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Effect of *Zingiber officinale* and propolis on microorganisms and endotoxins in root canals

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ABSTRACT

The purpose of this study was to evaluate the effectiveness of glycolic propolis (PRO) and ginger (GIN) extracts, calcium hydroxide (CH), chlorhexidine (CLX) gel and their combinations as ICMs (ICMs) against *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli* and endotoxins in root canals. Material and Methods: After 28 days of contamination with microorganisms, the canals were instrumented and then divided according to the ICM: CH+saline; CLX, CH+CLX, PRO, PRO+CH; GIN; GIN+CH; saline. The antimicrobial activity and quantification of endotoxins by the chromogenic test of Limulus amebocyte lysate were evaluated after contamination and instrumentation at 14 days of ICM application and 7 days after ICM removal. Results and Conclusion: After analysis of results and application of the Kruskal-Wallis and Dunn statistical tests at 5% significance level, it was concluded that all ICMs were able to eliminate the microorganisms in the root canals and reduce their amount of endotoxins; however, CH was more effective in neutralizing endotoxins and less effective against *C. albicans* and *E. faecalis*, requiring the use of medication combinations to obtain higher success.

Key words: *Zingiber officinale*. Propolis. Calcium hydroxide. *Candida albicans*. *Enterococcus faecalis*. *Escherichia coli*. Endotoxins.

INTRODUCTION

Endodontic infections present a polymicrobial nature, which include the presence of fungal species. Fungi are especially represented by *Candida albicans*[32]. *Enterococcus faecalis* has been frequently isolated among the microorganisms of endodontic infections, even in persistent infections after root canal treatment[8].

The predominance of anaerobic bacteria within this microbial diversity is also known, especially Gram-negative bacteria, which present endotoxins on their cell walls[14]. Endotoxins stimulate competent cells such as macrophages, neutrophils and fibroblasts, triggering the release of a large number of bioactive inflammatory chemical mediators or cytokines[18]. This fact leads to a series of biological effects with consequent inflammation, immune reaction and periapical bone resorption[18].

Although *Escherichia coli* is not commonly isolated from necrotic root canals, its endotoxin has been used in studies as a reference to evaluate the function of chemicals. This fact is related to the fact that the endotoxin from *Escherichia coli* presents the basic structure of the lipid component which represents the active center responsible for the toxicity of lipopolysaccharides (LPS)[3].

Calcium hydroxide (CH) has been widely used as an ICM during root canal treatment because of its antimicrobial properties[27], ability to induce remineralization[15] and especially its effective action on endotoxins[17,19,25,26].

The use of chlorhexidine (CLX) gel relies on its action on Gram-positive and Gram-negative aerobic and anaerobic microorganisms and yeasts[17,18]. On the other hand, it does not seem

to present any action on bacterial endotoxins[17,20]. Thus, the association of CH and 2% CLX has been recommended for intracanal dressing, since this combination has a broader spectrum of antimicrobial activity and efficacy on endotoxins in the root canal.

Currently, there are a growing number of studies in different medical specialties using natural substances, such as teas or extracts from various plants. Among the natural extracts used in dentistry, propolis stands out due to its anti-inflammatory, analgesic and antimicrobial properties[1,7,10,13,21,24]; and ginger (*Zingiber officinale*), due to its healing, anti-inflammatory and antimicrobial actions[23]. However, there are no studies on the therapeutic properties of these extracts in Endodontics.

The purpose of this study was to evaluate the action of glycolic propolis (PRO) and ginger (GIN) extracts, CH, CLX gel and their combinations used as intracanal dressing against *C. albicans*, *E. faecalis*, *E. coli* and endotoxins.

MATERIAL AND METHODS

Preparation of specimens

This study was approved by the Institutional Review Board of São José dos Campos Dental School, UNESP (protocol #06/2008-PH/CEP). Ninety-six human single-rooted teeth had their crowns sectioned and the length standardized at 16±0.5 mm. The initial instrumentation was performed in the full length of root canals, up to #30 K-file (Dentsply Indústria e Comércio Ltda., Petrópolis, RJ, Brazil), followed by irrigation with 3 mL of saline at each change of file. After preparation, the canals were filled with EDTA (Inodon, Porto Alegre, RS, Brazil) for 3 min and irrigated with 10 mL of saline. The apical region of each tooth was sealed with composite resin (Z-100; 3M ESPE, St. Paul, MN, USA) and the roots were externally sealed with a layer of epoxy adhesive (Araldite, Brascola, São Paulo, SP, Brazil), except for the cervical opening.

