

## Diffusion ability of endotoxin through dentinal tubules

### *Avaliação da difusão de endotoxinas através dos túbulos dentinários*

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**ABSTRACT:** The aim of this study was to evaluate the ability of endotoxin to diffuse through dentinal tubules towards the cement and to observe the period of time needed for it to reach the external root surface. Thirty single-rooted human teeth had their crowns and apices removed in order to standardize the root length to 15 mm. Teeth were instrumented until #30 K-file and made externally impermeable with epoxy adhesive, leaving 10 mm of the exposed root (middle third). The specimens were placed in plastic vials and irradiated (60Co gamma-rays). Then, they were divided into 2 groups (n = 15): G1) *Escherichia coli* endotoxin was inoculated into the root canal of the specimens and 1 ml of pyrogen-free water was put in the tubes; G2) (control): pyrogen-free water was inoculated into the root canals and 1 ml of pyrogen-free water was put in each tube. After 30 min, 2 h, 6 h, 12 h, 24 h, 48 h, 72 h and 7 days, the water of the tubes was removed and replaced. The removed aliquot was tested for the presence of endotoxin. Considering that the endotoxin is a B-lymphocyte polyclonal activator, at each experimental period, B-lymphocyte culture was stimulated with a sample of water removed from each tube and antibody (IgM) production was detected by ELISA technique. The results of IgM production were higher in groups of 24 h, 48 h, 72 h and 7 days in relation to the other studied groups, with statistically significant differences (ANOVA and Tukey's test  $p < 0.05$ ). Endotoxin was able to diffuse through the dentinal tubules towards the cement, reaching the external root surface after the period of 24 h.

**DESCRIPTORS:** Endotoxins; Tooth permeability; Dentin; Root canal.

**RESUMO:** O objetivo desta pesquisa foi avaliar *in vitro* a capacidade e o tempo necessário para a endotoxina se difundir pelos túbulos dentinários em direção ao cimento. Foram utilizados 30 dentes humanos unirradiculados, que tiveram suas coroas e seus ápices seccionados, padronizando-se o tamanho em 15 mm. Os dentes foram instrumentados até a lima K30 e impermeabilizados externamente com adesivo epóxi, deixando-se 10 mm de raiz exposta (terço médio). Os espécimes foram acondicionados em tubos plásticos e submetidos à radiação gama cobalto 60. Após a radiação, foram divididos em 2 grupos (n = 15): G1) foi inoculada uma solução de endotoxina de *Escherichia coli* no canal radicular dos espécimes e 1 mL de água apirogênica foi colocado no interior dos tubos; G2 (controle): foi inoculada água apirogênica nos canais radiculares e 1 mL de água apirogênica foi colocado em cada tubo. Após 30 min, 2 h, 6 h, 12 h, 24 h, 48 h, 72 h e 7 dias, a água do interior dos tubos foi removida e substituída por outra. A alíquota removida foi testada para se detectar presença de endotoxina através da produção de anticorpos (IgM) em cultura de linfócitos B, pois a endotoxina é um ativador policlonal dessas células. Os resultados foram submetidos à análise estatística ANOVA (5%) e teste de Tukey, em que foi verificado que a água removida dos tubos após 24 h, 48 h, 72 h e 7 dias induziu maior produção de anticorpos em relação aos demais grupos, com diferença significativa ( $p < 0,05$ ). Assim, a endotoxina foi capaz de se difundir pelos túbulos dentinários em direção ao cimento, atingindo a região externa da raiz após 24 h.

**DESCRIPTORIOS:** Endotoxinas; Permeabilidade dentária; Dentina; Canal radicular.

## INTRODUCTION

Bacterial infection is responsible for most pulpal and periapical inflammatory lesions<sup>10</sup>. Many factors affect the microbial growth and colonization in root canals, such as: nutrient concentration,

low tension of oxygen in canals with necrotic pulp and bacterial interactions<sup>23</sup>. These factors allow the predominance of certain species, in particular of the genera *Eubacterium*, *Peptococcus*, *Peptostrep-*

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*tococcus*, *Prevotella*, *Porphyromonas* and *Fusobacterium*<sup>23</sup>. Analyses of the microflora in infected root canals with necrotic pulps and apical periodontitis have showed a predominance of Gram-negative anaerobic bacteria<sup>14</sup>.

