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Antimicrobial Effect and pH of Chlorhexidine Gel and Calcium Hydroxide Alone and Associated with other Materials

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The purposes of this study were to evaluate the effectiveness of 2% chlorhexidine (CHX) gluconate gel, calcium hydroxide [$Ca(OH)_2$] and their combination with iodoform and zinc oxide powder as intracanal medications against select microorganisms, and to measure the pH changes caused by these medications. Antimicrobial activity was determined by the agar diffusion method. The zones of growth inhibition were measured and the results were analyzed statistically by Kruskal-Wallis test ($p<0.05$). The pH of the pastes was measured right after preparation, after 24 h and 1 week later. The largest mean zones of microbial inhibition were produced by 2% CHX gel, followed by $Ca(OH)_2$ + 2% CHX gel + iodoform, $Ca(OH)_2$ + 2% CHX gel, $Ca(OH)_2$ + 2% CHX gel + zinc oxide, and $Ca(OH)_2$ + water. The mean pH of all medications stayed above 12.0 during the whole experiment, except for CHX gel (pH=7.0). The results of this study showed that all medications had antimicrobial activity, but the most effective against the tested microorganisms were 2% CHX gel, followed by its combination with $Ca(OH)_2$ and iodoform.

Key Words: intracanal medicament, calcium hydroxide, chlorhexidine gel, microorganisms.

INTRODUCTION

Calcium hydroxide [$Ca(OH)_2$] is one of the most versatile medications in dentistry, especially for its use as an intracanal dressing in vital and non-vital teeth. $Ca(OH)_2$ therapy for nonvital teeth includes apexification procedures for teeth with incomplete root formation and repair of perforations from internal root resorption (1). In addition, $Ca(OH)_2$-based intracanal medications maintain a high pH, which is an important factor to prevent or inhibit inflammatory root resorption (2).

Several researchers and clinical investigators have studied the associations of $Ca(OH)_2$ with other antimicrobial substances, such as camphorated paramonochorophenol (3), chlorhexidine (4), iodine potassium iodine (5,6), erythromycin (7) and clindamycin or tetracycline (7). Athanassiadis et al. (8) reviewed several $Ca(OH)_2$ combinations and concluded that the choice for which intracanal medication to use during endodontic treatment is dependent on having an accurate diagnosis of the condition being treated.

The high pH of $Ca(OH)_2$ has a destructive effect on bacterial cell membranes and protein structure (9). It is well tolerated and is subsequently resorbed in the periapical region (10). It should ideally occupy the whole pulp space in order to act effectively as an intracanal dressing (11). However, $Ca(OH)_2$ cannot be considered as a universal intracanal medication, as it is not equally effective against all bacterial species found in the root canals (12).

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Chlorhexidine gluconate (CHX) has been used in endodontics and proposed as both an irrigant (13) and an intracanal medication (14). It is active against a wide range of microorganisms, such as Gram-positive and Gram-negative bacteria (14). One of the mechanisms that can explain its efficacy is based on the interaction between the positive charge of the molecule and the negatively charged phosphate groups on the bacterial cell wall, which allows the CHX molecule to penetrate into the bacteria with toxic effects (2).

The goal of associating $Ca(OH)_2$ and 2% CHX gel is to enhance the antimicrobial effectiveness, particularly against resistant microorganisms, such as *Enterococcus faecalis*, which are frequently found in infected root-filled teeth (6,15). Therefore, the purpose of this study was to evaluate the effectiveness of 2% CHX gel, $Ca(OH)_2$ and their association with iodoform (CHI_3) and zinc oxide powder as intracanal medications against select microorganisms. The pH changes caused by these medications were also assessed.

MATERIAL AND METHODS

Five medications/association of medications were evaluated: 2% CHX gel; $Ca(OH)_2$ + 2% CHX (1: 1; v:v); $Ca(OH)_2$+ 2% CHX + iodoform (1: 1: 0.01; v:v:v); $Ca(OH)_2$+ 2% CHX + zinc oxide (1: 1: 0.01 v:v:v); and $Ca(OH)_2$ + sterile distilled water (9:7; v:v). The tested substances were prepared based on the methodology proposed by Gomes et al. (14).

