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Antibacterial activity of intracanal medications based on calcium hydroxide and zinc oxide micro- or nanoparticles: an *ex vivo* study

Atividade antibacteriana de medicações intracanal à base de hidróxido de cálcio e óxido de zinco micro- e nanoparticulado: estudo ex vivo

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Resumo

Introdução: Nanopartículas e associações ao hidróxido de cálcio podem ser usados para aumentar a ação antimicrobiana. **Objetivo:** Avaliar a atividade antibacteriana sobre *Enterococcus faecalis* e o pH de medicações intracanal (MI) à base de hidróxido de cálcio (HC) e óxido de zinco (OZn) micro- e nanoparticulado, e suas associações com clorexidina (CHX) a 0,4%. **Material e método:** Canais radiculares de dentes humanos unirradiculados foram inoculados e incubados por 21 dias. Após coleta (C1), os canais radiculares foram preenchidos com os medicamentos durante 7 dias e as amostras foram coletadas imediatamente após a medicação (C2) e 7 dias depois (C3). Contagem de UFC mL^{-1} foi realizada. Os tubos de polietileno preenchidos com os medicamentos foram utilizados para a avaliação do pH após 3, 7, 14 e 28 dias. Os dados foram submetidos aos testes de ANOVA e Tukey ($p<0,05$). **Resultado:** Todas as MI promoveram eliminação de *E. faecalis* imediatamente após a medicação (C2). Todas as pastas promoveram similar aumento de pH. **Conclusão:** HC/OZn micro- ou nanoparticulado associado com CHX promove maior redução bacteriana nos canais radiculares e pH similar.

Descritores: Anti-infecciosos; hidróxido de cálcio; endodontia; *Enterococcus faecalis*; nanopartículas.

Abstract

Introduction: Nanoparticles and associations to calcium hydroxide can be used to increase antimicrobial activity. **Objective:** To evaluate antibacterial activity against *Enterococcus faecalis* and pH of intracanal medications (IM) based on calcium hydroxide (CH) and zinc oxide (ZnO) micro- or nanoparticles, and their association with 0.4% chlorhexidine (CHX). **Material and method:** Root canals from single-rooted human teeth were inoculated and incubated for 21 days. After sample (S1), the root canals were filled with the medications for 7 days and samples were collected immediately after medication (S2) and 7 days later (S3). Counting of CFU mL^{-1} was performed. Polyethylene tubes filled with the medications were used for the pH evaluation after 3, 7, 14 and 28 days. Data were submitted to ANOVA and Tukey tests ($p<0.05$). **Result:** All IM promoted elimination of *E. faecalis* immediately after medication (S2). All the pastes promoted a similar pH increase. **Conclusion:** CH/ZnO micro- or nanoparticles associated with CHX promoted greater bacterial reduction in the root canals and similar pH.

Descriptors: Anti-infective agents; calcium hydroxide; endodontics; *Enterococcus faecalis*; nanoparticles.

INTRODUCTION

Intracanal medication is used to control microorganisms in the root canal system (RCS) after root canal preparation. *Enterococcus faecalis* may survive after root canal procedures, contributing to endodontic treatment failure[1]. Calcium hydroxide is used as medication in teeth with pulp necrosis, promoting microorganism reduction in the RCS, favoring the endodontic treatment success[2]. Calcium hydroxide (CH) has antimicrobial effect, due to its dissociation into Ca^{+} and OH^{-} ions, promoting an alkaline pH[1,3] and interaction with the microorganism cell wall, and inactivating bacterial byproducts such as endotoxin[4-6].

The search for a better antimicrobial effectiveness of CH has been intense and several substances have been associated[1,2,4,7,8]. Association of CH with chlorhexidine is indicated in primary and secondary endodontic infections, increasing antimicrobial

activity against microorganisms resistant to calcium hydroxide, such as *E. faecalis* and *C. albicans*[9-11]. Delgado et al.[12] using root canals contaminated with *E. faecalis,* showed that CH and 2% chlorhexidine association were more effective when compared with CH medication. However, other studies have not shown benefits for association with CHX[3,4,13].

Ordinola-Zapata et al.[2] evaluated the physicochemical properties (pH, calcium ion release, radiopacity) and antimicrobial activity of CH pastes associated with different agents, including microparticles zinc oxide (ZnO). HC with microparticles ZnO showed proper results, but it did not improve the antimicrobial effectiveness of CH.