The specimens were randomly distributed and fixed with chemically cured acrylic resin (Dencor Articles Dental Classic, São Paulo, SP, Brazil), in 24-well cell culture plates (Costar, New York, NY, USA), with 12 teeth in each plate. All culture plates and all materials (cotton pellets, Eppendorf tubes, instruments, K files) were sterilized by Cobalt 60 gamma radiation (20 Kgy - 6 h)[4].

Contamination of specimens

The test microorganisms are described in Figure 1. Suspensions of microorganisms were prepared in sterile and pyrogen-free saline (Segmenta, Ribeirão Preto, SP, Brazil), containing 10^6 cells/mL confirmed using a spectrophotometer (Model B-582; Micronal S/A, São Paulo, SP, Brazil).

Under sterile laminar flow chamber, the root canal was contaminated with 10 μL of the *E. coli* suspension and 10 μL of Brain Heart Infusion Broth

Microorganism	Strain source	Wavelenght	Optical density
C. albicans	ATCC 18804	530 nm	0.284
E. faecalis	ATCC 29212	760 nm	0.298
E. coli	ATCC 25922	590 nm	0.324

Figure 1- Strains source, wavelength and optical density adopted for obtaining of standardized suspensions (10^6cells/mL)

n	Intracanal dressing	Manufacturer
12	Calcium hydroxide and saline	Biodinâmica Química e Farmacêutica Ltda, Ibiporã, PR, Brazil
12	2% chlorhexidine gel	Byofórmula – Farmácia de Manipulação, São José dos Campos, SP, Brazil
12	Calcium hydroxide and 2% chlorhexidine gel	Biodinâmica Química e Farmacêutica Ltda, Ibiporã, PR, Brazil
		Byofórmula – Farmácia de Manipulação, São José dos Campos, SP, Brazil
12	12% glycolic propolis extract	Apis Flora, Ribeirão Preto, SP, Brazil
12	Calcium hydroxide and 12% glycolic propolis extract	Biodinâmica Química e Farmacêutica Ltda, Ibiporã, PR, Brazil, Apis Flora, Ribeirão Preto, SP, Brazil
12	20% glycolic ginger extract	Apis Flora, Ribeirão Preto, SP, Brazil
12	Calcium hydroxide and 20% glycolic ginger extract	Biodinâmica Química e Farmacêutica Ltda, Ibiporã, PR, Brazil, Apis Flora, Ribeirão Preto, SP, Brazil
12	Pyrogen-free saline solution	Segmenta, Ribeirão Preto, SP, Brazil

Figure 2- Distribution of groups

(BHI broth) (Himedia Laboratories, Mumbai, India). A sterile cotton pellet was soaked in the BHI broth and placed at the cervical opening. The samples were stored in an incubator at 37±1°C with humid atmosphere for 7 days and 20 μL of BHI broth were added in the root canals every 2 days[31]. After 7 days, the root canals received 5 μL of the *C. albicans* suspension, 5 μL of the *E. faecalis* suspension and 10 μL of BHI broth. A cotton pellet soaked in BHI broth was placed at the cervical opening. The specimens were stored at 37±1°C with humid atmosphere for 21 days in an incubator, and 20 μL of BHI broth were added in the root canals every 2 days[30].

After 28 days of incubation, samples were collected from all specimens to confirm root canal contamination (Initial Sample - Si). All specimens were instrumented up to #50 K-file, using 3 mL of pyrogen-free saline between instruments. The first sample (S1) was collected from root canal immediately after biomechanical preparation.

Prior to ICM placement, the root canals were filled with 17% EDTA for 3 min and further irrigated with 10 mL of pyrogen-free saline. At this stage, the specimens were divided into 8 groups (n=12) according to the ICM (Figure 2).

The CLX, PRO, GIN medications and saline were taken to the root canals with 3-mL syringes until complete filling of the canal space. The CH+saline medication was obtained by mixing 0.1 g of CH with 100 μL of saline. The CH+CLX medication was performed at 1:1 ratio in a toothpaste consistency[9]. The PRO+CH ICM was obtained by mixing 0.1 g of CH with 100 μL of propolis and the GIN+CH ICM contained 0.1 g of CH mixed with 100 μL of ginger. All ICMs were applied in the root canal with the aid of a #30 K-file.

After ICM placement, a pyrogen-free cotton pellet was placed at the cervical opening. The teeth were stored at 37°C for 14 days. The ICM was removed after this period with a #50 K-file and 10 mL of pyrogen-free saline, and the root canal contents were collected (S2). Then, the canals were filled with pyrogen-free saline and stored in the incubator and another collection (S3) was performed after 7 days.