$Ca(OH)_2$ pastes were prepared using $Ca(OH)_2$ p.a. (Labsynth Produtos para Laboratório LTDA, Diadema, SP, Brazil). 2.0% CHX gel (Endogel, Itapetininga, SP, Brazil) is a gel base (1% natrosol) and CHX gel at pH 7.0. Natrosol gel (hydroxyethyl cellulose) is a nonionic, highly inert and water-soluble agent. Sterile saline and 1.0% natrosol were used as controls.

Agar Diffusion Method

The following microbial species were used: *Candida albicans* (NTCC 3736), *Enterococcus faecalis* (ATCC 29212), *Streptococcus sanguis* (ATCC 10556), *Streptococcus sobrinus* (ATCC 6715) and *Streptococcus mutans* (OMZ 175) grown on Brain Heart Infusion Agar (BHI, Lab M, Bury, UK). The clinical isolates of anaerobes, *Porphyromonas gingivalis* and *Prevotella intermedia*, were grown on Fastidious Anaerobe Agar (FAA, Lab M, Bury, UK). Five percent defibrinated sheep's blood was added to both culture media.

The same methodology proposed by Gomes et al. (12) was used in this study. All microorganisms were previously subcultured in appropriate culture media and under gaseous conditions to confirm their purity.

Tubes containing 5 mL of sterile saline were individually inoculated with the aerobes and facultative anaerobic strains. The suspension was adjusted spectrophotometrically at 800 nm ($O.D._{800}$) to match the turbidity of $1.5x10^8$ cfu mL^{-1} (equivalent to 0.5 McFarland standard). Glass flasks containing 50 mL of BHI agar (Lab M) at 46°C were inoculated with 500 µL of each microbial suspension, mixed and poured onto 130 mm-plates containing a previously set layer of Mueller Hinton agar (Oxoid, Unipath Ltd, Basingstoke, UK).

The clinically isolated anaerobes were suspended spectrophotometrically at 800 nm ($O.D._{800}$) to match the turbidity of $3.0x10^8$cfu mL^{-1} (equivalent to 1 McFarland standard). Sterile swabs were dipped into the bacterial suspension to inoculate pre-reduced 70 mm-plates containing 5% sheep-blood-FAA (Lab-M). Inoculum procedures were appropriate to provide a semi-confluent growth of the test microorganisms.

Sterile stainless-steel tubes, open at both ends, measuring 8.0 x 1.0 x 10 mm (inner diameter = 6 mm) were added to the surfaces of the media and filled with 40 µL of each test substance and controls (Fig. 1A). The plates were kept for 2 h at room temperature in appropriate gaseous conditions to allow agent diffusion through the agar, and then incubated at 37°C under proper gaseous conditions for an appropriate incubation time: aerobes, 24 h; facultative, 24-48 h in a CO_2 incubator (Jouan, Saint Herblain, France) in 10% CO_2 atmosphere; and anaerobes, 7 days in an anaerobic workstation (Don Whitley Scientific, Bradford, UK) in an atmosphere of 10% H_2, 10% CO_2 and 80% N_2. Pour- plate technique was performed according to Gomes et al. (14).

Zones of microbial growth inhibition around the cylinder containing the tested substances and the negative controls were measured and recorded after the incubation period (Fig. 1B). The inhibitory zone was considered to be the shortest distance (mm) between the outer margin of the cylinder and the initial point of microbial growth. The measurements were made with calipers accurate to 0.01 mm. Six replicates were made for each microorganism. In cases where no inhibition zone was formed, the cylinder was removed and the

presence or not of microbial growth on the surface of the medication was verified, as well as on the surface of the agar in contact with the medication. In addition, microbial samples were taken with sterile paper points moistened in sterile saline in these two sites: surface of the medication and agar surface. The paper point samples were plated out onto 5% blood agar plates, which were incubated at 37ºC for 7 days. If there was no microbial growth, it would mean that the microorganism was killed only by direct contact with the medication.