Nanoparticle substances may be used in the composition of intracanal medications and endodontic sealers, increasing antibacterial activity, due they present higher active surface area[1]. The incorporation of nanoparticles zinc oxide to CH can improve their radiopacity, consistency and antimicrobial activity without interferer with their other properties, such as hydroxyl ions release.

The aim of this study was to evaluate the antibacterial activity and pH of intracanal medications with CH and ZnO micro- or nanoparticles, and their association with 0.4% chlorhexidine (CHX). The null hypothesis is that the use of nanoparticle substances and association with CHX would not promote greater effect against *E. faecalis* than the microparticle formulations.

MATERIAL AND METHOD

Microbiological Evaluation

This study was approved by Ethics Committee of the Institution. The intracanal medications evaluated were: CH/ZnO microparticles (Sigma Chemical Co., St Louis, MO, USA); CH/ZnO nanoparticles (Physics Institute, USP, São Carlos, SP, Brazil); CH/ZnO micro + 0.4% CHX (Sigma Chemical Co., St. Louis, MO, USA); CH/ZnO nano + 0.4% CHX. The size of the nanoparticles (nano) used was 100-200 nm in solid state, obtained by sequential adsorption of polyelectrolytes. In addition to the intracanal medications, a negative control was used, with sterilized culture medium in the root canals, to confirm sterilization. Positive control was used, with no intracanal medication in the root canals, to confirm bacterial viability during the experiment.

Polyethyleneglycol 400 (Sigma Chemical Co., St. Louis, MO, USA) was used as vehicle for all medications. Intracanal medications were prepared using the components in the proportion: CH-2.5 g, ZnO-0.5 g and polyethyleneglycol 400-2.0 mL. For the medications with CHX, CH/ZnO paste was initially manipulated. The paste was weighed using a precision scale, and CHX was added until the final concentration in the paste was 0.4% CHX.

Preparation of Specimens

The specimens were prepared according to previous studies[10,14]. Sixty extracted single-rooted human teeth with a single straight root canal were selected. Crowns were removed using an Isomet 1000 device (Buehler Ltda, Lake Bluff, IL, USA) in order to standardize the length of each specimen at 15 mm and the foramen diameter was standardized with a size 25 K-file (Dentsply-Maillefer, Ballaigues, Switzerland).

The working length (WL) was established 1 mm short of the apical foramen and the root canal was instrumented up to a size 50 K-file (apical stop). Conventional needle irrigation was used with 5 mL of 1% NaOCl after each file. Subsequently, the root canals were filled with 17% trisodium EDTA (Biodinâmica, Ibiporã, PR, Brazil) for 3 minutes, followed by irrigation with 5 mL of saline solution. Following that, the root apices were sealed with light-cured composite resin (Z250 Universal Restorative; 3M ESPE, St. Paul, MN, USA) and all external root surfaces (except the root canal access) were made impermeable with two layers of epoxy adhesive (Araldite; Brascola Ltda., São Paulo, SP, Brazil).

The specimens (n=10) were randomly divided into eight 24-well cell culture microplates (Corning Incorporated, Corning, NY, USA). Specimens were attached to the wells with acrylic resin and sterilized by ethylene oxide (Acecil, Campinas, SP, Brazil). To verify the sterilization of the specimens, root canals were filled with sterile culture medium, incubated at 37 °C for 2 days and samples were collected from each root canal.

Contamination of the Specimens

These procedures were carried out in a laminar flow chamber (VecoFlow Ltda., Campinas, SP, Brazil). The root canals were contaminated with 20 µL suspension of *E. faecalis* (ATCC 29212) with 1 x 10^8 CFU mL^{-1} diluted in Tryptic Soy Broth - TSb medium (Difco, Detroit, MI, USA). The microplates containing the specimens were kept in microaerophilic environment at 37 °C for 21 days[10,14]. Every 2 days, sterilized culture medium (TSb) was added in the root canals.

After contamination, samples were collected from each root canal in order to confirm contamination (S1) using two sterile paper points (Tanari Industrial Ltda, São Paulo, SP, Brazil) in sequence and transferred to tubes containing sterile saline. Ten-fold serial dilutions were made and aliquots were seeded in triplicate onto Petri dishes containing Tryptic Soy Agar - TSa (Difco, Detroit, MI, USA) and incubated in microaerophilic environment at 37 °C for 48 hours. Bacterial growth was measured by the CFU mL^{-1} counts of *E. faecalis*, which were confirmed by colony morphology and Gram stain.

