In vitro evaluation of the action of irrigating solutions associated with intracanal medications on *Escherichia coli* and its endotoxin in root canals

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ABSTRACT

Objective: The purpose of this study was to evaluate the efficacy of auxiliary chemical substances and intracanal medications on *Escherichia coli* and its endotoxin in root canals. Material and Methods: Teeth were contaminated with a suspension of *E. coli* for 14 days and divided into 3 groups according to the auxiliary chemical substance used: G1) 2.5% sodium hypochlorite (NaOCl); G2) 2% chlorhexidine gel (CLX); G3) pyrogen-free solution. After, these groups were subdivided according to the intracanal medication (ICM): A) Calcium hydroxide paste (Calen®), B) polymyxin B, and C) Calcium hydroxide paste+2% CLX gel. For the control group (G4), pyrogen-free saline solution was used without application of intracanal medication. Samples of the root canal content were collected immediately after biomechanical preparation (BMP), at 7 days after BMP, after 14 days of intracanal medication activity, and 7 days after removal of intracanal medication. The following aspects were evaluated for all collections: a) antimicrobial activity; b) quantification of endotoxin by the Limulus Amebocyte Lysate test (LAL). Results were analyzed by the Kruskal-Wallis and Dunn's tests at 5% significance level. Results: The 2.5% NaOCl and CLX were able to eliminate *E. coli* from root canal lumen and reduced the amount of endotoxin compared to saline. Conclusions: It was concluded that 2.5% NaOCl and CLX were effective in eliminating *E. coli*. Only the studied intracanal medications were to reduce the amount of endotoxin present in the root canals, regardless of the irrigant used.

Key words: Endotoxins. Sodium hypochlorite. Chlorhexidine. Calcium hydroxide. Polymyxin B.

INTRODUCTION

The technological improvements in culture and microbiological identification in the 1980s showed predominance of anaerobic bacteria in root canals with necrotic pulp and chronic periapical lesion, especially Gram-negative microorganisms[9,13,27]. These bacteria contains endotoxins on their cellular wall, which are released during duplication or cell death and are composed of polysaccharides, lipids and proteins[9], also known as lipopolysaccharides (LPS). The A lipid is the molecular region of the endotoxin responsible for its toxic effect[16].

In the blood and tissues, the endotoxins bind to the CD-14 receptor on the surface of monocytes/macrophages and these cells, once activated, secret interleukins (IL-1α, IL-1β, IL-6, IL-8)[12] and α-tumor necrosis factor (TNF-α)[1]. The endotoxins can even increase the release of neurotransmitters and vasoactive substances at the periapical tissues and nerve-ending regions, causing pain[27], and stimulate the host cells to release the E2-prostaglandin[23], which has influence on the osteoclasts, perpetuating the periapical inflammatory reaction. Besides, the endotoxin can diffuse through the dentinal tubules

towards the cement in 24 h[18].

An important correlation between the endotoxin levels and the clinical symptomatology of the pulp and periapical tissues has been demonstrated[7,26], suggesting that endotoxins are one of the main etiological factors involved with pulpal and periapical pathologies[8,13,22].

During biomechanical preparation (BMP), several chemical substances have been used as irrigants. Due to a series of properties, sodium hypochlorite is today the most used during root canal instrumentation and irrigation[14,33]. The 2% chlorhexidine has also been used due to its antimicrobial effect, residual action and biocompatibility[11,28,34]. Although sodium hypochlorite (NaOCl) and CHX are commonly used in endodontics, the efficacy of these substances has not been observed on endotoxins during the biomechanical root canal preparation[2,19,29,33]. However, despite the antimicrobial effect of the chemical substances auxiliary to the BMP, some microorganisms and endotoxins can remain in the root canal system, dentinal tubules and apical resorption craters, requiring the use of intracanal medication.

Calcium hydroxide has been widely used as intracanal medicament due to its antimicrobial properties[14,32] and capacity to induce mineralization[10] and has been proven effective action on endotoxins[9,17,20,30].

The polymyxin B is a cationic antibiotic that act in the permeability cellular walls of Gram-negatives bacteria, causing bacteria death. Beyond the antimicrobial activity the polymyxin B is able to neutralize endotoxins, blocking some biologic effects caused by these[5,15].

