

Ex-vivo Effect of Intracanal Medications Based on Ozone and Calcium Hydroxide in Root Canals Contaminated with *Enterococcus faecalis*

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This *ex vivo* study evaluated the antibacterial effect of intracanal medications in root canals contaminated with *Enterococcus faecalis*. Fifty single-rooted human teeth were contaminated with *E. faecalis* (ATCC 29212) and incubated at 37°C for 21 days. The specimens were randomly divided into 5 groups according to the intracanal medication used: OZ-PG: ozonized propylene glycol; CH/CPMC: calcium hydroxide/camphorated paramonochlorophenol; OZ-PG/CH ozonized PG/CH; PC: positive control group (no medication); and NC: negative control group (no contamination). The samples were collected after 7 days (post-medication) and 14 days (final). Bacterial growth was checked by counting the colony-forming units (CFU). OZ-PG and CH/CPMC reduced significantly the CFU counts compared with PC in the post-medication and final samples, with no statistically significant differences among them. On the other hand, OZ-PG/CH did not reduce significantly the number of bacteria compared with PC. In conclusion, among the evaluated medications OZ-PG and CH/CPMC were the most effective against *E. faecalis*.

Key Words: calcium hydroxide, Endodontics, *Enterococcus faecalis*, ozone, root canal preparation.

Introduction

Resistant microorganisms may remain inside the root canals after biomechanical preparation, or even penetrate in the root canal system during or after the endodontic treatment. These microorganisms and their byproducts are able to promote persistent or secondary infections, leading to treatment failure. In these situations, the root canal microbiota is composed of fewer species than primary infections and Gram-positive bacteria predominate (1).

E. faecalis has high prevalence in root-filled teeth associated with periapical lesions and shows resistance against many antimicrobial agents. It possesses several virulence factors that contribute to its ability of surviving the effects of conventional root canal therapy (2). Additionally, this Gram-positive facultative anaerobe is able to invade dentine tubules and bind to collagen (3).

Among other applications in endodontics, calcium hydroxide (CH) is widely accepted as an interappointment medication during root canal treatment (4,5). However, this alkaline product is not able to kill all microbial species, especially *E. faecalis* (6). The combination of CH with camphorated paramonochlorophenol (CPMC) increases its antimicrobial activity (7,8). Nevertheless, CPMC is a potential irritant to vital tissues and it should be used with caution (9).

Ozone presents interesting biologic characteristics because it is a very reactive gas, which produces oxidizing

radicals in presence of water. They penetrate and attack the cell membranes, disturbing the osmotic equilibrium, allowing the oxidation of amino acids and fatty acids, which may cause cell lyses. This antimicrobial property has been used in the treatment of several diseases that involve bacteria, yeasts and virus (10).

The use of ozone in dentistry is still not very widespread, but it has attracted the interest of some researchers. This gas has been proven effective against several microbial species found in the oral cavity, and as a method of disinfecting dentures (11). In Endodontics, studies have focused on the use of ozone as an irrigating agent or an intracanal medication (4,12-15). Despite pioneering applications of ozone in Endodontics (16), no consensus has yet been reached about the experimental results (17). The purpose of this study was to evaluate the antibacterial effect of intracanal medications based on ozone and CH in root canals contaminated with *E. faecalis*.

Material and Methods

The study protocol was approved by the institutional Ethics Committee (Process #42/8). Fifty freshly extracted single-rooted teeth were used. They were previously radiographed in the mesiodistal plane and those with more than one root canal, with calcification, resorption or endodontic filling were excluded. Crowns were sectioned with a precision saw (Isomet 1000; Buehler Ltda, Lake

Bluff, IL, USA) and the root lengths were standardized to approximately 15 mm.

The root canals were enlarged 1 mm short of the root apex (14 mm) up to a size 50 K-file (Dentsply-Maillefer, Ballaigues, Switzerland) using the classic technique. Irrigation was performed with 1% NaOCl. After biomechanical preparation, the root canals were filled with 17% trisodium EDTA (Biodinâmica, Ibioporã, PR, Brazil), agitated with a size 50 K-file for 3 min, and then irrigated with 5 mL of saline to wash out the NaOCl and EDTA. The root apices were then sealed with composite resin (Z100; 3M, Sumaré, SP, Brazil) and all external root surfaces were made impermeable with epoxy adhesive (Araldite; Brascola, São Bernardo do Campo, SP, Brazil), except for the cervical access.