Experimental procedures and microbiological analysis

After confirmation of bacterial contamination, the root canals were prepared using 50 K-file and irrigated with 5 mL sterile saline. After that, the root canals were filled with 17% EDTA for 3 minutes and irrigated with sterile saline. The root canals were dried with sterile paper points, and then filled with the intracanal medications.

The root canals were filled with the medications by using a Lentulo (Dentsply Maillefer, Balaigues, Switzerland). Sterile cotton pellets were placed at the root canal access cavities and specimens were incubated at 37 °C in microaerophilic environment for 7 days. In positive control group, root canals were re-prepared, but did not receive intracanal medicaments.

After 7 days, the intracanal medication was removed and a new post-medication sample was performed (S2). Medication was removed using size 50 K-files, and the root canals were irrigated with 5 mL of sterile saline. After that, root canals were irrigated with

1 mL of a neutralizing agent specific for each evaluated medicament. Tween 80 and soya lecithin were used for medications with CHX and 0.5% citric acid was use to neutralize CH.

After irrigation with 1 mL of sterile saline solution, 2 paper cones were used for each specimen. The procedures were performed as described for initial harvest. The root canals were then filled with sterile saline and a sterile cotton pellet was placed at the root canal entrance.

The microplates containing the specimens were covered and incubated once again in microaerophilic environment at 37 °C for 7 days, after which a new sample was carried out (S3) according to the previously described steps, in order to recover microorganisms that had remained in the dentine tubules and root canal system.

Results were subjected to logarithmic transformation and analyzed using the GraphPad Prism 3.0 software (San Diego, CA, USA). Comparison between the experimental groups was performed by using ANOVA and Tukey tests. The significance was established at a 5% level ($P < 0.05$).

pH Evaluation

Intracanal medications were inserted into polyethylene tubes measuring 1 cm length and 1.5 mm in internal diameter. After this, they were immersed into 10 mL of distilled water in plastic flasks. The flasks were maintained at 37 °C. After 3 days of immersion, the tube/medication was removed and placed into a new flask with 10 mL of distilled water. The solution from each flask was used to evaluate the pH and the procedure was repeated in the time intervals of 7, 14 and 28 days.

The pH of the solution was determined by using a Digimed DM-21 (Digicrom Analítica Ltda., São Paulo, Brazil) pH meter. This appliance was previously calibrated with buffer solutions at pH 4, 7, and 10. The pH measurements were performed at 25 °C. The data obtained were submitted to ANOVA and Tukey tests and the significance was established at a 5% level ($p<0.05$).

RESULT

The results of the antimicrobial evaluation are presented in Table 1. S2 demonstrated that the intracanal medications were effective in eliminating *E. faecalis*. However, the bacteria remaining in the root canal systems provided recontamination of the main canal 7 days after medication removal, as was observed in the S3. In the S3, greater antimicrobial effectiveness was observed for medications associated with 0.4% CHX.

All the medications evaluated promoted a similar increase in pH during all the periods, showing higher pH than the control without medication ($p<0.05$) as demonstrated in Table 2.

Table 1. Comparison between medications at the samples S1, S2, and S3 (mean and standard deviation of CFU mL^{-1} log_{10})

Samples	Intracanal Medication				
	CH/ZnO micro	CH/ZnO nano	CH/ZnO micro + CHX	CH/ZnO nano + CHX	Control
S1	6.31[a] (0.28)	6.54[a] (0.27)	6.31[a] (0.32)	6.35[a] (0.26)	6.35[a] (0.21)
S2	0.0[a] (0.00)	0.0[a] (0.00)	0.0[a] (0.00)	0.0[a] (0.00)	6.57[b] (0.00)
S3	2.20[a] (0.23)	2.15[a] (0.23)	1.81[b] (0.19)	1.86[b] (0.18)	6.91[c] (0.20)

Microparticle (micro), nanoparticle (nano), calcium hydroxide/zinc oxide (CH/ZnO). Different letters on the same line indicate statistical difference between groups in the same sample.

