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QMix® irrigant reduces lipopolysaccharide (LPS) levels in an *in vitro* model

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ABSTRACT

The presence of endotoxin inside the root canal has been associated with periapical inflammation, bone resorption and symptomatic conditions. Objectives: To determine, *in vitro*, the effect of QMix® and other three root canal irrigants in reducing the endotoxin content in root canals. Material and Methods: Root canals of single-rooted teeth were prepared. Samples were detoxified with Co-60 irradiation and inoculated with *E. coli* LPS (24 h, at 37°C). After that period, samples were divided into 4 groups, according to the irrigation solution tested: QMix®, 17% EDTA, 2% chlorhexidine solution (CHX), and 3% sodium hypochlorite (NaOCl). LPS quantification was determined by Limulus Amebocyte Lysate (LAL) assay. The initial counting of endotoxins for all samples, and the determination of LPS levels in non-contaminated teeth and in contaminated teeth exposed only to non-pyrogenic water, were used as controls. Results: QMix® reduced LPS levels, with a median value of 1.11 endotoxins units (EU)/mL ($p<0.001$). NaOCl (25.50 EU/mL), chlorhexidine (44.10 EU/mL) and positive control group (26.80 EU/mL) samples had similar results. Higher levels were found with EDTA (176.00 EU/mL) when compared to positive control ($p<0.001$). There was no significant difference among EDTA, NaOCl and CHX groups. Negative control group (0.005 EU/mL) had statistically significant lower levels of endotoxins when compared to all test groups ($p<0.001$). Conclusion: QMix® decreased LPS levels when compared to the other groups ($p<0.001$). 3% NaOCl, 2% CHX and 17% EDTA were not able to significantly reduce the root canal endotoxins load.

Keywords: Endotoxins. EDTA. Chlorhexidine. Sodium hypochlorite.

INTRODUCTION

Microorganisms play an important role in the induction and maintenance of periapical diseases[15]. They have unique virulence factors, such as fimbriae, pilli, membrane receptors and endotoxins. Lipopolysaccharide (LPS) is an endotoxin that is present in the outer layers of Gram-negative bacteria cell walls, usually detected in root canal infections, eliciting biological responses associated with periapical inflammation[25] and bone resorption[10]. There is a positive correlation between LPS concentration in root canals and the development of symptomatic infections[19].

Endodontic therapy aims at the infection control, allowing the periapical healing. Several chemical substances have been used as adjuvant to the root canal mechanical preparation. Sodium hypochlorite (NaOCl) has been the most widely used root canal irrigant. NaOCl dissolves organic tissues and has a strong antimicrobial activity[27]. On the other hand, Chlorhexidine (CHX) is a biocompatible agent that has the antimicrobial action associated with substantivity[29]. Ethylenediaminetetraacetic acid (EDTA) is a chelating agent, allowing the smear layer removal[16]. EDTA favors the action of other irrigants into the dentinal tubules and root canal ramifications[3].

QMix® (Dentsply Tulsa Dental Specialties, Johnson City, TN, USA) is a novel irrigant that can be used as a final rinse. It is supposed to combine the antimicrobial and substantivity properties of

CHX with the smear layer removing properties of EDTA[8]. Moreover, QMix® contains a detergent that decreases surface tension and increases wettability in solutions[1]. Recent studies demonstrated that QMix® is effective against *Enterococcus faecalis*[28].

Previous studies have shown that root canal mechanical preparation plays an important role in reducing endotoxin load[21]. The effect of endodontic irrigants has also been established when in direct contact with LPS[4]. Nevertheless, it is not known if this effect is similar when endotoxins are within the root canal system. Therefore, the present *in vitro* study investigated the effects of auxiliary chemical and QMix® substances on endotoxins within the root canal space.

MATERIAL AND METHODS

This study was approved by the Research Board and the Research Ethics Committee from the Pontifical Catholic University of Rio Grande do Sul (PUCRS) (protocol numbers 0017/13 and 310.698, respectively).

Sample selection and root canals preparation

Fifty (50) single-rooted teeth were selected. Roots were sectioned at cementum-enamel junction, with a diamond disc (Dhpro, Paranaguá, PR, Brazil) under water-cooling. The working length (WL) was visually established, with a #10 hand instrument (Dentsply-Maillefer, Ballaigues, Jura-Nord Vaudois, Switzerland) that was inserted into the canal until its tip reached the apical foramen. The WL was determined 1 mm shorter to the apex. The root canals were prepared using the standardized K-files series (Dentsply-Maillefer, Ballaigues, Jura-Nord Vaudois, Switzerland), from a #10 K-file to a #60 K-file in the entire WL, to facilitate the LPS introduction and collection. At each change of instrument, the canals were flushed with 2 mL of NaOCl 1% (Biodinamica, Ibiporã, PR, Brazil). After preparation, canals were filled with EDTA 17% (Biodinamica, Ibiporã, PR, Brazil) for 3 minutes and irrigated with 5 mL of sterile saline solution. Canals were dried with paper points (Dentsply, Rio de Janeiro, RJ, Brazil). Each sample was fixed in epoxy resin (Loctite, São Paulo, SP, Brazil) in a well, in 12-well cell culture plates (Kasvi, Curitiba, PR, Brazil).