The specimens were randomly divided into five 24-well cell culture microplates (Corning Inc., Corning, NY, USA). Specimens were attached to the wells with acrylic resin (Clássico, São Paulo, SP, Brazil) and sterilized by gamma rays from a cobalt 60 source (Embrarad, Cotia, SP, Brazil).

The microbiological procedures were performed under aseptic conditions, in a laminar flow chamber. *E. faecalis* (ATCC 29212) was previously cultivated in Brain Heart Infusion broth (BHI; Difco, Detroit, MI, USA) at 37°C for 24 h. A bacterial suspension was prepared with sterile saline solution to match the turbidity of 1.5×10^8 CFU/mL (equivalent to 0.5 McFarland standard). Then, 10 μ L of the bacterial suspension and 10 μ L of BHI broth were inoculated into each root canal with the aid of an automatic micropipette (Gison, Villiers-le-Bel, France). The microplates containing the specimens were incubated at 37°C for 21 days. Throughout this period, BHI broth was added to the canals every 3 days. Six teeth did not receive bacterial suspension, only BHI broth (negative control group - NC).

Twenty-one days after inoculation, contamination was confirmed by seeding technique and CFU counting. This initial sample was performed using size 40 sterile paper points (Tanariman, Manacapuru, AM, Brazil) placed inside the root canal for 1 min and then transferred to test tubes containing 1 mL of sterile saline solution. The tubes were submitted to agitation for 1 min (Vortex Ap 56; Phoenix, Araraquara, SP, Brazil). After decimal serial dilutions, aliquots were seeded into Petri dishes containing BHI agar (Difco) and incubated at 37°C and 5% CO₂ for 24 h. Bacterial growth was measured by the CFU/mL counts of *E. faecalis*. Then, the root canals were dried with sterile paper points (Tanariman) and divided into three experimental and two control groups according to the used intracanal medications: OZ-PG (n=11): ozonized propylene glycol; CH/CPMC (n=11): commercial calcium hydroxide/camphorated

paramonochlorophenol paste (Calen PMCC; SSWhite, Rio de Janeiro, RJ, Brazil); OZ-PG/CH (n=11); PC (n=11): positive control group (contaminated teeth, without medication); and NC (n=6): negative control group (sterilized and non-contaminated teeth).

The propylene glycol vehicle (Labsynth, Diadema, SP, Brazil) was ozonized in 5 mL tubes submitted to ozone gas bubbles for 60 min, using an ozone generator (C-20 Model; Ozonic, São Bernardo do Campo, SP, Brazil), as proposed by Guinesi et al. (18). The ozonized product was disposed into empty anesthetic cartridges and taken to the root canals by a Carpule syringe and 27G needles. The powder/vehicle ratio used in the ozonized PG/CH paste was the same as that employed in the commercial CH/CPMC paste, which contains 48g% CH.

After 7 days, the root canals were irrigated with 5 mL of sterile saline solution and agitated with a size 50 K-file up to the working length in order to remove the intracanal medication. The post-medication samples were collected as previously described. The number of CFU/mL was calculated. Next, the specimens were filled with sterile saline and incubated for 7 more days. The final samples were collected and once again the number of CFU/mL was determined.

Results were submitted to logarithmic transformation and analyzed using the BioEstat 5.0 (CNPq, Brasília, DF, Brazil). Kruskal-Wallis and Dunn's tests were used for comparison among the groups. Friedman's test was used for comparison among the samples within each group. The significance level was set at 5% for all analyses.

Results

Table 1 shows the recovery of pure *E. faecalis* strains from all teeth in the initial sample performed 21 days after incubation, which revealed similar CFU/mL counts for all groups, except for the negative control.

