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ORIGINAL RESEARCH

Antimicrobial Activity and pH of Calcium Hydroxide and Zinc Oxide Nanoparticles Intracanal Medication and Association with Chlorhexidine

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ABSTRACT

Aim: To evaluate pH and antibacterial activity of pastes with calcium hydroxide [$Ca(OH)_2$] and zinc oxide (ZnO) microparticles (micro) or nanoparticles (nano) and association with 0.4% chlorhexidine against *Enterococcus faecalis*.

Materials and methods: The following pastes were analyzed: $Ca(OH)_2$/ZnO micro, (2) $Ca(OH)_2$/ZnO nano, (3) $Ca(OH)_2$/ZnO micro + 0.4% chlorhexidine, (4) $Ca(OH)_2$/ZnO nano + 0.4% chlorhexidine. Antibacterial activity against *E. faecalis* was evaluated by agar diffusion test. The direct contact test on planktonic cells of *E. faecalis* was performed for 30 and 60 seconds. Root canals from bovine teeth were filled with the pastes and pH was evaluated after 1, 7, 14, 21, 30 and 60 days. The data obtained were submitted to the statistical tests analysis of variance (ANOVA) and Tukey or Kruskal-Wallis and Dunn test, with a 5% significance level.

Results: Calcium hydroxide and zinc oxide nano, and the pastes with 0.4% chlorhexidine were more effective in agar diffusion test. In the direct contact test, the pastes with chlorhexidine showed the highest effect after 30 seconds. All pastes eliminated *E. faecalis* after 60 seconds. All pastes promoted an increase in pH. The highest increase in pH was observed with nanoparticle medications after 1 and 7 days ($p < 0.05$). After this period, the pastes presented similar pH increase.

Conclusion: It was concluded that calcium hydroxide and zinc oxide nanoparticles promoted greater initial alkalinization. The antimicrobial activity of the pastes against *E. faecalis* is favored by the association with chlorhexidine.

Clinical significance: Although nanoparticles of calcium hydroxide and zinc oxide promoted antibacterial effect, the activity against *E. faecalis* is favored by association with chlorhexidine.

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INTRODUCTION

The antimicrobial effect of calcium hydroxide [$Ca(OH)_2$] is related to hydroxyl ion release, which diffuse through dentinal tubules to external root surface.[1,2] The dissociation of calcium hydroxide promotes an increase in pH.[3] Hydroxyl ions are free radicals with high reactivity, acting on components from bacteria cell membrane, affecting their biologic activity.[2-6]

Calcium hydroxide is capable of dissolving organic tissue, inhibiting tooth resorption, inducing hard-tissue formation, stimulating osteoblast proliferation and inactivating bacterial lipopolysaccharide (LPS).[7-9] It presents antimicrobial effect against gram positive and negative bacteria and fungi from root canals.[10,11] However, calcium hydroxide has less effect on *Enterococcus faecalis*, a resistant microorganism observed in primary and/or secondary infections.[12-14] The association of calcium hydroxide with other antimicrobial agents has been proposed, such as camphorated paramonochlorophenol or chlorhexidine, in order to increase its antimicrobial effect.[12,15-17]

The association of calcium hydroxide with chlorhexidine promotes microorganism reduction and has been proposed as intracanal medication.[18] Lima et al, in 2012, evaluated the antibacterial effect of calcium hydroxide

pastes against *E. faecalis* in root canals of human teeth, and observed that the association with 0.4% chlorhexidine promoted a higher bacterial reduction when compared with pastes containing only calcium hydroxide.