Sterilization and detoxification

The specimens were irradiated with Co-60 gamma rays (EMBRARAD; Empresa Brasileira de Radiações, Cotia, SP, Brazil) for degradation of preexisting LPS[26]. The sterilization and detoxification of the instruments used in the experiment were performed in an oven at 250°C, for 30 minutes.

Contamination with endotoxin

The process of contamination of specimens with endotoxins was performed as previously described[26]. Inside a laminar flow chamber, 20 µL (1.000.000 UE/mL) of a solution containing *Escherichia coli* 055:B5 endotoxin (Lonza, Walkersville, MD, USA) was inoculated into the root canals of 45 specimens using a micropipette. Five teeth were not contaminated (control samples). Pyrogen-free cotton pellets were placed in the cervical portion of the canals in all samples. The plates containing the specimens were closed and incubated at 37°C under a humidified atmosphere for 24 hours.

Experimental groups

After the incubation period, samples were divided into the following groups, according to the irrigation solution: QMix®, 17% EDTA (Biodinâmica, Ibiporã, PR, Brazil), 2% CHX (Maquira, Maringá, PR, Brazil), 3% NaOCl (Farmácia Marcela, Porto Alegre, RS, Brazil) (n=9 *per* group). The initial count (ICo) of endotoxins was determined after the contamination (n=4), without irrigation. As control groups, non-contaminated teeth (negative control NCtrl, n=5) and contaminated samples rinsed with non-pyrogenic water (the flushing control – positive control PCtrl, n=5).

With disposable syringes (Descapack, São Paulo, SP, Brazil), the root canals were filled with each solution. After 3 minutes[28], the canal content was aspirated with a new disposable plastic syringe. Then, the root canal was filled with non-pyrogenic water. The root canal content was collected with three #45 paper points, which were immediately transferred to glass tubes, closed, and kept at -20°C until the analysis. For all control groups, root canals were filled with non-pyrogenic water, and the sample was collected. In order to certify the accuracy of LPS counting, the quantification of endotoxins levels in non-pyrogenic water and in the paper points used for sampling was determined.

The tubes containing paper points were filled with 1 mL of non-pyrogenic water, warmed (37°C±1°C) for 1 hour and vortexed (Phoenix, Araraquara, SP, Brazil) for 1 minute. The protocol previously described[20] was applied for endotoxins quantification. Briefly, the PYROGENT 5000 (Lonza, Walkersville, MD, USA) is a quantitative, kinetic assay for the detection of Gram-negative bacterial endotoxin. The sample is mixed with the reconstituted LAL reagent, placed in the photometer, and automatically monitored over time for the appearance of turbidity. The concentration of endotoxin in unknown samples can be calculated from a standard curve[19]. LAL reagent water (blank) was used as a negative control. All reactions were accomplished in duplicate to validate the test. 100 µL of the LAL Reagent Water blank, endotoxin standards, product samples, positive product

Table 1- Endotoxin values. ICo: Initial Count; NCtrl: Negative control; PCtrl: Positive control. p<0.001: significance using ANOVA. Different index letters represent statistical significant differences in the post-hoc procedure (Tukey test)

EU/mL	ICo^{c} (n=4)	$NCtrl_{a}$ (n=5)	$PCtrl^{c}$ (n=5)	$QMix_{e}$ (n=9)	$EDTA_{o}$ (n=9)	$CHX^{c}{}_{o}$ (n=9)	$NaOCl^{c}{}_{o}$ (n=9)
Median	33.75	0.005	26.8	1.11	176	44.1	25.5
Minimum	6.48	0.005	12.5	0.5	26.7	3.25	9.26
Maximum	60.7	0.064	31.7	4.68	370	147	106

