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Monitoring the effectiveness of root canal procedures on endotoxin levels found in teeth with chronic apical periodontitis

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ABSTRACT

Objective: The aim of this study was to monitor the effectiveness of root canal procedures by using different irrigants and intracanal medication on endotoxin levels found in root canals of teeth with chronic apical periodontitis. Material and methods: Thirty root canals of teeth with pulpal necrosis associated with periapical lesions were selected and randomly divided into groups according to the irrigants used: GI - 2.5% NaOCl, GII - 2% chlorhexidine (CHX) gel, and GIII - saline solution (SS) (all, n=10). Samples were collected with sterile/apyrogenic paper points before (S1) and after root canal instrumentation (S2), after use of 17% ethylenediaminetetraacetic acid (EDTA) (S3), and after 30 days of intracanal medication (Ca(OH)2+SS) (S4). A turbidimetric kinetic Limulus Amebocyte Lysate assay was used for endotoxin measurement. Results: Endotoxins were detected in 100% of the root canals investigated (30/30), with a median value of 18.70 EU/mL. After S2, significant median percentage reduction was observed in all groups, irrespective of the irrigant tested: 2.5% NaOCl (99.65%) (GI), 2% CHX (94.27%) (GII), and SS (96.79%) (GIII) (all $p<0.05$). Root canal rinse with 17% EDTA (S3) for a 3-minute period failed to decrease endotoxin levels in GI and a slight decrease was observed in GII (59%) and GIII (61.1%) (all $p>0.05$). Intracanal medication for 30 days was able to significantly reduce residual endotoxins: 2.5% NaOCl (90%) (GI), 2% CHX (88.8%) (GII), and SS (85.7%) (GIII, $p<0.05$). No differences were found in the endotoxin reduction when comparing S2 and S4 treatment groups. Conclusion: Our findings demonstrated the effectiveness of the mechanical action of the instruments along with the flow and backflow of irrigant enduring root canal instrumentation for the endotoxin removal from root canals of teeth with chronic apical periodontitis. Moreover, the use of intracanal medication for 30 days contributed for an improvement of endotoxin reduction.

Keywords: Endotoxins. EDTA. Chlorhexidine. Sodium hypochlorite. Root canal. Medications.

INTRODUCTION

Lipopolysaccharides (LPS), also known as endotoxins, present on the outer layers of the membrane of Gram-negative bacterial species, have been detected in 100% of the root canals with endodontic infection. Higher levels have been related to the severity of bone destruction in periapical tissues as well as to the development of clinical features of endodontic disease[5-9,13-14,21,25].

It has long been known that endotoxins found in pulp spaces and periapical areas are highly immunostimulatory, even at low concentrations[2,5]. This molecule is recognized by the human host as a foreign[3], eliciting a potent immune response that results in the release of different pro-inflammatory cytokines commonly involved in the pathogenesis of apical periodontitis[9-11]. In the light of this antigenic activity, detoxifying endotoxins from root canals during endodontic treatment may facilitate the

healing process of the periapical tissues[9,11-13].

Considering that necrotic teeth associated with periapical lesions contain an increased amount of endotoxins[2,22], there is a great effort in the search for effective clinical procedures for their removal.

Clinical studies have demonstrated that chemomechanical preparation (CMP) using hand K-files for apical preparation 1 mm from the radiographic apex together with either sodium hypochlorite (NaOCl) or chlorhexidine (CHX) used as auxiliary chemical substance, has little or no effect on LPS present in root canals[6,12,14], thus contributing to a reduction of only 50% of the LPS load. Conversely, root canal preparation with rotary instruments in the full extension of the root canal was capable to achieve a removal of 90% of the LPS content[11,13,25]. However, this virulence factor can still be detected in 100% of the root canal samples after instrumentation.