The size and molecular weight of chemical compounds used in the composition of intracanal medications, such as calcium hydroxide and zinc oxide, can affect their physical and biological properties. The paste radiopacity is favored by zinc oxide.[19-21] The zinc oxide nanoparticle may also promote antimicrobial effect.[22] Sheresta et al, in 2010, verified that nanoparticle zinc oxide reduced *E. faecalis* biofilm, and that its effectiveness was dependent on concentration and time of interaction.[23] Gómez-Ortíz et al, verified the *in vitro* antifungal effect of calcium hydroxide associated with zinc oxide nanoparticle against *Penicillium oxalicum* and *Aspergillus niger* by using electronic scanning microscopy (SEM) and X-ray diffraction (XRD).[24] Thus, nanoparticle substances may allow greater and faster penetrability into the dentinal tubules, favoring the chemical and antimicrobial effect in root canal systems.[22,25,26]

The aim of this study was to evaluate the pH and antibacterial activity of $Ca(OH)_2$/ZnO nanoparticles and their association with 0.4% chlorhexidine, against *E. faecalis*.

MATERIALS AND METHODS

Intracanal medications were manipulated at the time of application. Four experimental groups were analyzed, according to the intracanal medication:

1. Calcium hydroxide ($Ca(OH_2)$, Sigma Chemical Co, St Louis, MO, USA)/zinc oxide (ZnO, Sigma Chemical Co, St Louis, MO, USA) microparticles.
2. Calcium hydroxide/zinc oxide nanoparticles (Institute of Physics, USP, São Carlos, Brazil).
3. Calcium hydroxide/zinc oxide microparticles + 0.4% chlorhexidine (Sigma Chemical Co, St Louis, MO, USA), and
4. Calcium hydroxide/zinc oxide nanoparticles (Institute of Physics, USP, São Carlos, Brazil) + 0.4% chlorhexidine.

The size-range of the nanoparticles used was 100 to 200 nm, obtained by sequential adsorption of polyelectrolytes. The proportion of components in the intracanal medications was: 2.5 gm calcium hydroxide to 0.5 gm zinc oxide to 2.0 ml polyethylene glycol 400 (Sigma Chemical Co, St Louis, MO, USA).

For intracanal medications with chlorhexidine, the calcium hydroxide/zinc oxide paste was initially manipulated, and it was weighed. The volume was calculated, and the chlorhexidine added until a 0.4%. chlorhexidine concentration was obtained.

Agar Diffusion Test

For agar diffusion test, a suspension (3 × 107 cell/ml) of *E. faecalis* (ATCC 29212) was used, standardized by using spectrophotometry. After this, 20 µl of this suspension was seeded in petri dishes 20 × 100 mm, containing m-*Enterococcus* in agar culture medium (n = 3). Five equidistant wells 4 mm in diameter were made with the aid of a sterilized metal device, and these were immediately filled with the intracanal medications evaluated. One of the five wells was used as control group and it was filled with 2% chlorhexidine (Sigma Chemical Co, St Louis, MO, USA).

The plates were kept at room temperature for 2 hours for pre-diffusion of intracanal medications, and then incubated at 37°C for 24 hours under microaerophilic conditions. The inhibition haloes and diffusion were measured with a digital caliper. The data were submitted to the Kruskal-Wallis and Dunn tests, at a 5% level of significance.

Direct Contact with Planktonic Cells

The microbiological procedures were performed in triplicate, in an aseptic environment, within a laminar flow chamber (Veco Flow Ltda., Campinas, SP, Brazil). An amount of 250 mg of each intracanal medication was weighed, manipulated in a falcon tube, and 10 ml of distilled water was added to obtain a suspension of intracanal medication with a concentration of 25 mg/ml. The suspension was agitated and kept at rest for 24 hours at ambient temperature.

An *E. faecalis* (ATCC 29212) culture suspension was used, obtained in TSa plates (Tryptic Soy Agar, Difco, Detroit, MI, USA), by using seeding by exhaustion, 12 hours before each test. The inoculum purity was observed by using macroscopic and microscopic morphology (gram staining). Bacterial cell concentration was adjusted by spectrophotometry (Model 600 Plus, Femto, São Paulo, SP, Brazil). The bacterial suspension was used within a 60 minutes period after the adjustment to 3 × 107 colony forming units per ml (CFU/ml), standardized by spectrophotometry. As negative control, sterilized physiological solution was used, which served to confirm the initial number of CFU/ml.