This study evaluated of the BMP using NaOCl or chlorhexidine associated with calcium hydroxide, calcium hydroxide plus 2% chlorhexidine gel or polymyxin B on *Escherichia coli* and to quantify the endotoxins in root canals after use of these procedures.

MATERIAL AND METHODS

The present study was approved by the Research Ethics Committee of the São Paulo State University, São José dos Campos Dental School, Brazil (protocol n.067/2005). One hundred and twenty recently extracted human single-rooted teeth were used. They remained immersed in pyrogen-free saline solution (saline) (Aster Produtos Médicos Ltda., Sorocaba, SP, Brazil) prior to use. The crowns were crosscut with a carborundum disc and the length of the specimen was established at 16±0.5 mm. Initial root canal instrumentation was done up to K file #30 (Dentsply Maillefer, Petrópolis, RJ, Brazil), and the canals were irrigated with 1% NaOCl solution at each change of instrument. After biomechanical preparation, the canals were dried and filled with EDTA (Inodon, Porto Alegre, RS, Brazil) for 3 min and irrigated with 10 mL of saline. Next, the apical region of teeth was sealed with light-cured composite resin (Z-100. 3M, Sumaré, SP, Brazil) and the outer surfaces of the roots were rendered waterproof with an epoxy adhesive layer, except for the cervical opening region. These teeth were distributed randomly, according to anatomy to standardize the groups. The roots were joined to clear light-cured acrylic resin (Dencor Artigos Odontológicos Classico, São Paulo, SP, Brazil) in 24-well plates with (Costar, New York, NY, USA). The plates, instruments and materials used were sterilized by gamut radiation with cobalt 60 (20 KGy for 6 h) in order to neutralize the pre-existing endotoxins[4].

Escherichia coli (ATCC 25922) suspension in sterile and pyrogen-free saline solution containing 10^6 cells/mL was prepared, the root canals were contaminated with 10 μL of this suspension and with 10 μL of BHI broth and a pyrogen-free pellet soaked with BHI was placed in the root canal opening. After contamination, the specimens were stored at 37°C in relative humidity for 14 days and at each 3-day period the BHI broth was added to the root canals[14]. After the contamination period, collections were made of all specimens to confirm the root canal contamination (confirmation collection).

After confirmation, the specimens were divided into 3 experimental groups (n=36), according to the irrigant used: group 1: 2.5% NaOCl (Terapêutica Farmácia de Manipulação, São José dos Campos, SP, Brazil); group 2: 2% chlorhexidine gel [2% chlorhexidine in 0.8% natrosol gel (CLX) (Byoformula Farmácia de Manipulação, São José dos Campos, SP, Brazil)]; group 3 and group 4 (control): saline.

Biomechanical preparation was made with 1-mm retreat to K file #50 and step-back preparation up to K file #80. In groups 1, 3 and 4, 3 mL of irrigant were used at each change of instrument. In group 2, 2% CLX gel was placed inside the root canals to the top, the canals were instrumented and irrigated with 3 mL of saline; this procedure was done at each change of instrument.

After preparation, two collections were made from the root canal: one immediately after instrumentation (1st collection) and another 7 days later (2nd collection). The roots remained in saline and a pyrogen-free pellet in the root canal opening between the 1st and 2nd collections.

Next, groups 1, 2 and 3 were subdivided into 3 groups (n=12), according to the intracanal medication applied: A) calcium hydroxide paste (CHP) (Calen®, S.S. White, Rio de Janeiro, RJ, Brazil); B) polymyxin B solution (PmB) (10,000 IU/mL) (Ophtalmos Fórmulas Oficiais,

São Paulo, SP, Brazil); C) pro-analysis calcium hydroxide association (P.A.) (Biodinâmica Química e Farmacêutica Ltda., Ibiporã, PR, Brazil) with 2% CLX gel (CHP + 2% CLX gel) (Byoformula Farmácia de Manipulação). The ratio was 1:1, the consistency of the paste was similar to a toothpaste. In the group 4 (control group) (n=12), biomechanical preparation was made only with the saline and the intracanal medication was not used; the canals were kept with saline. Before placement of the intracanal medication, EDTA was used for 3 min followed by root canal irrigation with 10 mL of saline to cleaning of the root canals walls and better diffusion of intracanal medication.

