USE OF NANOPARTICULATE ZINC OXIDE AS INTRACANAL MEDICATION IN ENDODONTICS: PH AND ANTIMICROBIAL ACTIVITY

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ABSTRACT

The aim of this study was to evaluate the pH and antimicrobial activity of micro or nanoparticulate zinc oxide (ZnO) pastes with or without calcium hydroxide (CH). The following medications were evaluated: microparticulate ZnO + polyethylene glycol (PEG) 400; nanoparticulate ZnO + PEG 400; PEG 400; CH + microparticulate ZnO + PEG 400 and CH + nanoparticulate ZnO + PEG 400. The pH was assessed between 12 hours and 28 days, using a digital pH meter. The antimicrobial activity against Enterococcus faecalis (ATCC-9212), Candida albicans (ATCC-10231), Pseudomonas aeruginosa (ATCC-27853), Staphylococcus aureus (ATCC-6538) and Kocuria rhizophila (ATCC-9341) was determined in triplicate using agar diffusion test. The results were submitted to Kruskal-Wallis/Dunn and ANOVA/Tukey tests with 5% significance. The highest pH values were found for CH+ZnO, with higher values for nanoparticulate ZnO after 12 hours and 21 days ($p<0.05$). CH+ZnO medication promoted higher growth inhibition against P. aeruginosa and lower against E. faecalis. Calcium hydroxide pastes have higher pH and antimicrobial activity when associated with either micro- or nanoparticulate zinc oxide.

Keywords: *Nanoparticles;Calcium Hydroxide;Zinc Oxide; Disinfection*

USO DE ÓXIDO DE ZINCO NANOPARTICULADO COMO MEDICAÇÃO EM ENDODONTIA: PH E ATIVIDADE ANTIMICROBIANA

RESUMO

O objetivo do estudo foi avaliar o pH e atividade antimicrobiana de pastas de óxido de zinco micro ou nanoparticulado (OZn) com ou sem hidróxido de cálcio (HC). As medicações avaliadas foram OZn microparticulado + polietilenoglicol (PLG) 400; OZn nanoparticulado + PLG 400; PLG 400; HC + OZn microparticulado + PLG 400 e HC + OZn nanoparticulado + PLG 400. A análise do pH foi realizada entre 12 horas e 28 dias, usando um pHmetro digital. A atividade antimicrobiana frente Enterococcus faecalis (ATCC-9212), Candida albicans (ATCC-10231), Pseudomonas aeruginosa (ATCC-27853), Staphylococcus aureus (ATCC-6538) e Kocuria rhizophila (ATCC-9341) foi determinada, em triplicata, pelo teste de difusão em ágar. Os resultados submetidos ao teste Kruskal-Wallis e Dunn e ANOVA e Tukey com 5% de significância. Os maiores valores de pH ocorreram para HC+ZnO, sendo maior para ZnO nanoparticulado após 12 horas e 21 dias ($p<0,05$). A medicação de HC+ZnO promoveu maior inibição de crescimento sobre P. aeruginosa e menor sobre E. faecalis. As pastas de hidróxido de calico apresentam os maiores valores de pH atividade antimicrobiana com a associação de óxido de zinco micro ou nanoparticulado.

Palavras-Chave: *Nanoparticles, Calcium Hydroxide, Zinc Oxide, Desinfecção*

INTRODUCTION

Enterococccus faecalis is the most frequent microorganism in root canals with persistent infections after endodontic treatment[1-4]. It is capable of surviving with restriction on nutrients[3], in extremely alkaline pH [2, 4-6] and can adhere to root canal walls, forming biofilm and becoming more resistant to antimicrobials[2]. The microorganism penetration within deep dentinal walls[1] makes its elimination difficult. Thus, the use of intracanal medication has been indicated to complete disinfection of the root canal system[7-9].

Calcium hydroxide has been largely employed as intracanal medication because it has antimicrobial properties and is able to induce mineralization, as well as inactivation of endotoxins[7, 8,10]. In its pure form, its pH is 12.5 to 12.8[11]. Notwithstanding, *Enterococcus faecalis* may show resistance against it because of its pH homeostasis capacity[5].

