

LPS levels in root canals after the use of ozone gas and high frequency electrical pulses

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Abstract: The present study aims to verify the effect of ozone gas (OZY[®] System) and high frequency electric pulse (Endox[®] System) systems on human root canals previously contaminated with *Escherichia coli* lipopolysaccharide (LPS). Fifty single-rooted teeth had their dental crowns removed and root lengths standardized to 16 mm. The root canals were prepared up to #60 hand K-files and sterilized using gamma radiation with cobalt 60. The specimens were divided into the following five groups (n = 10) based on the disinfection protocol used: OZY[®] System, one 120-second-pulse (OZY 1p); OZY[®] System, four 24-second-pulses (OZY 4p); and Endox[®] System (ENDOX). Contaminated and non-contaminated canals were exposed only to apyrogenic water and used as positive (C+) and negative (C-) controls, respectively. LPS (O55:B55) was administered in all root canals except those belonging to group C-. After performing disinfection, LPS samples were collected from the canals using apyrogenic paper tips. Limulus Amoebocyte Lysate (LAL) was used to quantify the LPS levels, and the data obtained was analyzed using one-way ANOVA. The disinfection protocols used were unable to reduce the LPS levels significantly (p = 0.019). The use of ozone gas and high frequency electric pulses was not effective in eliminating LPS from the root canals.

Keywords: Endodontics; Endotoxins; Microbiology; Root Canal Therapy.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Microorganisms and their byproducts, such as bacterial LPS (endotoxin), play a key role in the development of apical periodontitis.^{1,2,3} LPS is released during multiplication or death of gram-negative bacteria, and is associated with many biological effects, such as release of pro-inflammatory mediators⁴ and induction of periapical bone resorption.⁵ The endotoxins can adhere to mineralized tissues⁶ and disseminate through the dentinal tubules,⁷ making root canal sterilization difficult if only chemo-mechanical preparation is used.⁸ Although the effects of different disinfection strategies^{9,10,11,12} on LPS have already been examined, none of them are fully effective as yet.

Electrofulguration and ozone gas equipment were tested for microorganisms, with satisfactory results.^{13,14,15,16} Electrofulguration system such as Endox[®], deliver electrical pulses into the root canal via a stainless steel surgical needle that works as an active electrode,¹⁵ thereby

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eliminating organic and inorganic content through steaming.¹⁷ Ozone is a very reactive gas that has the ability to oxidize cell walls and the cytoplasmic membrane of microorganisms.¹⁴

Till date, there have been no studies investigating the effects of this equipment on LPS. Thus, the objective of the present study was to assess *in vitro* the effect of ozone gas and high frequency electrical pulses on LPS levels in infected root canals.

Methodology

The present study was approved by the Ethics and Research Committee of *Pontifícia Universidade Católica do Rio Grande do Sul - PUCRS* (Protocols no. 5859 and 811.207).

All plates and materials used in this study were sterilized using cobalt 60 gamma radiation (20 kGy for 6 hours) (*Empresa Brasileira de Radiações - EMBRARAD*, Cotia, Brazil), as previously described.²

Sample selection and preparation

Fifty single-root premolars had their dental crowns sectioned in such a way that the root length was standardized to 16 mm. A 15 mm working length (WL) was established. The canals were manually prepared using the serial technique up to #60 hand K-files (Dentsply/Maillefer Instruments S.A., Ballaigues, Switzerland), and irrigated using 2% sodium hypochlorite (Iodontosul, Porto Alegre, Brazil).

The smear layer was removed using 17% trisodium EDTA (Iodontosul, Porto Alegre, Brazil) for 5 minutes after agitation in the root canal with a #60 hand K-file for one minute. Final irrigation was performed using 2 mL of 2% sodium hypochlorite, and the root canals were dried using sterilized paper points (Dentsply/Maillefer Instruments S.A., Ballaigues, Switzerland).

