

Effect of Calcium Hydroxide on Bacterial Endotoxin In Vivo

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The aim of this study was the histopathological evaluation of apical and periapical tissues in dog teeth that were submitted to bacterial endotoxin, associated or not with calcium hydroxide. After removal of the pulp from 60 premolars, the teeth were divided into four groups and were filled with bacterial endotoxin (group 1), bacterial endotoxin plus calcium hydroxide (group 2), saline solution (group 3), or had induced periapical lesions with no treatment (group 4). After 30 days, animals were killed and the teeth processed histologically. The inflammatory infiltrate, the thickness of the periodontal ligament, and the presence of resorption areas were similar for groups 1 and 4. Groups 2 and 3 were similar to each other. It can be concluded that the bacterial endotoxin caused a periapical lesion and that calcium hydroxide detoxified the lipopolysaccharides in vivo.

Improvement in microbiology techniques for culture and identification has shown that root canals in teeth with pulp necrosis and a chronic periapical reaction have a predominance of anaerobic microorganisms (1, 2), especially Gram-negative ones (3). This polybacterial infection is present not only in the lumen of the root canal and dentinal tubules, but also in the apical craters and the entire root canal system (2). Gram-negative microorganisms not only have different virulent factors and produce toxic products and sub-products in apical and periapical tissues, but also contain endotoxin in their cell wall. Endotoxin, which consists of lipopolysaccharides (LPS), is liberated during bacterial cell multiplication or death and is responsible for a series of important biological effects (4, 5). Its action on macrophages (6) triggers the release of a series of inflammatory, bioactive, chemical mediators, or cytokines (5), such as tumor necrosis factor (TNF) (4, 5) and interleukins-1 (5, 7), -6, and -8 (7). Endotoxin also induces fever (8), is mitogenic to B lymphocytes (5), activates the complement system (8) and the metabolism of arachidonic acid (5), and irreversibly adheres to mineralized tissues. These events lead to an inflammatory reaction and bone resorption in the periapical region. These

facts emphasize the important role of LPS in the pathogenesis of periapical lesions (4, 9, 10, 11).

A survey of the medical literature over the last 10 yr produced 22,450 articles dealing with endotoxin; however only four articles in dentistry (9, 10, 12, 13) evaluated the effects of LPS on apical and periapical tissues using experimental animals.

Treatment of root canals in teeth with pulp necrosis and a chronic periapical reaction should not only be concerned with bacterial death, but also the inactivation of endotoxin. Safavi and Nichols (14, 15), Barthel et al. (4), and Olsen et al. (16) studied, in vitro, the effect of calcium hydroxide on bacterial LPS, because LPS may remain in the root canals between intracanal dressing sessions. However, there are no in vivo studies reporting this problem.

The purpose of this study was to evaluate histopathologically the effect of pure endotoxin or endotoxin plus calcium hydroxide on the apical and periapical tissues in dogs.

MATERIAL AND METHODS

Endotoxin and Calcium Hydroxide Preparation

In a laminar air flow, 100 mg of *Escherichia coli* endotoxin (LPS B, *E. coli* 055:B5-Lipid A, 9.2%, Difco, Bacto, Detroit, MI) was suspended in 10 ml of phosphate-buffered saline. Half of the 10 mg/mL suspension was kept in sterile Carpules, and the other half was mixed with 2.75 g of calcium hydroxide p.a. (550 mg/mL, Merck, Whitehouse Station, NJ) and also kept in sterile Carpules.

Surgical Procedures

The second, third, and fourth mandibular premolars and the second and third maxillary premolars of three dogs (ages: 12–18 months; weights: 8–15 kg) were selected for treatment (total: 60 root canals). Twenty roots were used for each of the two experimental groups (groups 1 and 2) and 10 for each of the control groups (groups 3 and 4).

The animals were anesthetized intravenously with sodium thiopental (30 mg/kg body weight; Thionembutal, Abbot Laboratories, São Paulo, SP, Brazil). After isolation of the dental area with a rubber dam and disinfection of the operative field with 0.3% iodine/70% alcohol, crown occlusal access was made. The working length was determined to 2-mm short of the radiographic apex using #30 K-files. The root

pulp was removed and the root canal was irrigated with saline solution (Labormédica Indústria Farmacêutica Ltda., São José dos Campos, SP, Brazil) with a minimal volume of 3.6 ml at each instrument change. The apical foramen was enlarged by sequential use of #15 to #30 K-files (Maillefer, Ballaiques, Switzerland) to the radiographic apex (always with irrigation). After that, the instrumentation was performed to the working length up to a #50 K-file. A #30 K-file was used at the total length of the root to make sure that no dentin chips or other residues remained in the apical foramen. After irrigation, the root canals were dried by aspiration and sterile paper points and then filled with 14.3% buffered EDTA (pH 7.4; Odahcan-Herpo Productos Dentários Ltda., Rio de Janeiro, RJ, Brazil) for 3 min and then irrigated with saline and dried.