After 7 days, in the post-medication samples, CH/CPMC had the lowest CFU/mL counts. It was statistically similar to OZ-PG and NC, and different from the other groups.

Table 1. Bacterial counts (CFU/mL log₁₀) in the initial, post-medication and final samples

Group	Initial	Post-medication	Final
OZ-PG	6.82 ± 0.11 A,a	2.31 ± 1.85 AB,b	3.20 ± 1.22 AB,b
CH/CPMC	6.90 ± 0.10 A,a	0.62 ± 1.41 A,b	0.60 ± 1.34 A,b
OZ-PG/CH	6.89 ± 0.14 A,a	3.80 ± 1.34 BC,b	4.14 ± 0.35 B,b
Positive control	6.78 ± 0.38 A,a	4.68 ± 0.25 C,b	4.38 ± 0.72 B,b
Negative control	0 ± 0 B,a	0 ± 0 A,a	0 ± 0 A,a

OZ: ozonized. PG: propylene glycol. CH: calcium hydroxide. CPMC: camphorated paramonochlorophenol. Same uppercase letters in the same column and lowercase letters in the same row indicate statistically similar results (p>0.05).

The highest bacterial count was observed in PC, without significant difference from OZ-PG/CH (Table 1).

After 14 days, in the final sample, CH/CPMC had again the lowest CFU/mL counts. It was statistically similar to OZ-PG and the NC ($p>0.05$), and different ($p>0.05$) from the other groups. The highest bacterial count was observed in PC, without difference from OZ-PG and OZ-PG/CH (Table 1).

According to Friedman's test, all experimental groups and the positive control showed alterations in the bacterial counts throughout the procedures (Fig. 1). *E. faecalis* counts in the initial samples were significantly higher than in the post-medication and final samples, which were similar between themselves (Table 1). As expected, only NC did not show significant alterations throughout the procedures.

Discussion

Several methodologies can be used to assess the antimicrobial activity of endodontic medications. The experimental model adopted in this study allows simulating endodontic infection in the root canal system and dentinal tubules, closely resembling *in vivo* clinical situations (19).

E. faecalis was used for being frequently associated with post-endodontic therapy failure (2) and also because of its resistance to CH-based intracanal dressings (20). A 21-day period of contamination was adopted because it allows diffusion of the *E. faecalis* suspension through the dentinal tubules, as reported by Cardoso et al. (17). Contamination methodology was confirmed by the recovery of pure *E. faecalis* strains from all teeth in the initial sample, except for NC.

The *ex vivo* effect of ozonized PG and ozonized PG/CH as intracanal medications was evaluated against *E. faecalis*, in comparison with CH/CPMC. The medications remained within the root canals for 7 days and another sample was collected. Finding an interappointment medication with suitable bactericidal effect within a short period of time

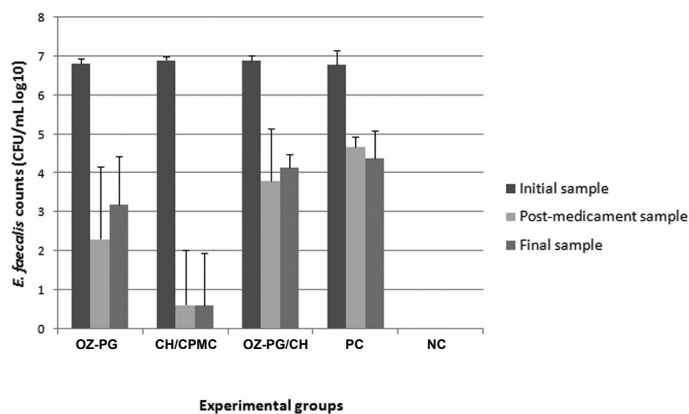


Figure 1. Chart showing the bacterial counts (CFU/mL log₁₀) in the initial, post-medication and final samples.

has been the focus of a current research worldwide.

In order to enhance the bacterial action of CH, its combination with other antimicrobial agents, such as CPMC, has been proposed (7,21,22). In the present study, a significant reduction in the bacterial counts was verified in the CH/CPMC group, in agreement with previous studies. According to Siqueira and Lopes (23), the use of CH with CPMC extends the antibacterial spectrum and kills bacteria faster than the mixtures of CH with inert vehicles.