The cell wall of Gram-negative bacteria contains endotoxins that are lipopolysaccharides (LPS) with potential cytotoxic action, released during bacterial multiplication or death<sup>2</sup>. Endotoxins are responsible for several important biological effects, such as: neutrophil chemotaxis; complement system activation; fever induction; structural, functional and metabolic alterations in many organs and cells; hemodynamic modifications<sup>9</sup>; B-lymphocyte polyclonal activation<sup>7,15</sup>; macrophage activation and release of several inflammatory mediators such as platelet activating factor (PAF), tumor necrosis factor (TNF), interleukins (IL-1, IL-6 and IL-8), superoxide (O<sub>2</sub><sup>-</sup>), nitric oxide and prostaglandin<sup>2,19,20</sup>; and also osteoclasts' attraction. Some studies suggested that endotoxins are the main etiologic factor involved in the pathogenesis of the periapical inflammation, including bone resorption<sup>17,26</sup>.

The effects of endotoxin on the periapical tissues of animals (monkeys, dogs and cats) are bone loss, and resorptive and inflammatory reactions in all experimental teeth<sup>13,17,22</sup>. Khabbaz *et al.*<sup>11</sup> (2001) demonstrated the presence of endotoxin in the vital pulp of symptomatic and asymptomatic teeth. Endotoxin content in infected root canals was higher among symptomatic teeth, teeth with radiolucent areas and with exudation than in those without these alterations<sup>8,21</sup>. Dahlén, Bergenholtz<sup>6</sup> (1980) observed that the endotoxic activity of the root canal samples was correlated with the presence and the number of Gram-negative bacteria in the root canal.

Elimination of microorganisms from the root canal system has been the major goal of endodontic treatment<sup>3</sup>. However, after bacterial death, endotoxin can remain and diffuse through dentin. Nissan *et al.*<sup>14</sup> (1995) reported that endotoxin is capable of passing through 0.5 mm of dentin in apical direction. Endotoxin is also able to penetrate through obturation materials<sup>25</sup> and can reach the periapical region faster (23 days) than bacteria (62 days)<sup>1</sup>. Horiba *et al.*<sup>8</sup> (1991) studied the endotoxin content in serial dentin samples of infected root canals. These authors observed that this content was significantly higher in samples from the pulpal surface to 300 µm in depth than in those taken farther toward the cementum side. However, little

attention has been given to the capacity and velocity of endotoxin diffusion toward the cement.

The aim of this study was to evaluate *in vitro* the ability of endotoxin to diffuse through dentinal tubules towards the cement and to observe the period of time needed for this substance to reach the outer root surface.

## MATERIALS AND METHODS

Thirty freshly extracted single-rooted human teeth were used in the study. These teeth were collected from patients of the School of Dentistry of São José dos Campos, São Paulo State University, Brazil, and the selection of teeth was made considering relative dimensions and similarity in morphology. Debris, calculus and soft tissue remnants on the root surfaces were cleaned using a Gracey curette (Duflex, Rio de Janeiro, RJ, Brazil) and the teeth were observed with a stereomicroscope (Zeiss, Oberkochen, Germany) to detect any cracks or damage in the root area. Compromised teeth were discarded. All teeth were stored in saline solution (NaCl 0.85%) (Merck, Darmstadt, Germany) until use. The selected specimens presented a diameter of approximately 4.5 ± 0.5 mm in the middle third, measured with a vernier caliper (Marberg, China). The apical 3 mm of each root were transversely sectioned using a water-cooled diamond disc (Jon, SP, Brazil) and the crown was sectioned at a point in order to standardize the root length to approximately 15 mm. Working length was determined by subtracting 1 mm from this measurement.