After application of intracanal medication, the roots remained in an oven at 37°C for 14 days. After that period, the medication was removed and the root canal contents were collected (3rd collection). The canals were filled with saline and after 7 days another collection was carried out (4th collection: 7 days after removal of intracanal medication).

All collections in root canals (confirmation; 1st collection: immediately after instrumentation; 2nd collection: 7 days after biomechanical preparation; 3rd collection after medication maintenance for 14 days; 4th collection: 7 days after removal of intracanal medication) were made in the same manner: the canals were filled with saline and 100 μL of the content were collected from the root canal with 1 mL syringes for microbiological and endotoxin count analysis.

To determine the antimicrobial activity of the auxiliary chemical substances and intracanal medications used, serial dilutions were made with samples double collected from the root canal and plated on plates containing BHI agar. Then, they were incubated in an oven at 37°C for 48 h and after that the counts of colony forming units (CFU/mL) of *E. coli* were performed.

In order to check the endotoxin neutralization, the chromogenic kinetic method of the *Limulus* amebocyte lysate test (LAL) (Cambrex, São Paulo, SP, Brazil) was used. A kinetic reader QCL (Cambrex) was used, connected to a computer with a Wink QCL software (Cambrex) specific for management, accomplishment and report writing. LAL test is the most considerable and trustworthy method for detention of endotoxin, allowing to quantify the endotoxin with higher precision. The curve-standard (concentrations between 0.005 and 50 EU/mL) was done according to fabricant. This software automatically calculates the parameters of the standard curve, and the values of the samples in endotoxin units (EU/mL).

All data were analyzed by the Kruskal-Wallis and Dunn's test at 5% significance level. Statistical analysis was carried out according to the collections made during the study: 1st collection, 2nd collection, 3rd collection and 4th collection.

The 1st and 2nd collections were analyzed comparing the auxiliary chemical substance used, whereas for the 3rd and 4th collections the groups were analyzed individually between themselves.

RESULTS

The microbiological analysis revealed that, upon the confirmation collection, there was microbial growth of all specimens; after biomechanical preparation with 2.5% NaOCl and 2% CLX gel the number of bacteria went under the detection limit. After application of the intracanal medication, the

Table 1- Mean of colony forming units/ milliliter (CFU/mL) in groups

Irrigation solution	Confirmation collection CFU/mL	1st collection CFU/mL	2nd collection CFU/mL	Intracanal medication CFU/mL	3rd collection CFU/mL	4th collection CFU/mL
				A ($Ca(OH)_2$)	0	0
2.5% NaOCl	$9.06x10^8$	0	0	B (PmB)	0	0
				C ($Ca(OH)_2$+CLX)	0	0
				A ($Ca(OH)_2$)	0	0
CLX	$2.22x10^8$	0	0	B (PmB)	0	0
				C ($Ca(OH)_2$+CLX)	0	0
				A ($Ca(OH)_2$)	0	0
Pyrogen-free saline solution	$3.08x10^8$	$1x10^5$	$4.4x10^6$	B (PmB)	0	0
				C ($Ca(OH)_2$+CLX)	0	0
Pyrogen-free saline solution	$3.31x10^8$	$6x10^4$	$4.2x10^6$	Pyrogen-free saline solution	$5.5x10^4$	$2x10^5$

CLX=chlorhexidine; PmB=polymyxin B; CHP=calcium hydroxide paste.

values of UFC/mL remained equal to zero, therefore there was no need to apply the statistical test to those results (Table 1).

In group 3 (pyrogen-free saline solution), there was microorganism reduction regarding the confirmation collection in all specimens. It was observed that, on the 2nd collection (7 days after the BMP), there was an increase in the number of microorganisms. It was also noted that, after application of intracanal medication, the number of bacteria went under the detection limit. However, in group 4 (control), in which no intracanal medication was used, the microorganisms remained viable throughout the study period.

Table 2 presents the values obtained in the quantification of endotoxins. It was observed that, on the 1st and 2nd collections, NaOCl and CLX were similar to each other ($p>0.05$) and different from pyrogen-free saline solution ($p<0.05$). The median obtained on the counting of EU/mL, as well as the homogeneous groups after the 3rd and 4th collections are displayed in Table 3.