The teeth were randomly fixed in 12-wells culture plates (Kasvi, Curitiba, Brazil) with Durepoxi® (Henkel, Düsseldorf, Germany). Each plate contained two teeth from each of the five experimental groups (Table).

Specimen contamination

This protocol was performed according to Signoretti et al.¹⁸ The teeth were inoculated with 30 µL of a solution containing the endotoxin *Escherichia coli* O55:B5 (Lonza, Walkersville, USA) inside a laminar flow chamber with the help of a micro pipette.

A solution containing LPS (80 EU/mL), previously diluted in apyrogenic water (50.37 EU/mL), was used to contaminate of all specimens except those belonging to group C- that were inoculated with 30 µL of apyrogenic water.

Apyrogenic cotton pellets were placed in the cervical portion of the canals in all samples. The plates containing the samples were sealed and incubated for 24 hours at 37°C temperature.

Disinfection Procedure

Prior to performing the clinical protocols, all root canals were filled with 10 µL of apyrogenic water. The disinfection protocols used in each experimental group were as follows:

- a. Group C+: the LPS contaminated canals did not undergo any disinfection protocol.
- b. Group C-: the canals had no previous LPS inoculation and did not undergo any disinfection protocol.
- c. Group OZY 1p: the tip of the OZY® system was introduced up to the working length of the root canal and one 120-second-long pulse was delivered, as described by Kustarsi et al.¹⁶

Table. Descriptive chart for the experimental groups and disinfection protocols used.

Experimental Group	n	Contamination/LPS	Clínic Protocol
C+	10	Yes	Without treatment
C-	10	No	Without treatment
OZY 1p	10	Yes	OZY® System (1 pulse - 120 s)
OZY 4p	10	Yes	OZY® System (4 pulses - 24 s each)
ENDOX	10	Yes	Endox® System

d. Group OZY 4p: the OZY® system was introduced up to the working length of the root canals and four 24-second pulses were delivered, according to Case et al.¹⁴ There was a 5 second interval between pulses.

An adapter was developed for the already available “Oto” tip to compensate for the unavailability of a specific tip for use with the OZY® system (Endox SRL, Italy). It was made of 420 surgical steel and had 0.20 mm diameter at the end and 30 mm length (Figure 1).

The OZY® system was operated at 5N intensity in accordance with the manufacturer’s instructions. The only variation between the OZY 1p and OZY 4p groups was in the device activation time.

a. Group ENDOX: the protocol used for the Endox® system (Lysis srl, Milan, Italy) was the same as that described by Lendini et al.¹⁷ The black probe of the device (measuring 30 mm in length and 0.20 mm in diameter) was introduced into the root canal. Two pulses were administered in the medium third of the root canal (5 mm short of WL), and two additional pulses in the apical third (in WL). Thus, four 600 kHz pulses were delivered, with standard time for each application being 1/10 of a second.

Determination of LPS Levels

The canals content were collected by holding three apyrogenic #60 paper points (Tanari®, Manaus,

Brazil) in position for 10 seconds. They were then transferred into glass tubes, sealed, and stored at -20°C until LPS levels quantification was performed.

Quantification of an apyrogenic water sample and the paper tips used was previously assessed in order to check the accuracy of LPS level estimation

The glass tubes containing the paper tips were filled with 1 mL apyrogenic water, warmed at 37°C for one hour, and finally centrifuged (Phoenix, Araraquara, Brazil) for 1 minute.

The chromogenic kinetic test of the turbidimetric LAL (Pyrogen 5000®, BioWhittaker, Cambrex Co., Walkersville, USA) was used to quantify the LPS levels in the root canals, as already described and applied in some studies.^{2,11}

The samples collected from the canals were mixed with the LAL reagent and automatically monitored over time with the help of a photometer until turbidity developed. The increase in optical density was measured by the reaction time, which is inversely proportional to the amount of LPS present in the sample.