Canals were enlarged until #30 K-file (Maillefer, Michigan, USA), so that the dentinal walls remained with 1.75 ± 0.25 mm in thickness in the medium third. Buccolingual and mesiodistal radiographs were taken and evaluated with a scale for confirming this thickness. Canals were irrigated with 5 ml of 1% sodium hypochlorite solution (Farmácia de Manipulação Aqua Marina, São José dos Campos, SP, Brazil) for each file used. The smear layer was removed with 17% EDTA (Farmácia de Manipulação Aqua Marina, São José dos Campos, SP, Brazil) for 3 min and specimens were washed with a final flush (10 ml) of phosphate buffered saline (PBS) (Merck, Darmstadt, Germany) and dried with paper points (Dentsply, RJ, Brazil). The apical region was sealed with light-cured resin composite (3M Dental Products, St. Paul, USA) and the external surface of the apical and cervical thirds was covered with epoxy adhesive (Araldite, Brascola, São Paulo, SP, Brazil), except the 10 mm of the root middle third.

All the roots were sterilized by autoclaving (Fanen, SP, Brazil) (20 min at 121°C) and placed in plastic vials (Eppendorf, New York, USA). The specimens and all the material used in the experiment such as gauze, cotton, scissors and micropipette tips were irradiated with 60 Co gamma-rays for degradation of the pre-existing LPS<sup>5</sup>.

Under sterile conditions (laminar air flow chamber – Veco, SP, Brazil), 1 ml of non-pyrogenic water (Charles River Endosafe, Charleston, SC, USA) was put in each plastic vial, so that the middle and apical thirds of the root were submerged. Then, the specimens were randomly assigned to 2 groups (n = 15): GE (experimental group): 10 µl (40 IU) of a standard solution containing *Escherichia coli* 055:B5 endotoxin (Sigma, St. Louis, USA) was inoculated in root canals; GC (control group): 10 µl of non-pyrogenic water was inoculated in root canals. All the teeth were sealed with pyrogen-free cotton balls (Apolo, MG, Brazil) and the plastic vials were capped.

For evaluation of the presence of the endotoxin on the external root surfaces, water inside the tubes was removed and evaluated at different times after the inoculation of the endotoxin in the root canals. Eight experimental groups were obtained: GE1) after 30 min; GE2) after 2 hours; GE3) after 6 hours; GE4) after 12 hours; GE5) after 24 hours; GE6) after 48 hours; GE7) after 72 hours and GE8) after 7 days. In the control specimens, which received only non-pyrogenic water in the root canals, the removal of the water from the vials was accomplished at two times: GC1) after 24 hours and GC2) after 7 days. Immediately after water removal, another aliquot of non-pyrogenic water was transferred to all the vials (n = 30), which were maintained at 37 ± 1°C under 100% humidity condition until the final period of 7 days.

Considering that the endotoxin is a B-lymphocyte polyclonal activator, at each experimental time B-lymphocyte culture was stimulated with a sample of water removed from each tube. Antibody production was detected by ELISA for verifying the presence of the endotoxin in the outer root surface.

### Antibody production in B lymphocyte culture

Four male Swiss mice (*Mus musculus*) with approximately six months of age were sacrificed and the spleen of each animal was removed and macerated in a pyrogen-free Falcon tube (Becton Dickinson & Co., NJ, USA) containing RPMI 1640 medium, pH 7.2 (Sigma, St. Louis, USA), to ob-

tain B-lymphocytes. The tube was centrifuged at 534.8 x g for 10 min and the pellet was resuspended in 30 ml of RPMI 1640 medium supplemented with 10% bovine fetal serum (Sigma, St. Louis, USA). In order to prevent contamination of the culture, sterile conditions were maintained by sterile laminar flow.

For cell viability verification, the trypan blue exclusion assay (0.4%) (Sigma, St. Louis, USA) and a buffer (Sigma Chemical Co., St. Louis, MO, USA; Reagen, RJ, Brazil; Vetec, RJ, Brazil; Labsynth, SP, Brazil) to promote erythrocyte rupture were used. The viable cell count was 4.22 × 10<sup>6</sup> cells/mL. Approximately 1 × 10<sup>6</sup> viable cells (250 µl) were transferred to each well of non-pyrogenic cell culture plates (24-well, Costar, NY, USA). These plates were incubated for 24 h at 37°C in a humidified atmosphere of 5% carbon dioxide (CO<sub>2</sub> – Nuair, Minnesota, USA). B-lymphocytes of each well of cell culture plates were stimulated with an aliquot of 30 µl of the water removed from each plastic tube, at the different periods of time. Fifteen wells containing only pure cell culture were considered the cell culture control group (GC3). The plates were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air for four days. The enzyme-linked immunosorbent assay (ELISA) was used to determine the total IgM levels in the culture supernatant.