All samples from the root canals were collected in the same way: the canals were filled with pyrogen-free saline using a 1-mL syringe and needle. The solution was stirred inside the canal using the needle and the root canal content was collected using another 1-mL syringe. This procedure was repeated until obtaining 100 μL from each canal, which were further transferred to 1.5-mL centrifugation Eppendorf microtubes containing 900 mL of pyrogen-free saline.

To determine the antimicrobial activity, the canal samples were serially diluted and plated in duplicate

Table 1- Percent reductions obtained in S2 and S3 in relation to Si: mean, standard deviation, median and homogeneous groups regarding the reduction of CFU/mL of microorganisms

Groups	n	S2 X Si								S3 X Si					
		C. abicans			*E. faecalis*			*E. coli*		*C. abicans*			*E. faecalis*			*E. coli*	
		Mean±SD*	Median	HG**	Mean±SD*	Median	HG**	Mean±SD*	Median	Mean±SD*	Median	HG**	Mean±SD*	Median	HG**	Mean±SD*	Median	HG**
$Ca(OH)_2$	12	90.64±22.52	100	A	96.81±11.06	100	A	100±0.00	100	83.32±26.7	100	AB	79.44±6.34	100	AB	100±0.00	100	A
CLX	12	100±0.00	100	A	100±0.00	100	A	100±0.00	100	100±0.00	100	A	100±0.00	100	A	100±0.00	100	A
$Ca(OH)_2$ + CLX	12	100±0.00	100	A	100±0.00	100	A	100±0.00	100	100±0.00	100	A	100±0.00	100	A	100±0.00	100	A
PRO	12	100±0.00	100	A	100±0.00	100	A	100±0.00	100	100±0.00	100	A	100±0.00	100	A	100±0.00	100	A
$Ca(OH)_2$ + PRO	12	100±0.00	100	A	100±0.00	100	A	100±0.00	100	100±0.00	100	A	100±0.00	100	A	100±0.00	100	A
GENG	12	100±0.00	100	A	100±0.00	100	A	100±0.00	100	100±0.00	100	A	100±0.00	100	A	100±0.00	100	A
$Ca(OH)_2$ + GENG	12	100±0.00	100	A	100±0.00	100	A	100±0.00	100	100±0.00	100	A	100±0.00	100	A	100±0.00	100	A
Saline	12	44.98±12.54	47.79	B	36.18±8.30	37.19	B	37.10±7.27	39.46	21.2±12.10	94.5	B	23.34±5.69	24.08	B	21.82±3.93	22,9	B

*SD: standard deviation (±)
**HG: homogeneous groups, different letters show statistical significant difference (p< 0.05)

using three culture media, selective for each tested organism: Sabouraud Dextrose agar with 0.1 mg/mL chloramphenicol (Vixmicina, União Química Farmacêutica S/A, Embu Guaçu, SP, Brazil) for *C. albicans* (0.1 g of chloramphenicol to each 100 mL of agar); Enterococcosel agar (Becton, Dickinson and Company, Sparks, MD, USA) for *E. faecalis*; and MacConkey agar with 15% of bile salts, crystal violet and NaCl (Himedia Laboratories, Mumbai, India) for *E. coli*.

The plates were incubated at 37°C for 24 h for determination of colony forming units *per* milliliter (CFU/mL) of *C. albicans*, *E. faecalis* and *E. coli* remaining in the root canal. The results were log transformed and analyzed statistically by the Kruskal-Wallis and Dunn tests, at 5% level of significance. Statistical analysis was based on the percent reduction of CFU/mL of each collection of microorganisms in relation to the initial sample (Si).

The neutralization of endotoxins was assessed by the kinetic chromogenic Limulus amebocyte lysate (LAL) assay (Cambrex, São Paulo, SP, Brazil). The concentrations of the standard curve (between 0.005 and 50 EU/mL) were performed following the manufacturer's instructions. Calculation of parameters of the standard curve and values of the samples of endotoxins (EU/ml) were performed automatically by the software.

The statistical analysis used in this study was based on the percent change related to the decrease or increase in the amount of endotoxins (EU/ml) of each sample in relation to the initial sample (Si). Data were analyzed by the Kruskal-Wallis and Dunn's statistical tests at 5% significance level.

RESULTS

Microbiological analysis

The analysis of S1 bacterial reduction in relation to Si was performed by the descriptive method. Since all groups in this phase received preparation with saline, 96 specimens were considered. The mean and standard deviation changes obtained for S1 in relation to Si (n=96) were the following: 43.98%±21.40 for *C. albicans*, 29.06%±9.84 for *E. faecalis* and 26.30%±14.43 for *E. coli*.