Because of the detoxifying activity of calcium hydroxide [$Ca(OH)_2$], its use as intracanal medication in infected root canals has been investigated[12-13,16-21,23-26,28]. Vianna, et al.[29] (2007) reported that root canal dressing for 7 days contributes to a reduction of endotoxin in only 1.4% compared to the reduction observed after root canal instrumentation. Furthermore, the application of $Ca(OH)_2$ for 14 days contributed to improve the removal of endotoxins by 12%[30]. This finding suggests that an extended period of intracanal medication could contribute to a better removal of endotoxins from infected root canals. Recently, Sousa, et al.[25] (2014) have demonstrated that $Ca(OH)_2$+2% CHX gel as root canal medicament for a 30-day period was able to significantly reduce endotoxins from root canals associated with acute apical abscesses. However, there is no currently published clinical study monitoring the effectiveness of root canal procedures, involving both chemomechanical preparation with different auxiliary chemical substances and the use of $Ca(OH)_2$ as root canal medication for an extended period of 30 days, aiming at the reduction of endotoxins from root canals of teeth with chronic apical periodontitis.

Therefore, this study was conducted to monitor the effectiveness of root canal procedures by using different irrigants and intracanal medication on endotoxin removal from root canals of teeth with chronic apical periodontitis.

MATERIAL AND METHODS

Patient selection

Thirty patients who attended the Piracicaba Dental School (SP, Brazil) for primary endodontic treatment were included in this study. A detailed dental history was obtained from each patient. Patients who had received antibiotic treatment during the previous 3 months or who had a general disease were excluded from the study. The selected teeth showed absence of periodontal pockets deeper than 4 mm. Their pulp chamber exhibited no visual communication with the oral fluid. The Human Research Ethics Committee of the Piracicaba Dental School approved a protocol describing the sample collection for this investigation, and all patients signed an informed consent form for their participation in this research.

All the selected teeth were single-rooted showing the presence of one root canal with pulp necrosis and radiographic evidence of apical periodontitis. A radiolucent area >4 mm was present in 11/30 patients. None of the patients reported spontaneous pain.

Sampling procedure

For degradation of preexisting endotoxins, all the materials used in the experiment were sterilized by gamma radiation with cobalt 60 (20 KGy for 6 hours)[4]. The method used for disinfecting the operative field has been previously published[5-6,9,13,14]. Initially, the teeth were isolated with a rubber dam. The crown and surrounding structures were disinfected with 30% H_2O_2 (volume/volume [V/V]) for 30 seconds, followed by 2.5% NaOCl for the same period of time and then inactivated with 5% sodium thiosulphate.

With regard to the access cavity preparation, a sterile/apyrogenic high-speed diamond bur (KG-Sorensen, Barueri, SP, Brazil) was used in conjunction with manual irrigation with sterile saline. Before entering the pulp chamber, the access cavity was disinfected according to the protocol described above. A new sterile/apyrogenic bur was used to access the canal.

Root canal length was determined by preoperative radiograph and then, the first endotoxin sample (s1) was taken by introducing a sterile/apyrogenic paper point #15 (Dentsply-Maillefer, Ballaigues, Switzerland) into the full length of the canal and left there for 1 minute. Then, the sample was placed in an apyrogenic glass and immediately suspended in 1 mL Limulus Amebocyte Lysate (LAL) water for further quantification of endotoxins.

After the first endotoxin sampling (S1), root canal length was confirmed by apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel) and the teeth were randomly divided into 3 groups according to the irrigant used for CMP: GI - 2.5% NaOCl (n=10), GII - 2% CHX gel (n=10), and GIII - saline solution (n=10).

The root canals were then prepared with Mtwo instruments (VDW, Munich, Germany) with permanent rotation at a speed of 300 rpm[13]. Mtwo instruments (10/.04, 15/.05, 20/.06, 25/.06,

30/.05, 35/.04 and 40/.04) were used up to full length of the root canal using a single-length technique, with gentle in-and-out movements while gradually forcing apically[13]. The use of each instrument was followed by the selected irrigant used for CMP.

In GI, root canal irrigation was performed with 5 mL of 2.5% NaOCl solution, simultaneously removed by suction. In GII, 1 mL of 2% CHX gel was introduced into the root canal with a 3 mL syringe and a 27-gauge needle (Ultradent Products, South Jordan, UT, USA) and, immediately after the use of each instrument, 5 mL of sterile/apyrogenic saline solution were used to wash the canal. In GIII, root canal irrigation was performed with 5 mL of saline solution, simultaneously removed by suction. All irrigation procedures were performed with a 27-gauge needle.