With the aim of increasing the action of calcium hydroxide on resistant microorganisms, its association with other substances has been indicated. The antimicrobial activity of zinc oxide against the strains *K. rhizofila, S. aureus, S. epidermidis, E.coli* has already been verified[12].

Nanotechnology applied to endodontics may contribute to controlling infection because of its biocidal action[13]. Zinc oxide nanoparticles may act as antimicrobial agents against a broad spectrum of microorganisms[14-22]. The production of reactive oxygen species (ROS) and the penetration of the nanoparticles in either the cytoplasm or the outer membranes may explain such capacity so that the antimicrobial activity of zinc oxide is inversely proportional to the size of its particles[17, 23]. Nanoparticulate zinc oxide shows ability to inhibit *C. albicans*[15] and pathogens such as *P. gingivalis, P. intermedia , F. nucleatun* and *A. actinomycetemcomitans*[19]. It also has an effect on reducing the biofilm of *Enterococcus faecalis*[18]. Additionally, the antimicrobial activity of an endodontic cement was increased by adding nanoparticulate zinc oxide, and the reduction of *Enterococcus faecalis* adhesion to root dentin was also verified[14].

The aim of this study was to evaluate calcium hydroxide pastes associated with either micro or nanoparticulate zinc oxide regarding pH and antimicrobial activity.

MATERIAL AND METHODS

Intracanal medications were divided into 5 groups according to their composition (Table 1). For groups 1 and 2, polyethylene glycol was added until a creamy consistency was obtained. For groups 4 and 5, 2.5 g of calcium hydroxide and 0.5 g of zinc oxide were used. They were manipulated using polyethylene glycol to obtain a creamy consistency.

pH assessment

Intracanal medication (n=10) was inserted in polyvinylchloride tubes (1cm long x 1mm inner diameter). The tubes were immersed in plastic flasks containing 10 ml deionized water, whose pH had previously been assessed. The flasks were closed and kept at 37ºC. The pH of the solution was determined with the aid of a pH meter (Digimed DM-21, Digicrom Analítica Ltd., São Paulo, Brazil), previously calibrated at a controlled temperature and kept at about 25ºC (Guerreiro-Tanomaru et al. 2012). The pH was analyzed after 12 hours, 24 hours; 3, 7, 14, 21 and 28 days. The data obtained were submitted to ANOVA and Tukey tests, with 5% level of significance.

Agar diffusion test

Standardized suspensions (1 x 10^6 CFU mL^{-1}) of *Enterococcus faecalis* (ATCC-9212), *Candida albicans* (ATCC-10231), *Pseudomonas aeruginosa* (ATCC-27853), *Staphylococcus aureus* (ATCC-6538) and *Kocuria rhizophila* (ATCC-9341) were obtained using a spectrophotometer. Triptic Soy Agar (TSa; DIFCO) culture medium and the microorganism inoculums were plated onto Petri plates in double layer. After solidification, five wells (4 mm diameter) were made and filled with the intracanal medications. For each medication and microorganism, 3 replicates were performed (n = 3).The plates were kept at room temperature for 2 hours to obtain the pre-diffusion of the substances, and then incubated at 37°C for 24 hours in microaerophily. After the incubation period, TTC gel was prepared with 1% agar (Difco) and 0.05% of triphenyltetrazolium chloride (Merck KgaA, Darmstadt, Germany. Aliquots of 5 ml were added to plates to colour the viable cells and facilitate the reading of the zones of inhibition[,24,26]. After the solidification, the samples were incubated at 37°C for 30 minutes. Images of the well-illuminated Petri dishes against a blue background, to contrast with the red color of the viable colonies, were digitized and the diameters of the zones of inhibition around each well were measured using the Image Tool software (UTHSCSA Image Tool for

Table 1: Division of the experimental groups.