The bacterial reductions obtained for S2 and S3 in relation to Si are presented in Table 1. For S2 and S3 evaluations, each experimental group (n=12) was individually assessed to determine variations of intracanal dressings.

For S2 (Table 1), all experimental groups were similar among them and different from the Saline group. However, even though the CH group was statistically similar to other groups, it seems that this medication was not able to eliminate completely *C. albicans* and *E. faecalis* in the root canals. This fact was also observed for S3, in which the CH group was similar to the Saline group.

Quantification of endotoxins

S1 exhibited significant reduction in the amount of endotoxins. The overall percent reduction obtained for S1 compared to Si (n=96) was 88.95%±44.33 (mean and standard deviation, median 98.33). However, in Table 2, the values are presented according to each experimental group (n=12). Analysis of group homogeneity was not performed for S1 because all groups went through the same procedure at this phase were and prepared using the same irrigant (saline). Regarding S2, CH group was similar to CLX+CH, PRO+CH, GIN and GIN+CH groups, presenting

Table 2- Percent change obtained in S2 and S3 in relation to Si: mean, standard deviation, median and homogeneous groups regarding the decrease or increase in the amount of endotoxins (EU/mL)

Groups	S2 X Si			S3 X Si		
	Mean±SD*	Median	HG**	Mean±SD*	Median	HG**
$Ca(OH)_2$	99.997±0.003	100	A	99.996±0.005	100	A
CLX	93.450±9.570	97.9	C	87.660±18.430	95.2	C
CLX + $Ca(OH)_2$	99.972±0.040	99.991	A B	99.959±0.048	99.971	A B
PRO	99.755±0.440	99.86	B C	98.781±3.300	99.934	A B C
PRO + $Ca(OH)_2$	99.994±0.011	99.999	A B	99.992±0.018	99.998	A
GENG	99.814±0.227	99.9	A B C	99.384±0.745	99.698	B C
GENG + $Ca(OH)_2$	99.925±0.158	99.997	A B	99.947±0.089	99.991	A B
Saline	67.000±67.700	90.8	C	-22.800±128.400	34.8	C

*SD: standard deviation (±)

**HG: homogeneous groups, different letters show statistical significant difference ($p < 0.05$)

the highest rates of reduction, being different from groups CLX, PRO and GIN. Saline group was similar to CLX, PRO and GIN groups. All groups containing CH were similar.

For S3, there were also similarities between groups containing CH [(CH, CH+CLX, CH+PRO, CH+GIN] and these groups were similar to the PRO group. CH group was statistically different from CLX and GIN groups. However, CLX and GIN were similar between them and to Saline group.

DISCUSSION

Microbiological analysis of the S1 (immediately after biomechanical preparation with saline) showed that there was a reduction of the test microorganisms. This reduction is related to the mechanical action of instruments and physical aids of the biomechanical preparation (irrigation, aspiration, flooding), since saline has no antimicrobial activity. Thus, it is important to use auxiliary chemicals with antimicrobial activity for a better removal of microorganisms from the root canal[2].

After application of the medication (S2), it was verified that only the CH group was not able to eliminate completely *C. albicans* and *E. faecalis* from root canals, with a mean percentage reduction of 90.64% for *C. albicans* and 96.81% for *E. faecalis*, in comparison to Si. This shows that these microorganisms present some resistance to CH[11,28,31]. The resistance of *E. faecalis* to CH is explained by the presence of a proton pump on their cell walls. This proton pump is able to acidify the cytoplasm, making this organism able to survive in an alkaline medium up to a pH of 11.1. However, when the pH reaches values equal to or greater than 11.5, there is activation of the proton pump, thus leading to death of the microorganism[5]. Moreover, *E. faecalis* is able to penetrate quickly and deeply into the tubules and is difficult to be removed after mechanical preparation[22]. Also, the pH values in the dentin bulk may not achieve the optimal pH for elimination of this microorganism because of the buffering capacity of dentin[11].

Because of this ability of *E. faecalis* to invade dentinal tubules, remain viable inside them and adhere to collagen in the presence of human serum, this microorganism is responsible for the persistence of endodontic infection and maintenance of chronic alterations in endodontically treated teeth[16].

The resistance of *C. albicans* may be related to its ability to adapt to different environmental conditions. This yeast has the ability to survive as a commensal in adapting to both acidic and basic pH[28]. Moreover, *C. albicans* has the capacity to undergo morphological transition, switching from yeast to hyphae, representing a shift from pathogenic to commensal state, presenting the ability to invade the host tissues and escape phagocytosis by macrophages. *C. albicans* also has the ability to form biofilms on different surfaces[28].