Because all variables should be tested in the same animal and in the different quadrants, each hemiarch was submitted in an alternate manner to the experimental protocols.

Group 1: Twenty root canals were each injected with 0.1 ml of the endotoxin preparation by using a threaded M.L. syringe (S.S. White Artigos Dentários Ltda., Rio de Janeiro, RJ, Brazil) with a long 27-gauge needle (Terumo, Tokyo, Japan).

Group 2: Twenty root canals were injected with 0.1 ml of the endotoxin plus calcium hydroxide by using a threaded M.L. syringe with a Calasept Kit needle (Scania Dental AB, Knivsta, Sweden).

Group 3: Ten root canals were injected with saline by using a Carpule syringe with a long 27-gauge needle.

Following these procedures, the pulp chambers of groups 1, 2, and 3 were sealed with a sterile cotton pledge and the teeth were sealed with zinc oxide–eugenol cement (IRM, S.S. White Artigos Dentários Ltda.) for a period of 30 days.

Group 4: Ten root canals were exposed to the oral environment for 5 days to allow microbial contamination. After this period, under general anesthesia, the pulp chamber was cleared of all debris and sealed with a cotton pledge and zinc oxide–eugenol cement to induce a periapical reaction (17).

All teeth were radiographed in a standard manner at 15-day intervals. Thirty days after the surgical procedure, the teeth were again examined radiographically and the animals killed by anesthetic overdose. The maxilla and mandible were dissected and sectioned to obtain individual roots.

Histological Procedures

The samples were washed and demineralized with EDTA in a microwave oven (Sharp, São Paulo, SP, Brazil). The roots were then washed in running water for 24 h, dehydrated by increasing concentrations of ethyl alcohol, cleared in xylol, and embedded in paraffin blocks. The serial 6- μ m wide longitudinal sections were stained with hematoxylin and eosin, Mallory Trichrome, and Brown and Brenn.

Histopathological and Statistical Analysis

The following parameters were analyzed subjectively: (a) intensity of inflammatory infiltrate (absent/mild, moderate, or severe); (b) type of inflammatory infiltrate (acute or chronic); (c) thickness of the periodontal ligament (normal/slightly increased, moderately increased, severely increased); (d) resorption of mineralized tissues—dentin, cementum, and alveolar bone (present or absent).

The results were analyzed statistically by the Mann-Whitney nonparametric test by using the software GMC7.7 (<http://www.forp.usp.br/restauradora/gmc/gmc.html>).

RESULTS

Group 1: LPS

The apical region of the 19 roots analyzed (one was lost during the histological procedure) had enlarged lacunae in the cementum that were empty or had disorganized connective tissue and inflammatory cells. All specimens had areas of resorption of apical cementum and absence of cementoblasts on the surface. The thickness of the periodontal ligament was severely increased in 16 roots, slightly increased in two roots, and moderately increased in 1 root. In this region, close to the root apex, a dense, diffuse inflammatory process could be seen, composed mainly of neutrophils. At a distance from the apical foramen, neutrophils and lymphomononuclear cells, mainly macrophages, could be seen in equivalent numbers with very little collagen matrix. In 12 roots the inflammatory infiltrate was severe and was moderate in the other 7. In 18 of the 19 specimens, the alveolar bone had extensive areas of active resorption, absence of osteoblasts, and in some samples, surface osteoclasts could be detected [Fig. 1 (A, B, and C)].