On the 3rd collection, immediately after intracanal medication, it was noted that groups G1A, G1B, G1C, G2A, G2C, G3A, G3C were significantly different from the control group G4 ($p<0.05$). The groups G1C, G2A, G2C and G3C presented the lowest endotoxin values, being equal to groups G1A, G1B, G3A and G3B ($p>0.05$). Even though groups G2B and G3B, which used the polymyxin B as intracanal medication, reduced the amount of endotoxin in the root canals, it was not enough to indicate statistically significant difference compared with the control group (G4) ($p>0.05$). However, group G2B was equal to groups G1A, G1B and G3A, and the G3B was equal to the others ($p>0.05$).

Upon the 4th collection, a statistically significant difference was also noted between groups. The

Table 2- Descriptive statistics of the median and homogeneous groups of quantity of endotoxin of *E. coli* (EU/mL) on the first and second collections

Irrigation solution	1st collection EU/mL		2nd collection EU/mL	
	Median	Homogeneous groups*	Median	Homogeneous groups*
NaOCl 2.5%	118.5	B	563	B
2% CLX gel	104.9	B	227	B
Pyrogen-free saline solution	444	A	6940	A

*different letters indicate statistically significant difference ($p<0.05$)

Table 3- Descriptive statistics of the median and homogeneous groups of quantity of endotoxin of *E. coli* (EU/mL) on the third and fourth collections

Irrigation solution	Intracanal medication	3rd collection EU/mL		4th collection EU/mL	
		Median	Homogeneous groups*	Median	Homogeneuos groups*
G1 NaOCl 2.5%	A ($Ca(OH)_2$)	14.25	BC	12.5	BC
	B (PmB)	19.45	BC	9.58	C
	C ($Ca(OH)_2$+CLX)	4.05	C	23.9	BC
G2 2% CLX gel	A ($Ca(OH)_2$)	7.19	C	16.9	BC
	B (PmB)	176	AB	67.5	ABC
	C ($Ca(OH)_2$+CLX)	11.35	C	17.2	BC
G3 Pyrogen-free saline solution	A ($Ca(OH)_2$)	12.8	BC	95.3	AB
	B (PmB)	37.5	ABC	33.2	BC
	C ($Ca(OH)_2$+CLX)	8.87	C	13.37	BC
G4 Pyrogen-free saline solution	Pyrogen-free saline solution	2160	A	2790	A

*different letters indicate statistically significant difference ($p<0.05$)

G1B presented lower endotoxin value but was statistically equal to groups G1A, G1C, G2A, G2B, G2C, G3B and G3C ($p>0.05$). The groups G2B and G3A exhibited reduced the amount of endotoxin in the root canals, yet it was not enough to indicate statistically significant difference in relation to group 4 (control). The other groups G1A, G1B, G1C, G2A, G2C, G3B, G3C presented considerable reduction of endotoxins compared to the control group G4 ($p<0.05$).

DISCUSSION

Even though the *Escherichia coli* is not commonly found in root canals with necrotic pulp, some studies found *E. coli* in root canals with periapical lesions[6,31]. Its endotoxin presents the basic structure of the lipid component, which represents the active center responsible for the toxicity of LPS.

Upon microbiological analysis after the biomechanical preparation (1st collection), it was seen that in the group irrigated with saline solution there was microorganism reduction compared with the confirmation collection. However, in groups instrumented with NaOCl and 2% CLX gel, the number of bacteria went under the detection limit. Even after 7 days of biomechanical preparation, with the canal filled with pyrogen-free saline solution, there was no bacterial growth confirming that the 2.5% NaOCl and the 2% CLX gel eliminated the *E. coli* in the root canal lumen. Orstavik and Haapasalo[21] (1990) verified that *E. coli* penetrates much less into the dentinal tubules than other species. It may count to its susceptibility to the irrigants agents used in this study.

In the present study, in the group instrumented with saline solution, only microorganism reduction was noted. This reduction was due to the physical (abundant irrigation and effective aspiration) and mechanical (root canal instrumentation) means, since the saline solution does not present antimicrobial activity. These results agree with those of Byström & Sundqvist[3] (1983), which showed that saline is ineffective.

The results obtained from this study when 2% CLX gel was used as auxiliary substance to the biomechanical preparation complied with other studies that used 2% CLX gel as chemical substance auxiliary to biomechanical preparation and showed the effectiveness of this substance on the microorganisms present in the root canal[25].