CPMC may complement the activity of CH by disrupting the bacterial cytoplasm membrane, denaturing proteins, and inactivating enzymes (7,21). Furthermore, CPMC in combination with CH forms calcium p-chlorophenolate, which promotes controlled and extended release of calcium and hydroxyl ions (24).

Noetzel et al. (4) evaluated the efficacy of CH, Er:YAG laser or gaseous ozone (either alone or combined with instrumentation and various irrigants) against *E. faecalis* in root canals. They verified that gaseous ozone applied directly into the root canals for 120 s had similar effect to CH paste applied for 7 days. In the present study, ozonized PG had similar effect to CH/CPMC when both were applied for 7 days. However, the results of these investigations cannot be directly compared because the form and dose of ozone were different. Moreover, the present results show that ozonized PG/CH had low effect against *E. faecalis*. There is no clear explanation for this finding, but the chemical interaction between ozone and CH probably affects the antimicrobial activity that these substances present separately. Further research addressing the chemical analysis of this combination is required.

While the half-life of ozone in gaseous form is ephemeral, its oil form allows storage for several months, avoiding the need of a generator (18). The mechanism of action of ozonized oils is related to their byproducts, the triozoneides, which generate hydrogen peroxide and other compounds. The ozonized oil can be stored up to 2 years under refrigeration. It takes from 1 h to 2 days to reach the desirable concentration at room temperature. Overall, 1 g of oil can absorb 160 mg of ozone (25).

In a previous research (13), ozonized oil was employed as an intracanal medication in dog's teeth, and the success rate was similar to that of CH/CPMC and greater than the single-visit treatment group. The authors investigated the state of the periapical tissues using a histological score system. The vehicles of the pastes are not the same of the ones employed in the present study, but the results also show promising application of ozone in Endodontics.

In order to verify if the medications based on

ozone have residual effect, microbiological samples were retrieved 14 days after the initial sample. The results were similar to those from the sample made 7 days post-medication for all experimental groups. Thus, there is no residual effect, indicating that microorganisms located inside dentinal tubules, lateral canals and apical deltas were not eliminated by any medication and they were able to re-colonize the main root canal.

In conclusion, ozonized PG and CH/CPMC reduced significantly the bacterial counts in comparison with PC in the post-medication and final sample. On the other hand, OZ-PG/CH was similar to PC in these samples. Thus, among the medications evaluated in this study, OZ-PG and CH/CPMC were the most effective against *E. faecalis*. These findings indicate a potential use of ozone as an intracanal medication. However, future studies evaluating biocompatibility, time of application and physicochemical properties should be performed to determine whether this substance is actually suitable for clinical use.

Resumo

Este estudo *ex vivo* avaliou o efeito antibacteriano de medicações intracanal em canais radiculares contaminados com *Enterococcus faecalis*. Cinquenta dentes humanos unirradiculares foram contaminados com *E. faecalis* (ATCC 29212) e incubados a 37°C durante 21 dias. Os espécimes foram aleatoriamente divididos em diferentes grupos de acordo com a medicação intracanal utilizada: PG-OZ: propilenoglicol ozonizado; HC/PMCC: hidróxido de cálcio/paramonoclorofenol canforado; PG-OZ/CH; CP: controle positivo (sem medicação); e CN: controle negativo (sem contaminação). As amostras foram coletadas após 7 dias (pós-medicação) e 14 dias (final). O crescimento bacteriano foi verificado através da contagem das unidades formadoras de colônias (UFC). PG-OZ e HC/PMCC reduziram estatisticamente o número de bactérias quando comparados com o CP nas amostras pós-medicação e final, sem diferenças estatísticas entre si. Por outro lado, PG-OZ/CH não reduziu significativamente o número de bactérias em comparação com o CP. Em conclusão, entre as medicações avaliadas, PG-OZ e HC/PMCC foram as mais eficazes contra *E. faecalis*.

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