Group 2: LPS Associated with Calcium Hydroxide

The apical and periapical region was normal in 18 of the 20 roots. Ramifications of the apical delta had normal connective tissue in 18 roots; in the other two, they were enlarged and empty. The apical cementum was regular, without active areas of resorption, except in two cases, showing surface cementoblasts and collagen fibers perpendicular to its surface. The periodontal ligament was normal or slightly thickened in 11 roots, moderate in 7, and severely thickened in only 2 roots. A slight to absent inflammatory infiltrate and intense formation of collagen fibers and other cells could be seen in the connective tissue of the periapical region, close to the apical area, in 18 of 20 samples. In half (nine) of these samples, the connective tissue was dense, with normal thickness of the periodontal ligament and occasional inflammatory cells, and formation of mineralized tissue in direct contact and adjacent to the apical opening. In two roots, in which the suspension was injected beyond the apical opening, there was mineralized tissue in direct contact with the extruded material. In only two cases, the surface of the apical cementum showed small areas of resorption without repair. The thickness of the periodontal ligament was increased, with severe inflammatory infiltrate and a reduced number of collagen fibers. There was connective tissue in the medullary spaces of the alveolar bone, with osteocytes inside and osteoblasts on the surface. Bone resorption was only observed in two cases [Fig. 2 (A, B and C)].

Group 3: Saline

In all 10 roots in this group, the apical delta ramifications were enlarged with normal connective tissue in their interior. The surface of the apical cementum was regular, and in only one case was there evidence of resorption without repair. The periodontal ligament was slightly thickened in six roots and moderate in four. The connective tissue in this region was less dense, with a discrete

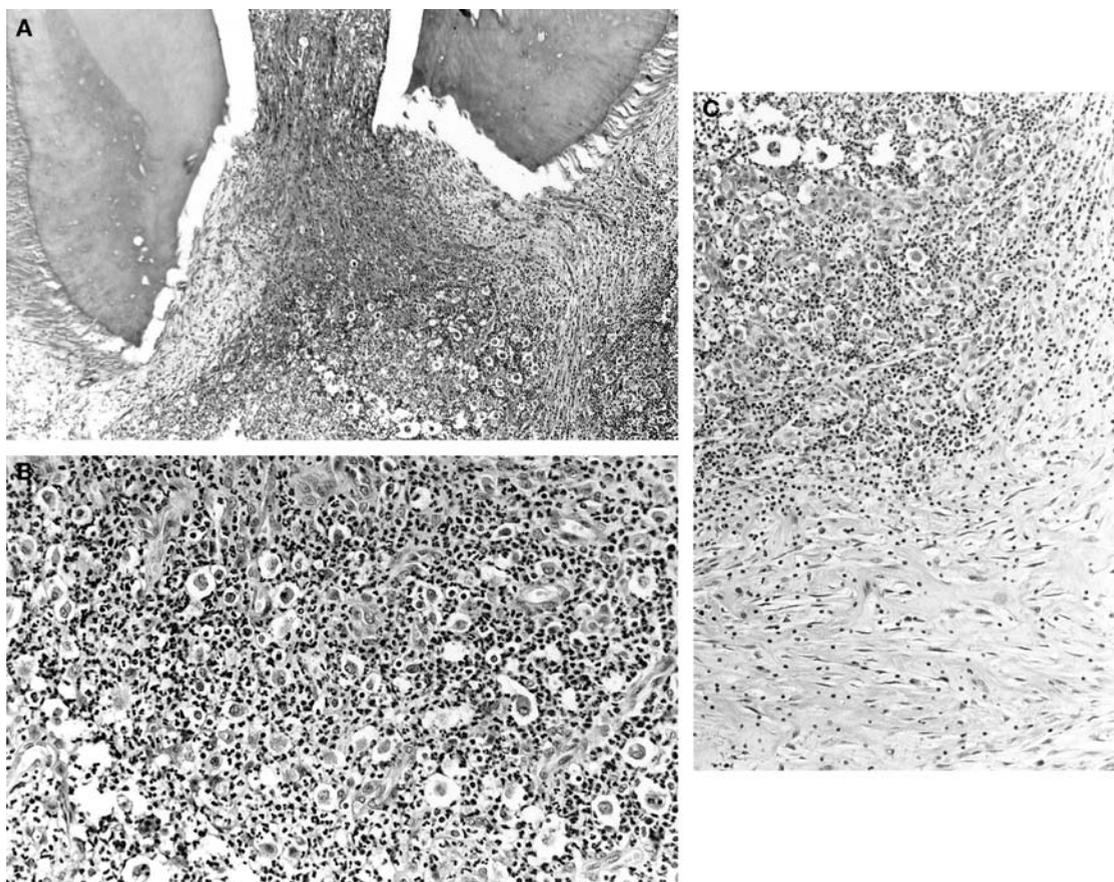


FIG. 1. Group 1 (LPS): (A) Intense inflammatory infiltrate in the interstitial tissue and periapical region. Resorption of cement with absence of cementoblasts (hematoxylin and eosin $\times 20$); (B) Concentrated presence of inflammatory cells with predominance of neutrophils close to the root apex (hematoxylin and eosin $\times 64$); (C) At a distance from the apical foramen, presence of neutrophils and mononuclear cells, with predominance of macrophages. Fibers and cells surround the inflammatory infiltrate (hematoxylin and eosin $\times 64$).

presence of collagen fibers and cells. There were few mononucleated cells in the periapical region of nine roots, and the inflammatory infiltrate was intense in only one. There was no resorption of dentin or bone in this group (Fig. 3).