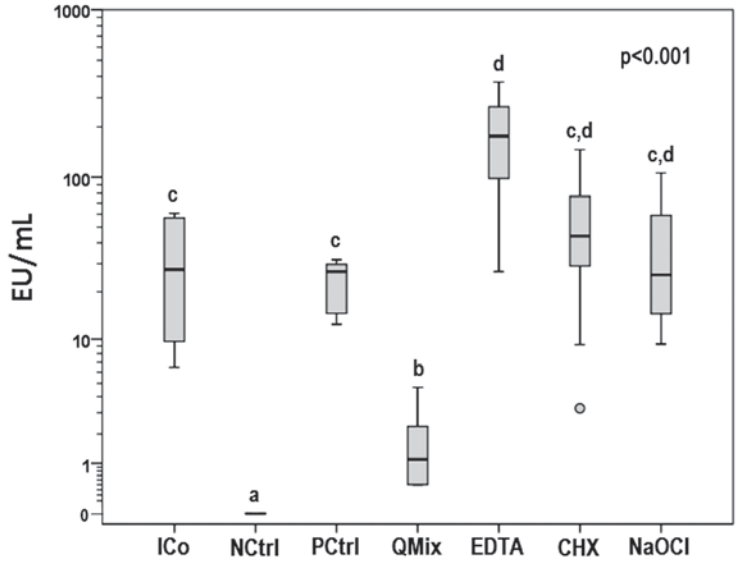

Figure 1- Endotoxin values. ICo: Initial count; PCtrl: Positive control; NCtrl: Negative control. Different index letters represent statistical significant differences in the *post-hoc* procedure (Tukey test)

controls (PPC: an aliquot of test sample spiked with a known amount of endotoxin) were carefully dispensed into the appropriate wells of a 96-well microplate (Corning Costar, Tewksbury, MA, USA). The filled plate was placed in the microplate Kinetic-QCL Reader and pre-incubated for ≥10 minutes at 37°C±1°C. After the incubation period, 100 µL of the PYROGENT - 5000 Reagent was dispensed into all wells of the microplate and the test was initiated.

According to the positive product control (PPC), the samples of the test groups need a 100-fold dilution to avoid the interference of the irrigants for the quantification assay. The ICo and PCtrl group samples need a 10-fold dilution. No dilution was required for the NCtrl group samples.

Statistical analysis

The collected data were log transformed, and one-way analysis of variance was applied on these data followed by the Tukey *post hoc* test. The level of significance was set to p<0.001. Data were analyzed using SPSS version 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Endotoxins in the water, as well as in the paper points used during the experiment, were quantified as <0.01 EU/mL.

Table 1 and Figure 1 show the endotoxin load for both control and test groups. QMix® reached the lowest levels of endotoxins among all test solutions (p<0.001). There was no significant difference among NaOCl, CHX and PCtrl group samples. Higher levels were found with EDTA, when compared to the PCtrl (p<0.001). There was no statistically significant difference among the endotoxin content for the EDTA, NaOCl and CHX groups. The NCtrl group presented statistically significant lower levels of endotoxins compared to all test groups (p<0.001).

DISCUSSION

The presence of endotoxin within the root canal has been associated with periapical inflammation[25], bone resorption[10] and symptomatic infections[19]. Its removal/neutralization from infected root canals during the endodontic treatment seems to be important for the healing process of the periapical tissues. This study evaluated the effect of auxiliary chemical substances in root canals infected with endotoxins, especially for final rinsing substances such as EDTA and QMix®.

LPS from most bacterial species is composed of three distinct regions: the O-antigen region, a

core oligosaccharide, and Lipid A[18]. In an aqueous environment, amphiphilic molecules like lipid A form supramolecular aggregate structures, changing the physical structure of LPS from monomeric molecules to multimeric aggregates. LPS in an aggregate structure has had a higher biological activity than monomerized LPS[24]. The LAL assay is not able to detect monomerized LPS[24]. Therefore, the lack of detection of LPS through the assay should not be considered as the absence of endotoxin. It could be associated with the presence of the low toxic monomerized LPS.

Some solutions, like NaOCl and EDTA, can interfere in the LAL reaction due to pH variations and chelating activity[7]. In the present study, the samples were diluted to avoid the interference of the irrigants in the analytic procedures. Furthermore, the absence of interferences was determined through the results observed in the PPC, which detects inhibition or enhancement of LAL through the addition of a known concentration of *E. coli* endotoxins to its sample, as recommended by the manufacturer (spike procedure). According to LAL assay manufacturer´s recommendations, the endotoxin recovered at PPC should equal the known concentration of the spike within 50% to 200%. To inactivate the LPS from teeth before their contamination, irradiation with Co-60 has been employed. The material employed in the experiment was detoxified through dry heat (250°C, for 30 minutes). The absence of endotoxins was determined before the analysis, and all materials showed negative results for the presence of endotoxins at the control procedures.