Analyzing the S3 results (7 days after ICM action), the microorganisms *C. albicans* and *E. faecalis* were still present in root canals in the CH group and in some specimens these microorganisms even increased from S2 to S3. Moreover, the CH group was similar to the Saline group, indicating that this medication alone is not effective for complete elimination of *C. albicans* and *E. faecalis* in the root canals. Thus, it is important to combine CH with another substance with higher ionic dissociation.

All other tested ICMs were all able to eliminate the microorganisms in the root canals. The 2% CLX gel was effective in eliminating microorganisms, as previously reported by Valera, et al.[29] (2010), who observed eliminating of *C. albicans* and *E. faecalis* after the use of 2% CLX gel.

The action of CLX occurs because this molecule is positively charged and the microbial cell walls are negatively charged, leading to electrostatic interactions and changing the osmotic balance of the cell. The increased permeability of the cell wall allows the CLX molecule to penetrate into the bacterium. When CLX is used at a high concentration (2%), precipitation occurs in the cytoplasm, with consequent death of the microorganism[9].

The propolis extract used in this study contained 5.63 mg/mL of flavonoids and was effective against the tested microorganisms. The antimicrobial activity of propolis extract on different microorganisms was verified by other authors[7,13]. The effectiveness of propolis on *E. faecalis* as an intracanal dressing for 10 days was investigated[21], and elimination of *E. coli* inoculated in root canals was observed after use of a 12% propolis glycolic extract[29].

However, the real mechanism of the antimicrobial action of propolis appears to be complex and not yet fully understood[13], and some authors attribute to flavonoids the higher antimicrobial activity present in this extract[1,10,13].

The ginger extract used in this study used the dehydrated rhizome of *Zingiber officinale Roscoe*, containing 1.79 mg/mL of flavonoids, with 1.50 mg/mL being the minimum standard for the whole extract. It was verified that this extract was effective in eliminating the microorganisms. Antimicrobial activity of ginger extract on three Gram-negative anaerobes, *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Prevotella intermedia* was observed[23]. The antimicrobial activity against *E. coli* was also reported[12]. However, the real mechanism of action of ginger extract has not yet been elucidated in the literature. It is known that the gingerol is its major active substance and is responsible for the beneficial properties of this

product[23].

In this study, similarly to other reports[9,29], the combination of CH and 2% CLX gel was effective against *C. albicans* and *E. faecalis*. This association is significantly more effective on these microorganisms than the CH paste alone, due to the synergic effect of the two medications[6].

The results of this study showed a significant reduction of endotoxins after using ICMs, with much smaller values compared with the Saline group, which received ICM. However, in groups with CH alone or in combination, there was significant reduction in the amount of endotoxins in the root canals, and in some specimens these ICMs inactivated the endotoxins. CH hydrolyzes the lipid A, resulting in high release of free fatty acids[25]. Moreover, after the use of CH, there is inhibition of prostaglandin E2 production in supernatants of monocytes stimulated by LPS[26]. Thus, the biological action of endotoxins requires the presence of hydroxylated fatty acids bonded to ester junctions; however, these bonds are disrupted by the treatment with CH.

The results also showed that chlorhexidine, propolis extract and ginger extract reduced the amount of endotoxins, yet they were unable to inactivate them completely in any specimen. CLX is a chemical commonly used in endodontic practice; however, it has little effectiveness on bacterial endotoxins[17,20]. In relation to the propolis extract, the present results agree with a previous study reporting that biomechanical preparation with propolis extract was able to reduce the amount of endotoxins from the root canals, yet not completely inactivating them[30]. However, the mechanism of action of this product is not yet elucidated.

In this study, all ICMs containing CH were more effective in neutralizing the endotoxins. Maekawa,et al.[17] (2011) and Valera, et al.[29](2010) evaluated the association of CH and CLX and found that the use of this combination for 14 days reduced significantly the amount of endotoxins in the root canal. However, there are no studies evaluating the association of CH with propolis or ginger, or the action of these medications on endotoxins when used alone. The mechanism of action of these combinations on endotoxins seems to be the same as that of CH, mentioned above.

This way, according to the results of this study, propolis or ginger extracts associated with CH can be used as an alternative ICM. However, further studies on the biocompatibility and chemical interaction between these products are required, as well as *in vivo* studies should be conducted to allow their use for endodontic therapy.

CONCLUSION

All ICMs were able to eliminate *C. albicans*, *E. faecalis* and *E. coli* in the root canals, except for the CH paste, which did not eliminate completely *C. albicans* and *E. faecalis*. All ICMs were able to reduce the amount of endotoxins in the root canals, but the ICMs containing CH were more effective.

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