Group 4: Periapical-Induced Reactions

In the apical region of the 10 roots, cementum lacunae were empty or contained necrotic debris and bacteria. The surface of the apical cementum was irregular, with resorption areas in all roots with rare cementoblasts on its surface. The interstitial connective tissue of the apical opening and periapical area had extensive necrotic areas and inflammatory cells, mostly mononucleated. There were microorganisms in all specimens. The thickness of the periodontal ligament was intensely increased in six roots, moderate in two, and slightly increased in two specimens. The connective tissue in the periapical region, close to the apical opening in four roots, showed intense infiltrate of mononuclear phagocytic inflammatory cells, permeated by neutrophils, edema, and intense fibrillar dissociation. At a distance from the root apex, the inflammatory infiltrate was moderate in five cases and presented vascular proliferation and considerable presence of macrophages. The alveolar bone had extensive areas of resorption in 9 of 10 roots, with few osteoblasts and osteocytes [Fig. 4 (A and B)].

Analysis of the Brown and Brenn-stained samples indicated that there was no bacteria in groups 1, 2, and 3, but there was a

considerable number of microorganisms in the entire root canal system of group 4.

Statistical Analysis

The inflammatory infiltrate, the thickness of the periodontal ligament, and the presence of resorption were statistically similar for groups 2 and 3 and for groups 1 and 4 (group 2 = group 3 \neq group 1 = group 4).

DISCUSSION

The classic research of Kakehashi et al. (18) showed the role of bacteria in the etiology of periapical reactions, but few studies have evaluated the isolated effect of LPS in direct contact with apical and periapical tissues. The results of this study show that after 30 days, even with the absence of bacteria, endotoxin in root canals may induce radiographically visible apical reactions and show intense inflammatory infiltrate, a large increase in the thickness of the periodontal ligament, and resorption of cementum and alveolar bone. This is comparable with that shown in the roots in group 4 (positive control) in which apical reactions were induced ($p < 0.05$). These observations are in accordance with those of Dahlén et al. (12) who reported that injecting *Fusobacterium nucleatum*