A curve was drawn using the endotoxins supplied by the kit, with known concentration as a parameter for estimating LPS levels present in the root canals All the samples collected for analysis and quantification were diluted ten times.

For test validation, the assays were performed twice in distinct wells in a 96-well microplate (Corning Costar, Cambridge, USA). One hundred µL of apyrogenic water was added to the negative control, 100 µL for standard endotoxin at different concentrations for the curve, and 100 µL from each sample for quantification. LPS level was measured according to the manufacturers’ instructions.

The microplate was incubated in enzyme immunoassay reader (Ultramark, Bio-Rad Laboratories Inc., Hercules, USA) for 10 minutes at 37 ± 1°C, and the reader was coupled with a computer with Wink QCL software version 4 (BioWittaker, Cambrex Co. Walkersville, EUA).

After incubation, 100 µL of LAL chromogenic kinetic reagent (Sigma Chemical Company St. Louis, USA) was added to each plate well to start quantification of LPS levels.

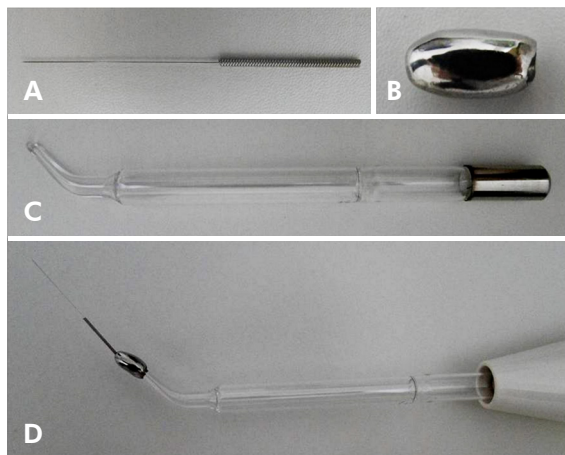


Figure 1. Adapter at the “Oto” tip of the OZY® system: (A) “endodontic” tip, (B) adapter, (C) “Oto” tip and (D) mounted OZ® system.

Statistical analysis

EU/mL measures were logarithmically transformed to reduce asymmetry and heteroscedasticity. The data were reported as geometric mean, maximum, and minimum values. The groups were compared using-way variance analysis (one-way ANOVA) on the logarithms, and the significance level was set at 5% ($p \leq 0.05$).

Results

The standard curve for LAL assay validation followed the linearity criteria ($r = 1$). The LAL assay showed that LPS was present in 100% of the root canals initially contaminated.

Results are shown in Figure 2. There were no significant differences ($p = 0.019$) in the reduction of endotoxin between the experimental groups undergoing disinfection and group C+. However, a significant difference was observed when the endotoxin concentration of the group C- specimens was compared with the other groups.

Discussion

Successful endodontic treatment of infected teeth involves elimination of microorganisms as well as inactivation of endotoxins and other toxic products.² Thus, considering the results observed when using the ozone gas system^{14,19} and high frequency electrical pulses^{13,15} on microbial strains, analysis of these systems on bacterial LPS was also thought to be pertinent.

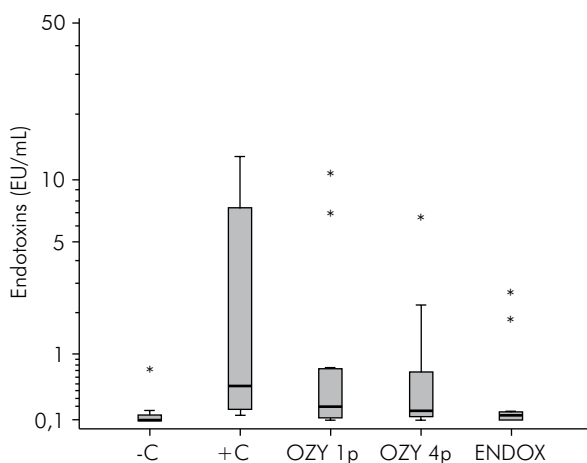


Figure 2. Boxplot distribution of endotoxin concentration in EU/mL for each experimental group.