ELISA was performed in six microplates (96-well, Hemobag, São Paulo, SP, Brazil). All the plates were coated with mouse anti-immunoglobulin M (Sigma, St. Louis, USA) at a concentration of 0.5 mg/ml. The plates were incubated for 2 h at 37°C and maintained in the refrigerator until use.

At the day of the experiment, plates were blocked with 0.5% gelatin (Sigma Chemical Co., St. Louis, MO, USA) (G) in phosphate buffered saline (PBS) (Merck, Darmstadt, Germany) and 0.2% bovine serum albumin (BSA) (Sigma, St. Louis, USA) (45 min at 37°C). Culture supernatants (100 µl) were added to each well and incubated at 37°C for 2 h, followed by washing with 0.1% Tween-20 in PBS (Merck, Darmstadt, Germany) (PBS-T). All the tests were performed in duplicate. Anti-mouse IgM peroxidase conjugate (Sigma, St. Louis, USA), diluted 1,000 times with PBS-GT (0.5% gelatin and 0.1% Tween-20 in PBS) (Merck, Darmstadt, Germany), was added to the plates, which were then incubated for 1 h at 37°C. After washing with PBS-T, peroxidase enzyme activity was detected by addition of the substrate, 50 µl of o-phenylenediamine (Sigma Chemical Co., St.

Louis, MO, USA) (1 mg/ml) in solution of 0.1 M citrate (Merck, Darmstadt, Germany) and 0.03% H<sub>2</sub>O<sub>2</sub> (Bioquímica, São José do Rio Preto, Brazil) for 10 min. Finally, the reaction was stopped with 2.5 N H<sub>2</sub>SO<sub>4</sub> (Merck, Darmstadt, Germany). The optical density values (OD) of the wells were read at an absorbance of 490 nm on a Bio-Rad Microplate reader Model 3550 (Bio-Rad Laboratories, Hercules, California). For each specimen, OD mean values were obtained and the results were analyzed statistically by ANOVA and Tukey's test. The level of significance was established at 5% ( $p = 0.05$ ).

## RESULTS

Optical density (OD) mean values and standard deviation obtained for each experimental group, according to the evaluated period, are presented in Table 1.

Stimulation of antibody (IgM) production in B-lymphocyte culture with aliquots of water removed from some experimental groups – GE1 (30 min), GE2 (2 h), GE3 (6 h), GE4 (12 h) – was similar to that of the control groups – GC1 (24 h), GC2 (7 days) and GC3 (pure cell culture) – ( $p > 0.05$ ), demonstrating the absence of endotoxin in the external root surface in these groups. The aliquots removed from the GE5 group (24 h) induced higher IgM production than those of all the other experimental and control groups ( $p < 0.05$ ), showing the presence of endotoxin in the external root surface after a period of 24 hours. The results obtained for groups GE6 (48 h), GE7 (72 h) and GE8 (7

days) were similar ( $p > 0.05$ ); and these results were higher than those obtained for control groups ( $p < 0.05$ ), also demonstrating the presence of endotoxin in these experimental groups. GC1 (24 h) and GC2 (7 days) control groups presented similar results in relation to GC3 control group (pure cell culture) ( $p > 0.05$ ), indicating the absence of the endotoxin.

## DISCUSSION

In this study, the endotoxin used was from *Escherichia coli*, since it is the standard endotoxin used in most of the studies<sup>2,13,22</sup>. Chemically, endotoxins are lipopolysaccharides that consist of three different parts: a non-polar lipid component, called lipid A, the core oligosaccharide and a heteropolysaccharide representing the surface antigen (O-antigen). The O-antigen is built up of a chain of repeated oligosaccharide units, which are strain specific and determinative for the serological identity of the respective bacterium<sup>16,18</sup>. The core oligosaccharide has a conserved structure with an inner KDO (2-keto-3-deoxyoctonic acid)-heptose region and an outer hexose region<sup>16</sup>. However, lipid A is the main component responsible for the endotoxic activity of LPS and its primary structure is well conserved among different species of Gram-negative bacteria<sup>12</sup>. Lipid A carries a total negative load due to the presence of three phosphoryl radicals and two to three carboxyl radicals associated with the inner region of the core, and another two phosphoryl radicals associated with the disaccharide part of the lipid<sup>16,18</sup>. Considering this, even if *E. coli* is not commonly found in root canals with necrotic pulps, its endotoxin presents the basic structure of the lipid component, which represents the responsible active center for the properties of LPS.