By using automatic pipettes, 1.4 ml of each intracanal medication suspension was put into polyethylene tubes. After this, an aliquot of 50 µl of *E. faecalis* suspension was added to the tube and agitated for 5 seconds in a vortex (Vortex AP 56, Phoenix, Araraquara, SP, Brazil). The contact times were 30 and 60 seconds.

After this period, serial decimal dilutions were made up to 10^{-4}. At this stage, aliquots of 100 µl of the mixture

were transferred to a second tube containing 0.9 ml of neutralizing agent. For calcium hydroxide-based intracanal medications, the neutralizing agent used was 0.5% citric acid. In the groups containing chlorhexidine, the following neutralizing agents were used: 0.5% citric acid and soy lecithin prepared with tween 80. These neutralizing agents were present in the first two tubes of the dilution. The other tubes contained 0.9 ml of saline solution, with the purpose of preventing the carry-over effect of the intracanal medications.

On conclusion of the serial decimal dilution up to the 4th dilution, aliquots of 20 µl of each of the dilutions were seeded in triplicate on the surface of TSa plates, and were incubated at 37ºC for 48 hours under microaerophilic conditions. The results of each plate in a mean of CFUs of the three areas of bacterial growth were obtained, using a number between 5 and 50 CFU/ml. From these means, the number of CFU/ml was calculated after each contact between the intracanal medication suspensions and the bacterial suspension.

The values obtained were transformed in log, by the formula $\log_{10}$ CFU/ml = X, and submitted to the analysis of variance (ANOVA) and Tukey statistical tests with a 5% level of significance.

Diffusion of Hydroxyl Ions

To evaluate the release of OH^- ions from the pastes, 72 bovine teeth were used. The tooth crowns were removed, maintaining a root length of approximately 15 mm. The roots were kept in a 2.5% sodium hypochlorite solution for 48 hours, and after this in distilled water. The root canals were instrumented 1 mm short of the root length up to file K#110 (Dentsply-Maillefer, Ballaigues, Switzerland). Root canal preparation in the cervical and middle thirds was performed with a Gates-Glidden No. 5 bur (Dentsply-Maillefer, Ballaigues, Switzerland).

The root canals were irrigated with 2.5% sodium hypochlorite solution (Instituto de Química, Araraquara, UNESP, Brazil) during the root canal preparation. Five mililiters were used at the beginning and 3 ml at each change of file. At the end, the root canals were filled with 17% ethylenediaminetetraacetic acid (ETDA) solution (Odahcam Dentsply, Petrópolis, RJ, Brazil) for 3 minutes. Next, a final irrigation was performed using saline solution.

The roots were stored in a flask containing distilled water at 37°C for 14 days, in order to stabilize ionic changes from dental surfaces. After this period, a cavity 4 mm long, 2 mm wide and 0.5 mm deep was made on root surface, between 5 and 9 mm from the apex, using a diamond drill #1052 (KG Sorensen Ind. e Com. Ltda, São Paulo, Brazil). This cavity was made in all the specimens to standardize the surface and thickness of exposed dentin. After this, the external root surface was sealed with a layer of epoxy adhesive (Brascola, Joinville, SC, Brazil), and a layer of nail varnish, except in the cavity preparation area. Tooth in the negative control group were completely sealed, including the cavity area. The specimens were measured and radiographed in order to verify the same dentin thickness between the cavity wall and root canal.

The intracanal medications introduced by coronary access into the root canals using a 3 ml BD plastic syringe (Benton, Dickinson and Company, Juiz de Fora, MG, Brazil) with a 1.20 × 40 needle (Benton, Dickinson and Company, Juiz de Fora, MG, Brazil). After this, the coronal access was sealed with temporary cement (Coltosol, Vigodent Coltene, Rio de Janeiro, RJ, Brazil). The samples were immersed in flasks containing 10 ml of distilled water. These flasks were closed and taken to an oven at 37°C. At the time intervals of 1, 7, 14, 21, 30 and 60 days, the pH of the water in each flask was analyzed, at the temperature of 25°C. The pH analysis was performed using a Digimed DM-21 (Digicrom Analítica Ltda., São Paulo, Brazil).