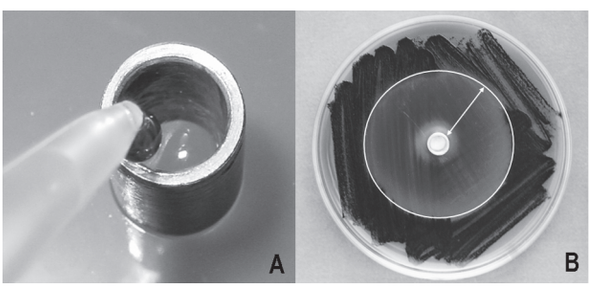

Figure 1. A: Stainless-steel cylinder containing a negative control (sterile water); B: Microbial growth (*P. intermedia*) and inhibition zone surrounded the cylinder containing $Ca(OH)_2$ + 2.0% CHX gel + IF after 48 h of anaerobic incubation.

pH Measurement

The pHs of the pastes were measured 5 min, 24 h and 7 days after preparation using a pH meter (Procyon, digital pH meter model AS 720, electrode A 11489, Procy Instrumental Científica, São Paulo, SP, Brazil). The pHs of all medications were measured in triplicate. pH was calibrated with solutions of known pH before and after measurements at each period.

Statistical Analysis

Data were analyzed statistically by Kruskall-Wallis non-parametric test using BioEstat software (CNpQ, 2000; Brasília, DF, Brazil). Mann-Whitney test were used to determine significant differences in susceptibility to intracanal medication among microbial species. Significance level was set at $p<0.05$.

RESULTS

Table 1 shows the mean zones of microbial growth inhibition (in mm) for each medication. All

Table 1. Mean zones of microbial growth inhibition (in mm) formed by the intracanal medications against the tested microorganisms.

	Candida albicans	*Streptococcus mutans*	*Enterococcus faecalis*	*Streptococcus sobrinus*	*Streptococcus sanguis*	*Prevotella intermedia*	*Porphyromonas gingivalis*	Mean values *per* medication
2% CHXG	0.25ab (±0.04)	15.50ab (±2.50)	0.60a (±0.08)	13.66a (±1.69)	0.80a (±0.08)	10.00a (±0.00)	11.83a (±0.84)	7.52a
$Ca(OH)_2$ + 2% CHXG	0.15b (±0.04)	11.00b (±1.00)	0.47ab (±0.09)	11.00ab (±0.81)	0.60ab (±0.08)	4.20b (±5.49)	0.41b (±0.14)	3.98a
$Ca(OH)_2$ + 2% CHXG + IF	0.36a (±0.04)	18.66a (±0.94)	0.03b (±0.04)	3.90bc (±4.31)	0.36b (±0.04)	10.00a (±0.00)	0.25b (±0.10)	4.79a
$Ca(OH)_2$ + 2% CHXG + ZO	0.26ab (±0.04)	18.00a (±0.00)	0.40ab (±0.08)	7.63b (±4.83)	0.56ab (±0.09)	0.55ab (±0.07)	0.38b (±0.10)	3.97a
$Ca(OH)_2$ + SW	0.00b (±0.00)	0.00c (±0.00)	0.00c (±0.00)	0.00c (±0.00)	0.00b (±0.00)	0.42b (±0.20)	0.42b (±0.02)	0.12b
Mean values *per* MO	0.20	12.63	0.30	7.24	0.46	5.03	2.66	

2%CHX = 2% chlorhexidine gluconate gel; $Ca(OH)_2$= calcium hydroxide; IF= iodoform; ZOE = zinc oxide; SW = sterile water; MO=Microorganism. Different lowercase letters indicate statistically significant differences ($p<0.05$) among the tested intracanal medications against the same microorganism (vertical direction). Standard deviation is shown in brackets.

medications were inhibitory at least by direct contact against all microbial strains. Microbial inhibition zones equal to zero indicated that the medication only had direct contact action. Negative controls (natrosol and distilled water) did not inhibit microbial growth.