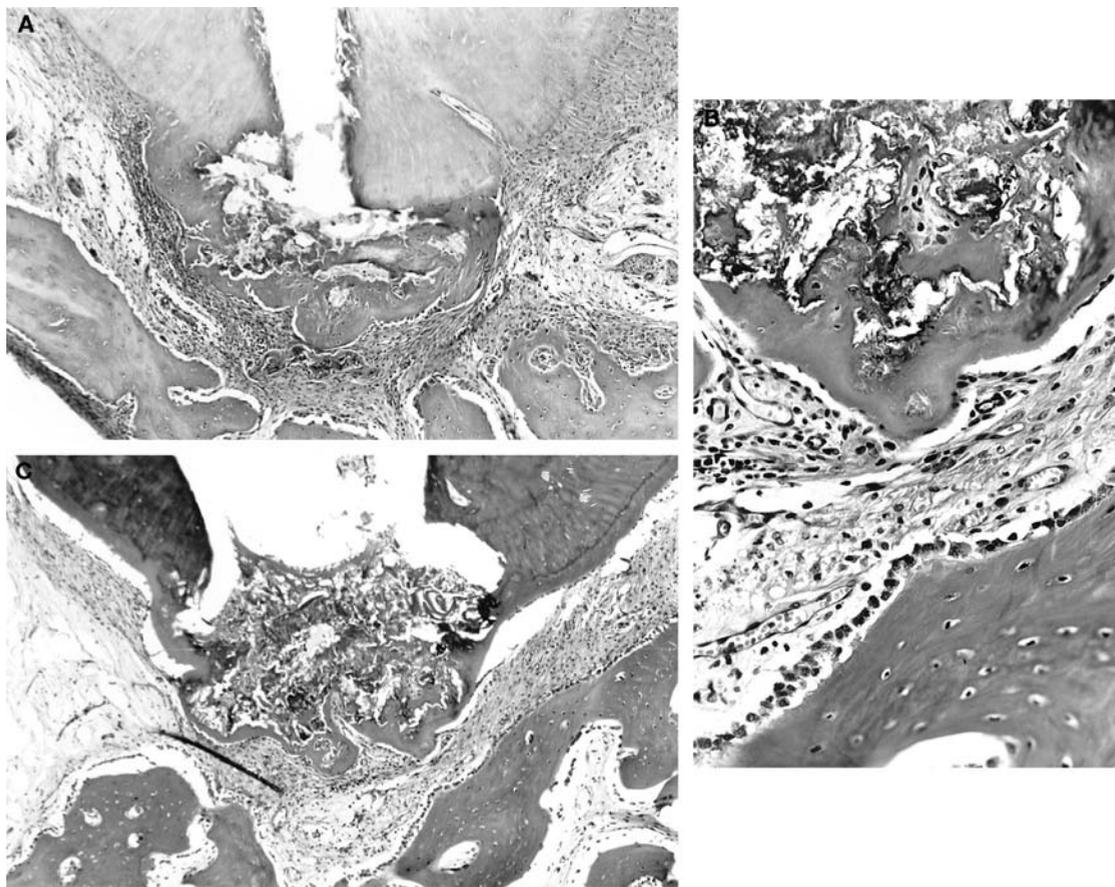


FIG. 2. Group 2 (LPS plus calcium hydroxide): (A) Formation of mineralized tissue in direct contact with overflow material. Normal periodontal ligament and alveolar bone (hematoxylin and eosin $\times 20$); (B) Periodontal space showing collagen fibers and cells. Normal alveolar bone with numerous osteoblasts (hematoxylin and eosin $\times 64$); (C) Formation of mineralized tissue in direct contact with overflow material. Normal periodontal ligament and alveolar bone (hematoxylin and eosin $\times 20$).

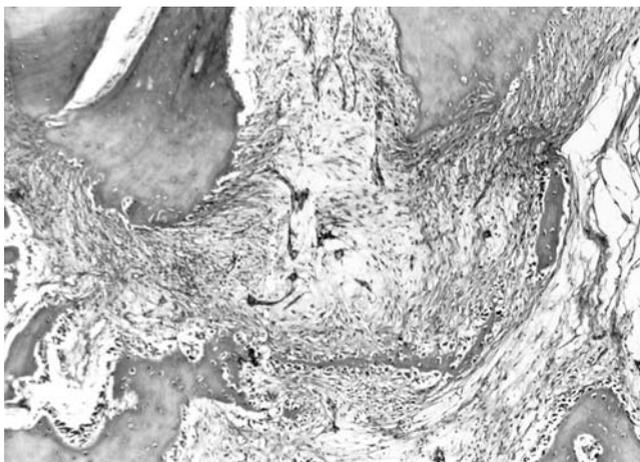


FIG. 3. Group 3 (saline): apical and periapical region showing intense formation of cells and collagen fibers and scant presence of mononuclear inflammatory cells. Normal periodontal ligament, cementum, and alveolar bone (hematoxylin and eosin $\times 20$).

endotoxin in the root canals of monkeys produces similar reactions 3 to 7 months later.

However, cellular differences were observed between groups 1 and 4. In group 4, there was a mixed inflammatory infiltrate,

mainly mononuclear, characteristic of a chronic process, whereas in group 1 inflammatory infiltrate with a predominance of densely clustered neutrophils occurred. This probably occurred because of the fast bacterial multiplication and release of endotoxins that induced organic defenses and led to a chronic condition in group 4; whereas the massive presence and the characteristic type of stimulation of LPS favored the development of an acute condition with infiltrate in group 1. Dwyer and Torabinejad (9) showed that *E. coli* endotoxin, injected into the teeth of cats, produced a basically neutrophil infiltrate also containing macrophages, plasmacytes, and lymphocytes with bone resorption. Using dogs and the endotoxin of *Salmonella minnesota*, Pitts et al. (13) showed that after 4 weeks it was possible to detect root and bone resorption and an inflammatory reaction with predominance of neutrophils. Similar results were reported by Mattison et al. (10) in dogs using the endotoxin of *Eikenella corrodens*.

The inflammatory infiltrate of group 4 was severe and diffuse, whereas that of group 1 was severe but circumscribed. The diffuse characteristic could be due to other bacterial products, such as hyaluronidase, collagenase, indole, H_2S , and toxic amines that dissociate collagen fibers and matrix. This would not occur with endotoxin only.