The results obtained were submitted to statistical analysis using ANOVA and Tukey test with a 5% significance level.

RESULTS

The results of the agar diffusion test (Table 1) demonstrated that the intracanal medications were effective against *E. faecalis*. The medication $Ca(OH)_2$/ZnO nano and pastes with 0.4% chlorhexidine presented higher activity than the $Ca(OH)_2$/ZnO micropaste ($p < 0.05$). In the test of direct contact with planktonic cells of *E. faecalis*, all the medications eliminated *E. faecalis* after 60 seconds. After 30 seconds, only the pastes with chlorhexidine in the formulation eliminated *E. faecalis*. Other pastes showed a reduction when compared with the control group (Table 2).

The data obtained in the hydroxyl ion diffusion evaluation (Table 3) demonstrated that all the medications increase the pH of the external root surface. The nanoparticle medications presented a higher pH after 1 and 7 days in comparison with the other pastes ($p < 0.5$). In the other evaluation periods, the pH increase was similar for all medications.

DISCUSSION

Bovine teeth were used for evaluating the diffusion of hydroxyl ions through dentin. Camargo et al, in 2006,

Table 1: Values of inhibition haloes in diffusion in agar against *E. faecalis*

Intracanal medication	*Mean value of haloes (mm)*
(1) Calcium hydroxide/zinc oxide microparticles	8.64[b]
(2) Calcium hydroxide/zinc oxide nanoparticles	11.00[a,b]
(3) Calcium hydroxide/zinc oxide microparticles + 0.4% chlorhexidine	18.83[a,b]
(4) Calcium hydroxide/zinc oxide nanoparticles + 0.4% chlorhexidine	18.52[a,b]
(5) Control group: 2% chlorhexidine solution	26.08[a]

Different letters in the same column represent statistically significant difference ($p < 0.05$)

Table 2: Data in log_{10} direct contact test of intracanal medication suspensions with *E. faecalis* after contact time of 30 seconds and 1 minute

Intracanal medication	*CFU/ml values in log_{10} after 30 seconds*	*CFU/ml values in log_{10} after 1 minute*
(1) Calcium hydroxide/zinc oxide microparticles	4.00[b]	0[b]
(2) Calcium hydroxide/zinc oxide nanoparticles	3.72[b]	0[b]
(3) Calcium hydroxide/zinc oxide microparticles + 0.4% chlorhexidine	0[c]	0[b]
(4) Calcium hydroxide/zinc oxide nanoparticles + 0.4% chlorhexidine	0[c]	0[b]
Saline	7.69[a]	7.58[a]

Different letters in the same column represent statistically significant difference ($p < 0.05$)

verified that human and bovine teeth are similar to evaluate pH.[26] The results of the present study, by means of ionic dissociation through bovine dentin, demonstrated that medications with nanoparticle substances promoted a higher level of alkalization in the shortest periods of evaluation (1 and 7 days).

Hydroxyl ions must promote the dentinal alkalinization, despite the buffer effect of dentin.[28,29] The nanoparticle substances favored the faster hydroxyl ions diffusion, probably due its higher concentration of particles per volume, determining an elevated availability of hydroxyl ions and favoring penetration into the dentinal tubules, thereby increasing the pH of the external medium of the root surface in a shorter time.[30] Komabayashi et al described the particle size of the calcium hydroxide microparticles as making it possible to penetrate directly into the dentinal tubules.[31] The nanoparticles used in this study had a particle size range between 100 and 200 nm, around 1000 times smaller than the calcium hydroxide microparticle. Therefore, nanoparticles favored the faster alkalinization of dentin.