The diameter of the microbial growth inhibition zones of 2% CHX gel against the tested microbial strains ranged from 0.25 to 15.5 mm, being ≥10 mm against *S. mutans*, *S. sobrinus*, *P. intermedia* and *P. gingivalis*. $Ca(OH)_2$ + sterile water was the least effective medication, showing mean microbial growth inhibition zones <1 mm or inhibiting microbial growth only by direct contact (Table 1). The tested microorganisms presented different susceptibilities to the intracanal medications. While *C. albicans*, *E. faecalis* and *S. sanguis* were the most resistant, *S. mutans* showed significantly larger inhibition zones (Table 1).

Table 2 shows the mean pHs of the substances evaluated in this study. The mean pH of all medications was >12.0 throughout the experiment, except for CHX gel (pH=7.0).

DISCUSSION

Common clinical signs, such as exudation, hemorrhage, perforation, root resorption, trauma or incomplete root formation, may indicate the need for intracanal dressing (11). In these situations, it is beneficial to clean and shape the canals and fill them with a medication, such as a $Ca(OH)_2$ paste, densely into their full length, in order to increase the antimicrobial efficacy (9).

$Ca(OH)_2$ is widely used in endodontics. It acts as a physical barrier in the canal preventing root canal reinfection, interrupting the nutrient supply to the remaining bacteria, and thus delaying recontamination (3).

However, the mechanism that makes $Ca(OH)_2$ effective is unclear and its inefficacy against certain microorganisms, such as *E. faecalis*, has been reported (12). The findings of this study show that $Ca(OH)_2$ had antimicrobial activity only by direct contact against most tested microorganisms.

Pastes prepared with $Ca(OH)_2$ and sterile water has an alkaline pH (>12.0), as confirmed in the present study. However, the low solubility and diffusibility of $Ca(OH)_2$, as well as its dentin buffering ability, may make it difficult to reach a pH capable of eliminating bacteria located within dentinal tubules or enclosed in anatomical variations (15). It has also been reported in experimental studies that when $Ca(OH)_2$ reaches the peripheral dentin, its pH drops to around 6.0-7.4, which may have a harmful effect on marginal periodontal healing cells (16,17).

Studies have suggested that CHX gel is an effective intracanal medication due to its broad antimicrobial spectrum (13-15), which is in agreement of the findings of the present study. The present results showed that the 2% CHX gel produced the largest mean inhibition zones and was effective against all microorganisms (Table 2). Even at the highest concentrations, it has very low toxicity. Also, it adsorbs onto dental tissues and mucous membranes resulting in its prolonged gradual release at therapeutic levels (i.e. substantivity) (18).

Furthermore, CHX may be particularly beneficial in the treatment of inflammatory root resorption (2). However, it is not an effective intracanal barrier, needing to be changed frequently as it diffuses through the dentinal tubules, leaving an empty canal (4). It is also radiolucent, making its visualization difficult inside the canal.

In vitro studies have demonstrated that the associations with some substances, such as $Ca(OH)_2$, urea and sulphate lauryl sodium, reduce the antimicrobial activity of CHX (19). Accordingly, in the present study, it was observed that the antimicrobial action of CHX was reduced when it was combined with $Ca(OH)_2$. On the other hand, the antimicrobial activity of $Ca(OH)_2$ increased with this association. The goal of this association is to add antimicrobial properties to $Ca(OH)_2$. Furthermore, the presence of $Ca(OH)_2$ in a paste formulation allows formation of a more stable physical barrier, which will remain in the root canal for a longer period and thus delay recontamination. Additionally, the pres-

Table 2. Mean pH values obtained with the tested the intracanal medications.