The test of B-cell stimulation was used in this study to observe the presence of the endotoxin in the samples based on the mitogenic effect of LPS on B-lymphocytes<sup>7</sup>. Oliveira *et al.*<sup>15</sup> (2005), in a previous study, reported the use of this methodology to evaluate the effect of intracanal medication on LPS. In the presence of endotoxin, B-lymphocytes proliferate and produce antibodies that are detected by ELISA. Endotoxin is a T-independent antigen and has the capacity of activating various B cell clones. Considering this, we accomplished the cell culture using mice spleen<sup>15</sup>, which represents an important lymphoid secondary organ. To observe antibody production in the presence of the endotoxin, an interval of 4 days after the placement

**TABLE 1** - Optical density (OD) mean values and standard deviation obtained for each group.

Groups	Mean values (OD)	Standard deviation
GE1 (30 min)	222.8	25.9
GE2 (2 h)	221.2	27.7
GE3 (6 h)	225.9	18.6
GE4 (12 h)	232.5	19.7
GE5 (24 h)	509.6	23.2
GE6 (48 h)	402.0	21.1
GE7 (72 h)	409.4	26.8
GE8 (7 days)	423.0	21.9
GC1 (24 h)	227.4	20.6
GC2 (7 days)	229.5	25.6
GC3 (pure cell culture)	213.0	25.9

GE: experimental group; GC: control group.

of the samples in the cell culture was maintained, because it is considered a period of time which is enough for the proliferation and transformation of the cells into plasmocytes. The polyclonal activation of the B-cells *in vitro* and in mice increases the synthesis of IgM more than that of IgG<sup>4</sup>. In this study, the increase in the production of IgM antibodies was detected using an anti-mouse IgM peroxidase conjugate by enzyme immunosorbent assay.

Higher antibody production could be observed in GE5 (24 h), GE6 (48 h), GE7 (72 h) and GE8 (7 days) groups. These results indicate that the endotoxin presented the capacity to diffuse through the dentinal tubules, in agreement with the reports of Nissan *et al.*<sup>14</sup> (1995). These authors studied the ability of bacterial endotoxin to diffuse through human dentin in apical direction and concluded that endotoxin was capable of passing through 0.5 mm of dentin in a period that ranged from 15 min to 4 1/2 h, so that the dentin may act as a semipermeable barrier, thus allowing the passage of bacterial products to the pulp. In the present study, we evaluated the ability of the endotoxin to diffuse through dentinal tubules toward the cement. Considering that the specimens presented dentinal walls of approximately 1.75 mm in thickness, the requested time for the endotoxin to reach the outer root surface was of 24 h. Horiba *et al.*<sup>9</sup> (1990) evaluated the endotoxin content in a series

of samples of dentin from the pulpal surface of infected root canals to the cementum, independently of the time, and observed that the endotoxin content was significantly higher in samples from the pulpal surface of dentin to 300  $\mu$ m in depth than in those samples from 301 to 800  $\mu$ m toward the cementum side.

The present study demonstrated that the endotoxin has the capacity to diffuse deeply through dentinal tubules towards the cement. These data could be of great importance in endodontic practice since they suggest that dentinal tubules can be LPS reservoirs. Endotoxins, if not detoxified during the treatment, could diffuse through dentinal tubules, return to the canal or penetrate obturation materials and reach the periapical tissues, inducing or maintaining a periapical lesion. In this way, due to the depth of diffusion of endotoxins inside the dentinal tubules, we believe that the concomitant use of irrigation agents and intracanal medications with capacity of detoxifying LPS is essential<sup>15,24</sup> in order to warrant the success of endodontic treatments.

## CONCLUSIONS

Considering the results obtained in this study, it can be concluded that endotoxin diffused through dentinal tubules toward the cement, reaching the outer root surface after the period of 24 h.

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