Groups	Intracanal Medication
Group 1	Microparticulate Zinc Oxide* + Polyethylene glycol 400
Group 2	Nanoparticulate Zinc Oxide ** + Polyethylene glycol 400
Group 3	Polyethylene glycol
Group 4	Calcium Hydroxide* + Microparticulate Zinc Oxide + Polyethylene glycol 400
Group 5	Calcium Hydroxide + Nanoparticulate Zinc Oxide + Polyethylene glycol 400

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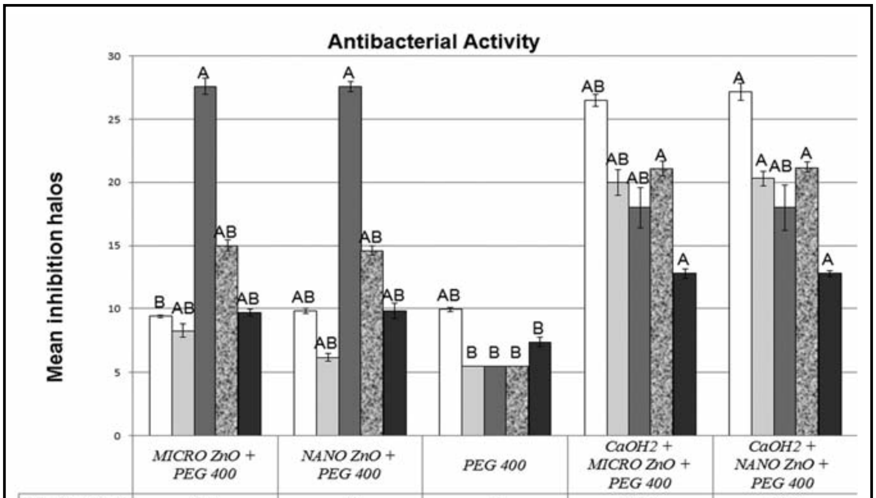
Windows, version 3.0).The results were submitted to Kruskal-Wallis and Dunn tests, with a 5% level of significance.

RESULTS

Table 2 shows the values obtained during the pH assessments at 12h, 24h, 3, 7, 14, 21 and 28 days. The highest values were verified in the calcium hydroxide pastes with added zinc oxide; the paste containing nanoparticulate zinc oxide was higher than that with microparticulate ZnO only after 12 hours and 21 days ($p<0.05$). Pure polyethylene glycol 400 exhibited intermediate values and differed significantly from the zinc oxide pastes ($p<0.05$).

In the evaluation of the antimicrobial activity through the agar diffusion test, the largest zones of inhibition were observed in the groups with calcium hydroxide with addition of either micro- or nanoparticulate zinc oxide. *C. albicans, E. faecalis* and *S. aureus* had the largest zones of inhibition in the associations of calcium hydroxide with zinc oxide, although without statistical difference when compared to zinc oxide pastes ($p>0.05$). Fig. 1 illustrates the means and standard deviation of the zones of growth inhibition of the microorganisms for each intracanal medication evaluated.



	MICRO ZnO + PEG 400	NANO ZnO + PEG 400	PEG 400	CaOH2 + MICRO ZnO + PEG 400	CaOH2 + NANO ZnO + PEG 400
P. aeruginosa	9,4	10	10	26,5	27,2
C. albicans	8,3	6,2	5,5	20	20,3
K. rhizophila	27,6	27,6	5,5	18	18
S. aureus	15	14,6	5,5	21,1	21,2
E. faecalis	9,7	9,8	7,4	12,8	12,8

Fig. 1: Means and standard deviations of microorganism growth inhibition zones evaluated through agar diffusion test. Different letters indicate statistically significant differences among the materials for each microorganism (p< 0.05).

Table 2: pH values verified in each group* at 12 and 24 hours, 3, 7, 14, 21 and 28 days.