Medications	Initial	24 h	7 days
2%CHXG	7.0	7.0	7.0
$Ca(OH)_2$ + 2%CHXG	12.76	12.41	12.51
$Ca(OH)_2$ + 2%CHXG + IF	12.26	12.00	12.57
$Ca(OH)_2$ + 2%CHXG + ZO	12.49	12.45	12.44
$Ca(OH)_2$ + SW	12.0	12.45	12.44

2%CHXG = 2% chlorhexidine gluconate gel; $Ca(OH)_2$= calcium hydroxide; IF=iodoform; ZOE = zinc oxide; SW = sterile water.

ence of CHX adds substantivity to the formulation maintaining the canal free of microorganisms, even after having been removed from the canal (18)

The pH of this combination was higher than those obtained with CHX or $Ca(OH)_2$ alone. It is interesting that most bacteria present in the root canal system grow best at a pH around 6.5 to 7.5, and most microorganisms are destroyed at pH 9.5, though a few can survive at pH 11 or higher (20). The high pH of the medications may also neutralize the acids secreted by osteoclasts and this may help preventing further destruction of mineralized tissue.

$Ca(OH)_2$ and 2% CHX gel were mixed with iodoform and zinc oxide powder in order to enhance radiopacity and consequently improve their ability to be observed after placement inside the canal. Their antimicrobial activity was less than the combination alone, but still greater than $Ca(OH)_2$ plus sterile water. Pastes with iodoform showed more antimicrobial activity than the ones with zinc oxide.

Most studies on $Ca(OH)_2$ antimicrobial activity uses the agar diffusion method, which only indicates the potential of a medication to eliminate the microorganism and is directly related to its dissociation and diffusion of the medium. Therefore, the inhibition zones may be more related to material solubility and diffusibility in agar than to its actual efficacy against the microorganisms. Regarding $Ca(OH)_2$, its antimicrobial activity is related to its high pH, which in turn precipitates this medicament on agar, preventing its diffusion. These facts may explain the poor performance of $Ca(OH)_2$ using the agar diffusion method. However, studies using other methodologies (14) have also found that $Ca(OH)_2$ pastes mixed with sterile water are not as efficient as $Ca(OH)_2$ + 2% CHX gel or 2% CHX gel alone, as intracanal medications, the latter being the most effective to eliminate endodontic pathogens. These results are consistent with the findings of the present work.

The outcomes or this study indicate that $Ca(OH)_2$ associated with 2% CHX, with or without iodoform or zinc oxide, when used as an intracanal medication provides antimicrobial action and is able to maintain an ideal pH. Even though such medication enhanced by radiopacifiers can be used in vital or necrotic pulps, its main application is in traumatized teeth with necrotic pulp and incomplete apex formation, to induce apexification. The presence of a radiopacifier, such as iodoform or zinc oxide, allows better visualization of the medication after filling the root canal with the medication. When treating teeth with open apex, it should be considered that the barrier of calcified tissue will be formed across the apex, which, in turn, will facilitate the placement of a permanent root canal filling material (1).

Further clinical trials are necessary to confirm the results of *in vitro* studies, addressing the effectiveness of medications against microorganisms of the endodontic microbiota.

RESUMO

O objetivo do estudo foi avaliar, *in vitro*, a efetividade antimicrobiana da clorexidina gel 2% (CHX) e hidróxido de cálcio, isoladamente e associados com iodofórmio e pó de óxido de zinco como medicamentos intracanais frente a microrganismos e medidos pHs das diferentes medicações. A atividade antimicrobiana foi determinada pelo método de difusão em ágar. As áreas de inibição de crescimento foram medidas e os resultados estatisticamente analisados utilizando-se o teste de Kruskal-Wallis ($p<0,05$). O pH das pastas foi mensurado após a manipulação, após 24 h e após uma semana. Os resultados mostraram que a maior zona de inibição foi da CHX gel 2%, seguida pelo $Ca(OH)_2$ + 2% CHX gel, $Ca(OH)_2$ + 2% CHX gel + iodofórmio, $Ca(OH)_2$ + 2% CHX gel +óxido de zinco, $Ca(OH)_2$ + água. A média de pH de todos os medicamentos intracanais foi de 12 durante todo o experimento, exceto com CHX gel 2% (pH=7,0). Estes resultados permitiram concluir que todos os medicamentos tiveram atividade antimicrobiana, no entanto, a maior foi da CHX gel 2%, seguido da associação com o $Ca(OH)_2$ e iodofórmio.