After root canal preparation, NaOCl was inactivated with 5 mL of sterile 5% sodium thiosulphate for 1 minute, which then was removed with 5 mL of sterile/apyrogenic saline solution. The chemical activity of CHX was inactivated with 5 mL of a solution containing 5% Tween 80 and 0.07% (v/v) lecithin for 1 minute, which was then removed with 5 mL sterile/apyrogenic saline solution.

After neutralization of the auxiliary chemical substances, a second endotoxin sample (s2) was taken from the root canals. Removal of the smear layer was performed with 3 mL of 17% ethylenediaminetetraacetic acid (EDTA) solution for 3 minutes, followed by a final flush with 5 mL of sterile/apyrogenic saline solution. Next, the third endotoxin sample (S3) was taken.

Subsequently, the canals were filled with freshly prepared paste of $Ca(OH)_2$ in saline solution (1:1). The paste was inserted into the canals by using a sterile/apyrogenic Lentulo bur (Dentsply-Maillefer, Ballaigues, Switzerland). Special attention was paid to complete fill the root canal with the medication. A cotton pellet was used to condense the paste at the canal orifice level and a periapical radiography was performed to ensure the quality of the root canal fill. Next, the access cavities were sealed with light-cured resin composites (3M Dental Products, St Paul, MN, USA). After 30 days of intracanal medication, the canals were aseptically accessed under rubber dam isolation according to the protocol for disinfection, as previously described. The medication was removed with a file size 45, 0.02 taper against the lateral walls of the root canal and copious irrigation with 10 mL of saline solution. Afterwards, a final root canal sampling (S4) was performed.

Determination of endotoxin concentration

The turbidimetric test (Pyrogent 5000® Lonza, BioWhitaker, Inc, Walkersville, MD, USA) was used to measure endotoxin concentrations in the root canals according to the LAL technique, as previously published by the authors[11,13,14]. As a parameter for calculation of the amount of endotoxins in the root canal samples, a standard curve was plotted by using the endotoxin of known concentration supplied by the kit (100 EU/mL), with its dilutions reaching final concentrations (0.01, 0.1, 1, 10 EU/mL) according to the manufacturer's instructions. Firstly, the endotoxin samples were suspended in 1 mL of LAL water supplied by the kit and agitated in vortex for 60 seconds. Next, 100 mL of the blank, followed by the same volume of the standard endotoxin solutions (0.01, 0.1, 1, 10 EU/mL) and 100 mL of the samples, were immediately added in duplicate to a 96-well microplate with their respectively positive controls. To avoid inhibition or enhancement of LAL, a known concentration of *Escherichia coli* endotoxin was added to the clinical samples, as recommended in the manufacturer's instructions (spike procedure). Both unspiked and spiked samples (positive control) had their endotoxin concentrations automatically calculated. The amount of endotoxins recovered was equal to the known spike concentration, ranging from 50% to 200%. A 96-well microplate (Corning Costar, Cambridge, MA, USA) was placed on a heating block at 37°C and maintained there at this temperature throughout the assay. The test procedure was performed according to the manufacturer's instructions. The absorbance of endotoxins was individually measured by using an enzyme-linked immunosorbent assay plate reader (Ultramark, Bio-Rad Laboratories, Inc, Hercules, CA, USA). During calculation of the endotoxin concentration, the microplate reader monitors the absorbance of each well of the microplate continuously at 340 nm by means of the WinKQCL software (BioWittaker, Cambrex Co, Walkersville, MD, USA). The WinKQCL software automatically performs a log/log linear correlation of the reaction time of each standard curve with its corresponding endotoxin concentration, including printing the standard curve parameters. If the absolute value of the correlation coefficient (r) is 0.980, a polynomial model can be used to construct a standard curve and, in turn, predict endotoxin concentrations of the samples.