At 14-day and longer time intervals (up to 60 days), all the calcium hydroxide pastes were similar, with pH increase, irrespective of the particle size and association with chlorhexidine. These results are in agreement with those of Duarte et al, who verified in an *in vitro* study that pastes based on calcium hydroxide and chlorhexidine promoted calcium and hydroxyl ion release in the studied periods.[32]

The vehicle used in the composition of the paste has an influence on its dissociation into hydroxyl and calcium ions. The polyethylen glycol 400 used in the present study allows the progressive dissociation of calcium hydroxide, even in longer periods.[33,34] Freire et al demonstrated a high pH for calcium hydroxide pastes associated with polyethylene glycol and/or chlorhexidine, even in the presence of dentin for the period of 21 days, in agreement with the results of the present study.[3]

Calcium hydroxide can be placed by different techniques: syringe-lentulo spiral and the syringe-spreader.[35] Calcium hydroxide has low activity against *E. faecalis*. This microorganism is resistant and has the capacity to survive the adversities, such as nutrient deficiency.[36-39] *E. faecalis* isolates were recovered from patients with dental diseases especially necrotic pulps.[40] The association of calcium hydroxide with chlorhexidine increases its antibacterial property against *E. faecalis* without compromising the alkalinity of the medium.[41-43] This fact was also observed in the present study.

There are some factors that may interfere in the results of the agar diffusion test, such as the type of culture medium used, size, diffusivity and solubility of the particle tested. In the present study, the groups that contained only calcium hydroxide and zinc oxide were

Table 3: pH values of medications in different time intervals (means and standard deviation)

	Intracanal medication				
Period	*$Ca(OH)_2$/ZnO micro*	*$Ca(OH)_2$/ZnO nano*	*$Ca(OH)_2$/ZnO micro + chlorhexidine*	*$Ca(OH)_2$/ZnO nano + chlorhexidine*	*Control*
1 day	7.11 (0.09)[b]	7.38 (0.14)[a]	7.09 (0.07)[b]	7.49 (0.08)[a]	6.41 (± 0.13)[c]
7 days	7.16 (0.13)[b]	7.39 (0.14)[a]	7.01 (0.31)[b]	7.42 (0.07)[a]	6.38 (± 0.10)[c]
14 days	7.56 (0.35)[a]	7.82 (0.40)[a]	7.57 (0.28)[a]	7.82 (0.34)[a]	6.42 (0.12)[b]
21 days	7.76 (0.39)[a]	7.64 (0.50)[a]	7.85 (0.60)[a]	7.61 (0.27)[a]	6.43 (0.15)[b]
30 days	7.84 (0.27)[a]	7.73 (0.22)[a]	7.89 (0.22)[a]	7.74 (0.33)[a]	6.48 (0.24)[b]
60 days	8.31 (0.36)[a]	8.13 (0.35)[a]	8.18 (0.27)[a]	8.04 (0.25)[a]	6.76 (0.24)[b]

Different letters in the same line represent statistically significant difference ($p < 0.05$)

those that showed the lowest inhibition halo values. However, the association of calcium hydroxide/zinc oxide (both microparticles and nanoparticles) with 0.4% chlorhexidine presented the largest halo of growth inhibition of *E. faecalis*. These results may be related to the antibacterial activity and greater diffusion capacity of chlorhexidine in agar medium. Evans et al, studying the mechanism of resistance of *E. faecalis* to calcium hydroxide in bovine dentin, verified that the microorganism becomes sensitive to the association of calcium hydroxide with chlorhexidine.[44] Chlorhexidine requires a short time to perform its antibacterial action against *E. faecalis* due to its mechanism of action.[45,46] Its cationic molecule interacts with the negative charge of the phosphate groups present in the bacterial cell wall, causing damage to metabolism and consequently bacterial death.[47]

In general, the nanoparticles of calcium hydroxide and zinc oxide provide with greater alkalinization potential in shorter periods (up to 1 week). However, the action of these pastes against *E. faecalis* was shown to be favored by the association with chlorhexidine.

CONCLUSION

Within the limits of the present study, it can be concluded that the nanoparticles of calcium hydroxide and zinc oxide promoted greater initial alkalinization. The antimicrobial activity of the pastes against *E. Faecalis* is favored by the association with chlorhexidine.

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