Sampling methods to study the root canal of microbial communities have been discussed in the current literature[2,22]. Their limitations can also affect sampling procedures of endotoxin. In the present study, root canal sampling was performed with paper points, as previously reported[10,20], to simulate the clinical limitations that are imposed during sampling. It should be emphasized that paper points were not able to entirely remove the endotoxins that are in the dentinal tubules and root canal irregularities. In this study, it was inoculated 20 mL of a 1.000.000 UE/mL solution containing *Escherichia coli* endotoxin into the root canals. It was observed that a median value of 33.75 EU/mL was recovered in the initial sampling (ICo). Therefore, endotoxins can be distributed in the entire root canal system. It might be suggested that endotoxins can be trapped in the deep dentin layers, isthmus and irregularities. Similar and broad-covering sampling methods should be developed to allow a broad endotoxin recovery from root canals, for both *in vitro* and *in vivo* studies.

Because of the high toxicity of endotoxin, some substances have been tested to obtain its inactivation. Sodium hypochlorite has been widely used as an auxiliary chemical substance. Besides its bleaching, deodorant and tissue dissolution effects, sodium hypochlorite have been proven as an effective disinfectant[27]. On the other hand, chlorhexidine is a biocompatible agent that has the antimicrobial action associated with substantivity[29]. However, a clinical study concluded that NaOCl and CHX have no detoxifying effect on endotoxins, and that the removal of more than 47% of the LPS content was related to the mechanical action of the instruments in dentin walls accomplished by the flow and backflow of the irrigants[11]. In the same way, the results of the present study have demonstrated that sodium hypochlorite and chlorhexidine are not able to detoxify root canals infected with LPS. These results are consistent with the findings of previous studies that evaluated different irrigants in direct contact with LPS, and the same behavior was observed[4,6].

Ethylenediaminetetraacetic acid is a chelating agent that promotes the smear layer removal[16]. It favors the action of other irrigants into the dentinal tubules and root canal ramifications[3]. When an aqueous solution of LPS was mixed with EDTA, there was little breakdown of LPS[4]. On the other hand, in the present study, there was a high level of endotoxins for the EDTA group when compared to the positive control group. A previous study[5] observed that EDTA can exert a chelating action in the calcium present in the lipid A portion of the endotoxin molecules. Therefore, the action of EDTA on exposing the deep layers of contaminated dentin may improve LPS release by dentin, increasing its recovery rates. Furthermore, EDTA can enhance the endotoxin release by *E. coli* cells after a brief exposure without changing its biological activity[17]. In the present study, isolated endotoxin has been employed to contaminate the samples. Therefore, this effect may be determinant especially for clinical studies that evaluate the content of endotoxins inside root canals infected with free LPS and bacteria.

QMix® has been employed after the root canal preparation as a final rinse to improve root canal cleaning and disinfection[28]. It comprises an aqueous solution of EDTA, chlorhexidine and N-cetyl-N,N,N-trimethylammonium bromide[13]. A previous study[28] employed a 3-minute period to evaluate the antimicrobial effect of QMix® in a direct exposure test. Furthermore, it was demonstrated that QMix® promoted additional antimicrobial action, especially in longer periods (>1 minute)[23]. There is no study that reported the effect of QMix® over the endotoxic content within root canals. According to the results, QMix® had the potential to reduce LPS content from the root canal when compared to the other irrigants. The presence of a high amount of surfactant and EDTA in QMix® composition may explain the great ability of this solution to remove biofilm cells from a substrate[12]. Thus, the presence of EDTA can

enhance the LPS removal from the samples, due to its ability to expose the infected inner dentin and to its potential to bind to the calcium present in the lipid A. Additionally, tensioactive agents may favor LPS removal, emulsifying endotoxins, and favoring the physical action of irrigant solutions on its removal[9,14]. Furthermore, cetrimide, as a cationic detergent, interacts with endotoxin, which has a negative charge[18]. Also in this sense, non-polar interactions occur between the Lipid A of the endotoxin and the detergent[18]. Thus, the charge characteristics and the interaction of the detergent with the endotoxin seem to be important in reducing LPS levels.

CONCLUSIONS

Based on the results, it is possible to conclude that the chemical action of NaOCl, CHX and EDTA has not been able to reduce the LPS load inside the root canal system. The physical action of irrigants associated with mechanical instrumentation may be necessary to achieve LPS reduction. QMix® seemed to reduce the LPS load inside the root canal system. Further studies should be performed to determine if this QMix® property may enhance the ability of the chemomechanical preparation to reduce the total endotoxic load from root canals.

Conflict of interest

The authors deny any conflict of interest related to this study.

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