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REFERENCES

1. Anthony DR, Gordon TM, Del Rio CE. The effect of three vehicles on pH of calcium hydroxide. Oral Surg 1982;54:560-565.
2. Lindskog S, Pierce AM, Blomlöf. Chlorhexidine as a root canal medicament for treating inflammatory lesions in the periodontal space. Endod Dental Traumatol 1998;14:186-190.
3. Siqueira Jr JF, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999;32:361-369.
4. Gomes BPFA, Sato E, Ferraz CCR, Teixeira FB, Zaia AA, Souza-Filho FJ. Evaluation of time required for recontamination of coronally sealed canals medicated with calcium hydroxide and chlorhexidine. Int Endod J 2003;36:604-609.
5. Cwikla SJ, Bélanger M, Giguère S, Progulske-Fox A, Vertucci

FJ. Dentinal tubule disinfection using three calcium hydroxide formulations. J Endod 2005;31:50-52.

6. Siren EK, Haapasalo MP, Waltimo TM, Ørstavisk D. *In vitro* antibacterial effect of calcium hydroxide combined with chlorhexidine or iodine potassium iodine on *Enterococcus faecalis*. Eur J Oral Sci 2004;112:326-331.
7. Molander A, Dahlén G. Evaluation of the antibacterial potential of tetracycline or erythromycin mixed with calcium hydroxide as intracanal dressing against *Enteroccus faecalis in vivo*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;96:744-750.
8. Athanassiadis B, Abbott PV, Walsh LJ. The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. Aust Dent J 2007;52:S64-S82.
9. Camões ICG, Salles MR, Cheviatarese O, Gomes GC. Influence on pH of vehicle containing glycerin used with calcium hydroxide. Dent Traumatol 2003;19:132-138.
10. Spangberg LSW. Intracanal medication. In: Ingle JI, Bakland LK (Editors). Endodontics, 4th ed. Baltimore: Williams & Wilkins, 1994. p. 627-640.
11. Martin DM, Crabb HS. Calcium hydroxide in root canal therapy. A review. Brit Dent J 1977;142:277-283.
12. Gomes BPFA, Ferraz CCR, Vianna ME, Rosalen PL, Zaia AA, Teixeira FB, Souza-Filho FJ. *In vitro* antimicrobial activity of calcium hydroxide pastes and their vehicles against selected microorganisms. Braz Dent J 2002;13:155-161.
13. Ferraz CCR, Gomes BPFA, Zaia AA, Teixeira FB, Souza-Filho FJ. *In vitro* assessment of the antimicrobial action and mechanical ability or chlorhexidine gel as an endodontic irrigant. J Endod 2001;27:452-455.
14. Gomes BPFA, Vianna ME, Senna NT, Zaia AA, Ferraz CCR, Souza-Filho FJ. *In vitro* evaluation of the antimicrobial activity of calcium hydroxide combined with chlorhexidine gel used as intracanal medicament. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:544-550.
15. Gomes BPFA, Souza SFC, Ferraz CCR, Teixeira FB, Zaia AA, Valdrighi L et al.. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against *Enterococcus faecalis* in bovine root dentine *in vitro*. Int Endod J 2003;36:267-275.
16. Tronstad L, Andreasen JO, Hasselgren G, Kristersson L, Riis I. pH changes in dental tissues after root canal filling with calcium hydroxide. J Endod 1981;7:17-21.
17. Blomlöf L, Lindskog S, Hammerström L. Endodontic infection and calcium hydroxide-treatment. Effects on periodontal healing in mature and immature replanted monkey teeth. J Clin Period 1992;19:652-658.
18. Komorowski R, Grad H, Wu XY, Friedman S. Antimicrobial substantivity of chlorhexidine treated bovine root dentin. J Endod 2000;6:315-317.
19. Hauman CHJ, Love RM. Biocompatibility of dental material used in contemporary endodontic therapy: a review. Part 1. Intracanal drugs substances. Int Endod J 2003;36:75-85.
20. Bonesvoll P. Influence of ionic strength calcium, sodium dodecyl sulphate and urea on the retention of chlorhexidine in the human mouth after mouth rinses. Arch Oral Biol 1977;22:273-279.

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