	MICRO ZnO + PEG 400	NANO ZnO + PEG 400	PEG 400	$CaOH_2$ + MICRO ZnO + PEG 400	$CaOH_2$ + NANO ZnO + PEG 400
12 h	7.548 (0.228) D	7.995 (0.089) C	8.167 (0.166) C	10.66 (0.156) B	11.05 (0.136) A
24 h	7.796 (0.079) B	7.881 (0.145) B	8.013 (0.187) A	10.46 (0.086) A	10.53 (0.082) A
3 days	7.608 (0.162) B	7.654 (0.251) B	7.621 (0.212) B	10.94 (0.045) A	10.99 (0.052) A
7 days	7.212 (0.135) D	7.752 (0.160) C	8.235 (0.184) B	10.79 (0.158) A	10.96 (0.086) A
14 days	7.783 (0.1026) C	7.834 (0.061) C	8.252 (0.071) B	10.98 (0.065) A	11.06 (0.070) A
21 days	7.887 (0.149) D	7.851 (0.084) D	8.267 (0.107) C	10.56 (0.39) B	10.97 (0.053) A
28 days	8.074 (0.28) B	7.946 (0.207) B	8.098 (0.167) B	10.61 (0.39) B	10.72 (0.053) A

•Mean (standard deviation). Different letters indicate statistically significant differences among groups at each experimental time ($p<0.05$).

DISCUSSION

The agar diffusion method is used to evaluate the antimicrobial activity of dental materials because it is a simple method enabling the evaluation of endodontic cements, intracanal medications and irrigant solutions against several microorganisms[25, 26]. However, the results may be influenced by the physical-chemical properties of the material evaluated, nature of the culture medium, composition, pH and thickness of the medium[27].

The use of calcium hydroxide as intracanal medication contributes to the reduction of the endodontic infection[28, 29] and periapical repair[30]. The antimicrobial activity of nanoparticulate zinc oxide on several microorganism strains has been demonstrated by several studies[16-18, 20-22]. Yamamoto verified that the small size of the particles and the powder concentration increases the antimicrobial activity of the zinc oxide on *S. aureus* and *E. coli*[22]. In our study, either micro- or nanoparticulate zinc oxide when only added to polyethylene glycol 400 showed similar mean values for zones of inhibition, regardless of the size of the particles, but they were not higher than calcium hydroxide.

Notwithstanding, calcium hydroxide produced larger growth inhibition zones when associated with nanoparticulate zinc oxide than with microparticulate zinc oxide. Nanoparticle size may favor the capacity to diffuse through agar; however, no significant difference was found regarding antimicrobial activity. These results suggest that further studies are needed on the antimicrobial activity of the pastes.

Nanoparticulate zinc oxide promotes the growth inhibition of six species of microorganisms, including *E. faecalis*, with the highest antimicrobial activity against *S. Aureus*[31], in accordance with Raghupathi et al., 2011[17]. In the presence of planktonic *S. aureus* cell suspension, antibacterial effect increased as zinc oxide particle diameter decreased[23].

The nanoparticles of zinc oxide showed good performance on the reduction of *E. faecalis* biofilm, maintaining antibacterial activity for up to 90 days[18]. Bacterial death in contact with zinc oxide nanoparticles can be explained by damage to the cell membrane followed by depression of the activity of some enzymes of the membrane[32].

Enterococcus faecalis was the microorganism with the highest resistance to the action of calcium hydroxide-based medication. This result may be related to its survival capacity in environments with high pH levels[5, 6]. Calcium hydroxide in aqueous medium can increase pH up to 12.5[33]. pH values between 10.5 and 11 were verified in the calcium hydroxide-based intracanal medications in this study. The presence of either micro- or nanoparticulate zinc oxide with added calcium hydroxide did not influence the pH values, except at 21 days, when the microparticulate composition was less alkaline.

The different vehicles for calcium hydroxide in intracanal medications such as polyethylene glycol 400, camphorated paramonochlorophenol and distilled water did not influence the pH values of the pastes[34]. However, the use of a viscous vehicle in the composition of calcium hydroxide-based paste can continue releasing of hydroxyl ions[35], maintaining higher pH levels for longer periods of time. The results of the study suggested that calcium hydroxide pastes with micro or nanoparticulate ZnO can favor the antimicrobial activity of calcium hydroxide-based intracanal medication. However, calcium hydroxide medication without zinc oxide has not been studied, warranting further studies.

It is concluded that high values of pH and antimicrobial activity are verified in $Ca(OH)_2$ medications with either micro- or nanoparticulate ZnO, suggesting that these associations can be used as intracanal medications.

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