Statistical analysis

The data obtained with LAL assay were statistically analyzed using SAS Software, version 9.1 (SAS Institute, Cary, NC, USA). The Kolmogorov-Smirnov's test showed that the distributions of the studied variables deviated from normality. Friedman's test was performed to compare the levels of endotoxin at different sampling times. Wilcoxon's test was used when significant differences were found. Comparison between the experimental

Table 1- Distribution of the median values of endotoxins (EU/mL) found in primarily infected root canals at all different sampling times

Groups	Initial (S1)	After root instrumentation (S2)	After 17% EDTA (S3)	After medication (S4)
	Median (CB 95%)	Median (CB 95%)	Median (CB 95%)	Median (CB 95%)
NaOCl (I)	25.80 (13.75 – 51.47)	0.08 (0.03 – 0.15)	0.1 (0.1 – 0.23)	0.01 (0.01 – 0.03)
CHX (II)	18.65 (8.41 – 44.35)	0.66 (0.48 – 1.31)	0.27 (0.14 – 0.85)	0.06 (0.01 – 1.2)
SS (III)	13.95 (-2.08 – 73.53)	0.18 (-4.85 – 13.33)	0.07 (0.03 – 0.15)	0.01 (-0.01 – 0.19)

S1- Before instrumentation; S2- After instrumentation; S3- After use of 17% ethylenediaminetetraacetic acid (EDTA); S4- after 30 days of intracanal medication (Ca(OH)2+SS); CB (confidence bound)
CHX=chlorhexidine
SS=saline solution

Table 2- Distribution of the median percentage values of endotoxin reduction found in primarily infected root canals after different sampling times

S1 to S2 (%)	S2 to S3 (%)	S3 to S4 (%)
Median (CB 95%)	Median (CB 95%)	Median (CB 95%)
99.65 (99.28 – 99.83)^{Aa}	No improvement	90 (88.92 – 100.00)^{Aa}
94.27 (65.13 – 101.99)^{Aa}	59 (46.10 – 64.20)^{Ab}	88.8 (84.06 – 119.59)^{Aac}
97.22 (74.77 – 105.73)^{Aa}	61.1 (51.04 – 69.79)^{Ab}	85.7 (83.08 – 100.16)^{Aac}

*Different letters means statistically significant differences ($p<0.05$)

groups (GI, GII and GIII) was performed by using the Kruskal-Wallis' test. Significance level was set at 5% ($p<0.05$).

RESULTS

Table 1 provides an overview of the individual median values of EU/mL found in root canals of teeth with chronic apical periodontitis at different sampling times. Particular information of the median percentage values of endotoxin reduction at the different sampling times is shown in Table 2. For validation of the LAL assay, the standard curve fulfilled the criteria of linearity (r=1) for all assays, as reported by the guidelines. The LAL assay indicated that endotoxins were present in 100% of the root canals investigated (30/30), with a median value of 18.7 EU/mL, ranging from 0.16–82.1 EU/mL.

After root canal instrumentation (s2), a significant median percentage reduction was observed in all groups, irrespective of the irrigant tested: 2.5% NaOCl (99.65%) (GI), 2% CHX (94.27%) (GII), and SS (96.79%) (GIII) (all $p<0.05$) (Table 2). No differences were found among the irrigants used.

Root canal rinse with 17% EDTA (s3) for a 3-minutes period failed to decrease endotoxin levels in GI. However, after its use, there was a slight reduction of endotoxins in in GII (59%) and GIII (61.1%) (all $p>0.05$).

Intracanal medication for 30 days (S4) was able to significantly reduce residual endotoxins: 2.5% NaOCl (90%) (GI), 2% CHX (88.8%) (GII), and SS (85.7%) (GIII, $p<0.05$) comparing to S3. However, no differences were found in the endotoxin reduction when comparing S2 with S4.

Comparing the results gotten after intracanal medication with those found in the baseline (s1), a higher percentage of endotoxin removal was achieved as follows: 2.5% NaOCl (99.9%) (GI), 2% CHX (99.4%) (GII), and SS (99.9%) (GIII) (Table 2).

DISCUSSION

The present study monitored in the same teeth the levels of endotoxins after each endodontic procedure, providing particular information on the effectiveness of every phase of the root canal therapy. Furthermore, the results found will allow a further development of clinical protocols to enhance detoxification.

For this purpose, LAL assay was used to measure the levels of endotoxins and to evaluate the effect of root canal procedures on its removal. Among the LAL methods, the turbidimetric method (Pyrogent-5000®), which presents a wide range of detection (0.01 to 100 EU/mL), proved to be

sensitive and effective in recovering endotoxin from endodontic samples even at very low concentrations.

