garcia-Godoy, & Hargreaves, 2007). Dental material research has been driven by an understanding of the physicochemical characteristics, toxicity limitations, and biocompatibility of the new materials with dental and other oral tissues (Schweikl et al., 2005). Here, we used a formulation of semisolid devices based on the use of binary hydrophilic polymer gels (i.e., BTS) that may offer several advantages with respect to clinical performance in pulp protection. The polymers employed in this study, thermoresponsive P407 and highly mucoadhesive C934P, were chosen because of their capacity to form a BTS in aqueous solvent, facilitating insertion and improving the intimacy of contact and retention time of the formulation into the dental space (Fabri et al., 2011). The molars filled with the BTS presented a moderate to intense inflammatory process, with abscesses or necrosis detected in 50% of the teeth. In a cavity preparation with any pharmacological treatment, the inflammatory response in the teeth filled with only BTS was even worse. An initial irritant action is expected and appears to be important for pulp regeneration after injury. For example, CaOH₂, a capping material that has been used in traditional pulp therapy because of its alkalinity (pH 12), induces focal necrosis upon contact with dental pulp, stimulating reparative dentine formation (Hu, Zhang, Qian, & Tatum, 1998). Our results are consistent with other studies that investigated the effects of CaOH₂ in dental pulp capping (Tziafas,

Smith, & Lesot, 2000; Dammaschke, Stratmann, Fischer, Sagheri, & Schäfer, 2010). First molars filled with CaOH₂ showed a mild inflammatory response 3 days after the cavity preparation. The effects of CaOH₂ were similar to L-NAME, a NOS inhibitor. The effect of L-NAME was somewhat unexpected because systemic or local administration of L-NAME has been shown to increase vascular pulp resistance (Lohinai et al., 1995; Kispélyi et al., 2005), which could culminate in a worsening of the inflammatory response. Further experiments using other doses of L-NAME need to be conducted to elucidate the effect of this compound on indirect pulp capping.

The effects of 0.015% sildenafil were similar to those obtained with the BTS. Three days after the cavity preparation, only a mild inflammatory response was detected, and the odontoblast layer was organized. However, sildenafil at a 10-fold higher concentration of 0.15% produced a severe inflammatory response, with necrosis detected in all of the molars evaluated. Therefore, the pharmacological effects of sildenafil varied according to its concentration. Nitric oxide has been shown to bi-directionally modulate various physiological and pathological processes. In inflammatory processes, for example, NO at low concentrations potentiated activity, whereas a high concentration inhibited both the activity and expression of cyclooxygenase-2 (Swierkosz, Mitchell, Warner, Botting, & Vane, 1995). Nitric oxide has also been shown to maintain the physiological tone of blood vessels in oral tissues (Da Silva et al., 2008), although an increase in NO-NOS-cGMP pathway activity could generate oxygen-derived free radicals, such as superoxide anions, intensifying the inflammatory process. Therefore, NO at low concentrations appears to play an anti-inflammatory role in dental pulp inflammation. At greater concentrations, in contrast, NO could act as a proinflammatory mediator. The molecular and cellular interactions between inflammation and regeneration are complex, and a fine balance may exist that can result in synergy or aggravation of the pulp healing response.

Conclusion

The present study described the use of a BTS that contained sildenafil and L-NAME in indirect pulp capping. The effects of sildenafil at a concentration of 0.015% were similar to CaOH₂, reducing inflammatory processes 3 days after the cavity preparation. In contrast, sildenafil at 0.15% intensified the inflammatory process. These results suggest that the level of NO-NOS-cGMP pathway

activity may be a determinant in the healing process of dental pulp healing. Further investigations of the effects of the BTS in the dental pulp response and possible molecular mechanisms related to the effects of NO in the healing of dental pulp after cavity preparations should be conducted.

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