Table 2. Comparison of mean pH values (standard deviation) of medications in different periods

Period	Intracanal Medication				
	CH/ZnO Micro	CH/ZnO Nano	CH/ZnO Micro + CHX	CH/ZnO Nano + CHX	Control
3 days	10.22[a] (0.56)	10.63[a] (0.51)	10.55[a] (0.35)	10.70[a] (0.13)	6.72[b] (0.23)
7 days	10.87[a] (0.35)	10.70[a] (0.21)	10.94[a] (0.17)	10.71[a] (0.14)	679[b] (0.31)
14 days	11.08[a] (0.13)	10.93[a] (0.22)	11.08[a] (0.03)	10.96[a] (0.07)	6.97[b] (0.21)
28 days	11.05[a] (0.17)	10.91[a] (0.25)	11.07[a] (0.26)	11.00[a] (0.08)	6.80[b] (0.12)

Microparticle (micro), nanoparticle (nano), calcium hydroxide/zinc oxide (CH/ZnO). Different letters on the same line indicate statistical difference between groups in the same period.

DISCUSSION

The aim of using intracanal medications is to provide effect against microorganisms remaining after root canal preparation. Calcium hydroxide (CH) promotes destruction of bacterial cytoplasmic membrane, and microbial DNA due to its high pH[4,7,15].

Evaluation of the pH demonstrates hydroxyl ions release providing alkalinization, essential to antibacterial effect of CH[8]. The results of present study show that CH and ZnO size (micro-or nanoparticles) did not interfere in the pH values up to 28 days, since the observed values were similar for the medications. Also, the CHX addition did not decrease the hydroxyl ion dissociation from medications, maintaining the alkalinity, in agreement with Duarte et al.[16].

E. faecalis biofilm induced in root canals for 21 days, allowed the propagation of the microorganisms into dentinal tubules and root canal system. Therefore, the methodology allowed evaluation of the antibacterial effect of the medications against the microorganisms into root canal systems[10]. The evaluated medications were effective in eliminating *E. faecalis* from main canal, as demonstrated in the post-medication sample (S2). However, the elimination of *E. faecalis* from root canal system was not completely observed, since recolonization of the main root canal was observed 7 days after medication removal. The lower number of CFU mL^{-1} in the medications associated with CHX was demonstrated in comparison with the medications without CHX. In root canals contaminated with *E. faecalis*, Lucena et al.[17] verified that CH was not effective in its elimination, in addition there was an increase in the percentage of bacterial viability from 26.3% to 55.8%. However, CHX was effective, with a reduction of 40% in bacterial viability.

The association CH/CHX was shown to increase the antimicrobial effect, reducing the application time required when compared with the use of CH alone, against the resistant fungi and bacteria colonizing the root canal systems[9,10,18]. The results of the present study demonstrated that the association with CHX favored the antibacterial activity of CH against *E. faecalis*. These results may be related to the reduction in *E. faecalis* adhesion to dentin[19,20], and its sensitivity to CHX[10,17,21,22].

The electrostatic interaction between the positively charged nanoparticles and negatively charged bacterial cells has been associated with the increase in membrane permeability and loss of bacterial membrane function[23]. Shrestha et al.[24] evaluated nanoparticles of ZnO and chitosan against *E. faecalis* biofilm, and observed that ZnO was capable of reducing and destructuring the biofilm, with its efficiency being dependent on concentration and interaction time. ZnO is used in the composition of CH pastes due its radiopacifying and antibacterial properties. Narayanan et al.[25] showed that antibacterial activity of nanoparticle ZnO is proportional to its concentration. However, the results of the present study did not demonstrate increased antimicrobial effect of nanoparticles in comparison to microparticle substances.

Aguiar et al.[7] evaluated intracanal medications based on calcium hydroxide and showed that the intracanal medications with 0.4% CHX eliminated planktonic cells of *E. faecalis* after 30 seconds of contact. Also, they observed higher pH values for calcium hydroxide and zinc oxide nanoparticulated pastes after 1 and 7 days, disagreeing with the present study. It can be related to different methodologies used for pH evaluation.

CONCLUSION

Medications that presented CH/ZnO associated with 0.4% CHX was more effective against the *E. faecalis* biofilm present in the root canal system. The use of CH and ZnO micro- or nanoparticles showed no influence on the antimicrobial effect and hydroxyl ions release.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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