Even though the chemical substances used in biomechanical preparation have shown antimicrobial activity on *E. coli*, they were not able to eliminate the endotoxins present in the root canal. On the 1st collection, it was seen that 2.5% NaOCl and 2% CLX gel showed lower amounts of endotoxin when compared with the control group where only the pyrogen-free saline solution was used as irrigant auxiliary to root canal instrumentation. These results comply with other studies that also observed that biomechanical preparation with NaOCl and 2% CLX gel is not enough to inactivate the endotoxin[19], requiring the complementary action of intracanal medications. It was also seen that, after 7 days (2nd collection), the amount of endotoxins was higher than the 1st collection. It shows that the biomechanical preparation with NaOCl or CLX, even eliminating the *E. coli* in the root canals, left the endotoxins released with bacterial death remain in the dentinal smear layer, requiring the use of intracanal medication in order to inactivate these endotoxins.

After the use of intracanal medication, the results showed considerable reduction of endotoxin with all medications tested when compared with the 2nd collection. In some specimens the calcium hydroxide paste and the association of calcium hydroxide and the 2% CLX gel were able to inactivate the endotoxins. The polymyxin B reduced the endotoxin count, yet it was not able to inactivate them completely in any specimen and did not differ from the control group upon the 3rd and 4th collections in groups irrigated with CLX and on the 3rd collection in the group irrigated with PFS. Similar results were found by Tanomaru, et al.[33] (2003), who used a similar methodology and reported that biomechanical preparation with NaOCl and CLX solutions could not inactivate the effects of endotoxins; however, the calcium hydroxide inactivated the effects of endotoxins.

In vitro studies have already shown that calcium hydroxide can inactivate the endotoxins. Safavi and Nichols[24] (1993) saw that calcium hydroxide hydrolyzes the A lipids resulting in high release of free fatty acids. In 1994 these authors identified[23] the E2 prostaglandin production in floating monocytes stimulated by the LPS; however, the E2 prostaglandins were not identified in floating elements that were previously treated with calcium hydroxide. Yet, Nelson-Filho, et al.[17] (2002) and Silva, et al.[30] (2002) observed in dog´s teeth that the endotoxin caused radiographically and histopathologically visible periapical lesion, yet when associated with calcium hydroxide that endotoxin lost its toxic action.

In the present study, it was seen that the only intracanal medication that differed from the control in all groups was CHP+CLX (groups G1C, G2C and G3C). The calcium hydroxide associated with CLX was able to neutralize endotoxins due to its calcium hydroxide action. Besides, that association brings together the antimicrobial properties of CLX and the mineralization potential and neutralization of endotoxins by calcium hydroxide.

As for the effectiveness of polymyxin B, it was

seen that groups G2B (BMP: CLX; MIC: PmB) and G3B (BMP: PFS; MIC PmB) were equal to the control group on the 3rd and 4th collections and 3rd collection, respectively. It shows that polymyxin in these groups was not able to considerably reduce the amount of endotoxins. These results can be explained by the fact that polymyxin B is an antibiotic, since Oliveira, et al.[19] (2007) saw the effective neutralization of the endotoxin after biomechanical preparation with polymyxin B. However, Oliveira, et al.[20] (2005), when using polymyxin B as intracanal medication for 7 days, noted a reduction in endotoxin counts. That difference between polymyxin B action as irrigant and as intracanal medication occurs because polymyxin B is an antibiotic with immediate local action, losing its effectiveness after some h in the root canal. Morrison and Jacobs[15] (1976) found that polymyxin B has the capacity to bind with high affinity to an A lipid portion, thus altering the tridimensional conformation of the LPS molecule. This conformational alteration possibly restrains the complex endotoxin-polymyxin B from binding to the CD14 receptor in the monocytes, inhibiting the release of inflammatory mediators[5].

CONCLUSION

Root canal biomechanical preparation with 2.5% NaOCl and 2% CLX gel as auxiliary chemical substances promoted the elimination of *E. coli* in the root canal lumen and considerably reduced the amount of endotoxin compared to saline. Only the intracanal medications were able to reduce the amount of endotoxin present in the root canals, regardless the irrigant used during biomechanical preparation.

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