In our study, endotoxin was recovered from all root canal samples collected from infected root canals, as reported in previous studies[6-9,13-14,21,25]. At the baseline samples, endotoxin was detected in a median value of 18.7 EU/mL. After chemomechanical preparation, statistically significant percentage values of endotoxin reduction were achieved by the root canal instrumentation with rotary Mtwo files accomplished by 2.5% NaOCl (99.65%), 2% CHX gel (94.27%) as well as saline solution (96.79%). It must be highlighted that the root canal preparation with an inert irrigant (saline solution) obtained a great percentage of reduction with no differences among the antimicrobial substances tested. In the face with the "no-detoxifying" ability of NaOCl and CHX, it seems reasonable to assume that the chemical action of these irrigants is critical for the removal of intracanal bacteria[14,29], but not a relevant factor regarding endotoxin removal. In accordance with previous studies[6,11,13,15,25], our results showed that the mechanical action of the endodontic instruments associated with the physical action of the root canal irrigation seems to play a role in the endotoxin removal from infected root canals.

It is worth to mention that the apical limit of the root canal instrumentation exerts a direct influence in endotoxin removal[11]. Particularly in cases of pulpal necrosis associated with a periapical lesion, as evaluated by this study, the establishment of the working length up to the full extension of the root canal, rather than 1 mm from the apical foramen, might have ensured a better debridement by the mechanical action of the instruments compared to previous investigations[6,14,29].

During and after root canal instrumentation it is necessary to remove both the organic and inorganic remnants by the use of chelating agents [i.e., ethylenediaminetetraacetic acid (EDTA)][17]. EDTA strongly chelates with Ca2+ and other divalent cations[1], demineralizes dentin and penetrates into deeper layers (130 μm) of dentin not prepared chemomechanically[25]. Due to its properties, we hypothesized that EDTA might contribute for the endotoxin removal from root canal walls during endodontic treatment. However, no significant differences were observed in the percentage values of endotoxin reduction after the use of 17% EDTA when compared with the reduction obtained after root canal instrumentation, agreeing with the findings of Sousa, et al.[25] (2014).

It is reported that the use of EDTA alone is not effective for complete removal of the smear layer, in both young and old dentin, but the combination with NaOCl seems to be the best cleansing approach[17]. Surprisingly, our results showed that 17% EDTA failed to decrease endotoxin levels in the NaOCl group after smear layer removal. It is known that the decalcifying efficacy of acids and chelating agents depends largely on application time, the pH and concentration of the solution, and the hardness of dentin[18]. In addition, sclerosis of dentin is another important factor that affects the efficiency of demineralizing agents[27].

In this study, root canal preparation was able to drastically reduce endotoxins, despite not eliminating all content. It is known that minimal amounts of endotoxins can express potent bioactivity and thus maintain an inflammatory process[9,11-13]. Calcium hydroxide is arguably the most commonly used intracanal medication and seems to be clinically beneficial when endotoxin is suspected in a canal[2]. Due to its very alkaline pH of 10–12, $Ca(OH)_2$ neutralizes LPS by hydrolysis of ester bonds in the lipid A chains. The slightest variation of the fatty acid chains of the bioactive molecule's lipid A portion renders the entire molecule biologically inactive[2].

The ability of calcium hydroxide medication for 30 days to improve the endotoxin reduction from root canals of teeth with chronic apical periodontitis was demonstrated by the present study. After 30 days of $Ca(OH)_2$ medication, the levels of endotoxins were reduced to a median percentage value of 99.9% (NaOCl and SS) and 99.4% (CHX) with no differences between the endotoxin reduction achieved immediately after root canal preparation. However, an extended use of the intracanal medication for 30 days in chronic cases, as shown by the present study and in acute cases as evaluated by Sousa, et al.[25] (2014), compared with a 7-day medication by Vianna, et al.[29] (2007), seemed to be important for a better *in vivo* endotoxin reduction activity of $Ca(OH)_2$.

CONCLUSION

In conclusion, our findings demonstrated the effectiveness of the mechanical action of instruments together with the flow and backflow of irrigant enduring root canal instrumentation for endotoxin removal from root canals of teeth with chronic apical periodontitis. Moreover, the use of intracanal medication for 30 days contributed for improvement of the endotoxin reduction.

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