The endotoxin used in the present study was obtained from *Escherichia coli* as it is the standard endotoxin employed in most research studies.^{12,20} LAL assay was used to quantify LPS levels as it is extremely sensitive to measurement.^{2,10}

The turbidimetric LAL assay indicated that the endotoxins were present in all previously contaminated samples. The concentrations ranged from 0.1 to 12.8 EU/mL in the positive control group, and this was in accordance with Jacinto et al.²¹ who reported endotoxin concentrations ranging between 2.3 and 22.1 EU/mL. This range in values can be attributed to the sensitivity of the test that allows detection of slight variations, and to the differences in the dental anatomy of species. However, the LPS concentration obtained was inferior to the approximate value of 50.37 EU/mL injected into each root canal. This can be justified by the fact that the samples were collected from the main canal and not from the deeper dentinal tubular region. According to Horiba et al.,⁷ the endotoxin is capable of penetrating into the dentinal tubules four times deeper than that of the microorganisms, reaching 800 μm in depth, mainly due to its low molecular weight. Thus, there is a need for auxiliary strategies that complement mechanical instrumentation to perform effective root canal disinfection.

None of the clinical protocols performed herein promoted significant reduction of endotoxin levels in relation to the positive control group. This can be justified by the fact that the principle anti-microbial action^{14,15} of the two systems involves causing damage to the structure of the microbial coating. However, LPS does not present a coating structure and it is mainly composed of specific polysaccharide O, a central nucleus, and a lipid component A,²² thereby yielding these systems ineffective against it. This was confirmed by Cardoso et al.²³ who showed that ionized water was unable to neutralize the root canal endotoxins. The results suggest presence of endotoxins in the ozone water group as well the saline solution control group. Another factor that may corroborate the results obtained in this study is the ephemeral ozone gas half-life.²⁴ The only techniques that have been tested so far and have showed a certain degree of effectiveness against LPS are (a) the use of calcium hydroxide as an intra-canal medication^{12,25,26} as it hydrolyses lipid A and changes

it into chains of fatty acid and nontoxic sugars,²⁷ and (b) the application of laser Nd:YAG.⁹

The results obtained in this study reinforce the importance of instrumentation in addition to any disinfection protocol,^{11,28,29} especially with regard to bacterial LPS that strongly adheres to the dentinal wall⁶ and makes it necessary to use endodontic instruments for its removal. Some studies have reported endotoxin decrease up to 44.4%,³⁰ 59.99%,²⁸ and 57.98%¹⁰ after chemo-mechanical preparation.

Martinho et al.¹¹ noticed 98.06% decrease in the endotoxin after performing canal preparation with NiTi (Mtwo[®]) rotatory tools under irrigation with 2.5% sodium hypochlorite solution, and 96.27% decrease when the rotatory preparation was associated with irrigation using apyrogenic saline solution. According to the authors, a basic factor that must be taken into account when performing chemo-mechanical preparation to decrease bacterial LPS is the enlargement the apical third. Enlargements over size 30 instruments are recommended for removal of endotoxin as well as

infected dentin. Furthermore, this approach allows for deeper irrigation and increases the probability of opening secondary canals and apical deltas. However, this was contradicted by Martinho et al.²⁹ who tested different systems with different designs, conicities, and tapers such as WaveOne[®], Reciproc[®], Protaper[®], and Mtwo[®] and did not observe any statistically significant differences in the decrease of bacterial LPS. The percentage of LPS reduction observed with each system was 95.15%, 96.21%, 97.98%, and 96.34%, respectively.

Conclusion

Based on the results of this study, the clinical protocols used for disinfection were unable to reduce LPS levels. The use of ozone gas and high frequency electric pulses was not effective in the elimination of LPS in root canals.

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