In this study, group 3 was used as a negative control to evaluate tissue response to the surgical procedure, because the enlargement of the root apex in itself triggers a local inflammatory reaction. Group 3 had mononuclear residual cells or no cells at all, close to the apical opening and also at a distance. The thickness of the

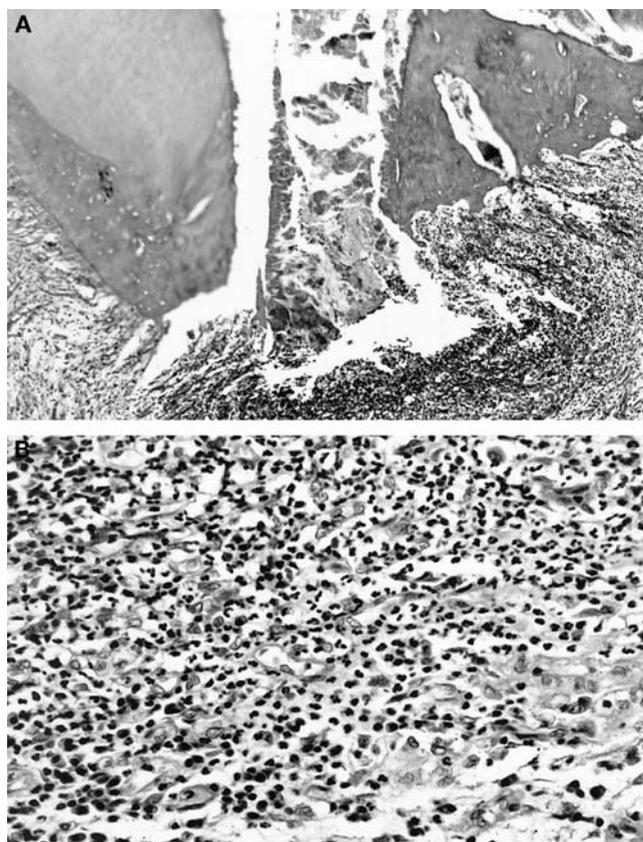


FIG. 4. Group 4 (experimentally induced periapical lesions): (A) Irregular apical root cementum, with evidence of resorption areas. Concentrated intense inflammatory infiltrate close to apical opening. Dilated apical delta ramifications containing necrotic debris (hematoxylin and eosin $\times 20$); (B) Mixed and diffuse inflammatory infiltrate, densely clustered (hematoxylin and eosin $\times 64$).

periodontal ligament was normal or slightly increased in six specimens and moderate in four. These results are in agreement with those reported by Dahlén et al. (12), Dwyer and Torabinejad (9), Pitts et al. (13), and Mattison et al (10).

According to Leonardo et al. (2), teeth with and without radiographically visible periapical periodontitis are different pathological entities that need different types of treatment. In teeth with radiographically visible periapical lesions, they recommend the use of antibacterial dressings between sessions. However, it seems unwise to use drugs that are only effective on bacteria, because these bacteria will release massive amounts of LPS, perpetuating inflammatory processes and bone resorption.

Recently, Safavi and Nichols (14) reported that, *in vitro*, calcium hydroxide hydrolyzes lipid A, which is the toxic component of the endotoxin; they also concluded that after lipid A hydrolysis, this potent toxic agent is converted to fatty acids and amino sugars that are not toxic (15). Barthel et al. (4) and Olsen et al. (16) also observed that calcium hydroxide could detoxify bacterial LPS *in vitro*.

In this study, histopathological analysis of group 2 roots showed a slight to absent inflammatory infiltration in 18 samples and a small increase in the thickness of the periodontal ligament in 11 roots and a moderate increase in 7. These results were statistically similar to those of the negative control group in which the root canals were filled with saline ($p < 0.05$).

A relevant finding of this study was the presence of mineralized tissue close to the apical opening in nine roots of group 2. It is

known that bacteria or its products must be absent if mineralization is to occur. Indeed, bacteria were not found in the samples of this group using Brown and Brenn stain.

This study shows that bacterial endotoxin—LPS—induces the development of a periapical lesion, in the teeth of dogs, and suggests that calcium hydroxide detoxifies bacterial endotoxin—LPS—*in vivo*, even in the high concentrations used. This quality adds to the excellent results of calcium hydroxide already obtained in clinical practice, and thus, it should be used in antibacterial dressings between sessions, in teeth showing pulp necrosis, and radiographically visible apical reactions.

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