In vitro evaluation of the antimicrobial activity of calcium hydroxide combined with chlorhexidine gel used as intracanal medicament

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The aim of this study was to investigate the antimicrobial activity of calcium hydroxide (Ca(OH)₂) combined with 2% chlorhexidine gluconate (CHX) gel against endodontic pathogens and to compare the results with the ones achieved by Ca(OH)₂ mixed with sterile water and by CHX gel alone. Two methods were used: the agar diffusion test and the direct contact test. Ca(OH)₂ + 2% CHX gel produced inhibitory zones ranging from 2.84 to 6.5 mm, and required from 30 seconds to 6 hours to eliminate all tested microorganisms. However, 2% CHX gel showed the largest microbial growth zones from 4.33 to 21.67 mm, and required 1 minute or less to inhibit all tested microorganisms. A paste of Ca(OH)₂ plus sterile water inhibited only the microorganisms with which it was in direct contact and required from 30 seconds to 24 hours to kill all tested microorganisms. In conclusion, 2% CHX gel + Ca(OH)₂ showed better antimicrobial activity than Ca(OH)₂ manipulated with sterile water. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:544-50)

One of the major factors associated with endodontic failure is the persistence of microbial infection in the root canal system and periradicular area.1 The chances of a favorable outcome of root canal treatment are significantly higher when microorganisms have been effectively eradicated before sealing the root canal system. However, microorganisms may persist in the root canal after root filling, increasing the risk of treatment failure.2,3

The intracanal dressings tend to be effective against microorganisms that may have resisted the root canal preparation. Besides, medicaments control persistent exudation and destructive action of osteoclasts present in external dental resorption.4

Ca(OH)₂ is believed to to have many of the properties of an ideal root canal dressing, mainly due to its alkaline pH.5,6 It is bactericidal7 and neutralizes the remaining tissue debris in the root canal system.8 Ca(OH)₂ also promotes an alkalinizing osteogenic environment on the surrounding tissues through the continuous release of OH⁻ ions.9,5 Furthermore, Ca(OH)₂ mediates the neutralization of lipopolysaccharides10 and thus helps in cleansing the root canal.8

Estrela et al.11 claimed that Ca(OH)₂ inhibits bacterial enzymes by means of hydroxyl ions of the bacteria’s cytoplasmic membrane, generating the antibacterial effect. It activates tissue enzymes such as alkaline phosphatase, leading to the mineralizing effect. The chemical and biological dynamics that occur in the ionic dissociation of Ca(OH)₂ and its effect through tissue and bacterial cellular alterations deserve careful discussion and investigation.

For calcium hydroxide to act effectively as an intracanal dressing, it should ideally occupy all the pulp space thereby diffusing into areas inaccessible to in-
strums. Its effectiveness is linked to the diffusion of hydroxyl ions through the dentinal tubules and accessory canals into areas where bacteria and their byproducts may be harbored. In addition to acting as a physical barrier, the calcium hydroxide dressing may both prevent root canal re-infection and interrupt the nutrient supply to the remaining bacteria. Its alkalizing pH (around 12.5) promotes a destructive effect on cell membranes and protein structure. However, Ca(OH)₂ cannot be considered as a universal intracanal medicament, since it is not equally effective against all bacteria found in the root canal. Indeed, several studies have reported the failure of Ca(OH)₂ to eliminate enterococci effectively as they tolerate high pH values, varying from 9 to 11.

Chlorhexidine has been widely used in periodontics. It has antimicrobial activity against Gram-negative and Gram-positive microorganisms. The antimicrobial effect of chlorhexidine is related to the cationic molecule binding to negatively charged bacterial cell walls, thereby altering the cell’s osmotic equilibrium. Its use in endodontics has been proposed as an irrigant as well as an intracanal medicament, since it is not equally effective against resistant microorganisms such as Enterococcus faecalis, Staphylococcus aureus, Candida albicans, Porphyromonas endodontalis, Porphyromonas gingivalis and Prevotella intermedia. Chlorhexidine gluconate gel, and both intracanal medicaments are two aerobes, one facultative and three strict anaerobic microorganisms commonly isolated from infected root canals, as follows: Candida albicans (ATCC 10556), Staphylococcus aureus (ATCC 25923), Enterococcus faecalis (ATCC 29212) were grown on Brain Heart Infusion Agar plates (BHI, Lab M, Bury, UK); and Porphyromonas endodontalis, Porphyromonas gingivalis and Prevotella intermedia grown on Fastidious Anaerobe Agar plates (FAA, Lab M, Bury, UK). In both media, 5% defibrinated sheep blood was added.

The aim of the combined medicaments (Ca(OH)₂ plus 2.0% CHX gel) is to add antimicrobial properties to calcium hydroxide. Ca(OH)₂ has the ability to induce hard tissue formation and to cause intracanal occlusion, has tissue dissolving capability, and also mediates the neutralization of lipopolissacharides. However, it is not equally effective against all bacteria found in the root canal. Adding chlorhexidine could increase its antimicrobial activity and make the medicament more effective against resistant microorganisms such as Enterococcus faecalis.

The objective of this study was to investigate the antimicrobial activity of Ca(OH)₂ pastes, 2% chlorhexidine gluconate gel, and both intracanal medicaments combined against the following selected microorganisms: Enterococcus faecalis, Staphylococcus aureus, Candida albicans, Porphyromonas endodontalis, Porphyromonas gingivalis and Prevotella intermedia.

**MATERIALS AND METHODS**

- The substances tested were:
  - Ca(OH)₂ plus 2.0% CHX gel (1:1).
  - 2.0% CHX gel
  - Ca(OH)₂ plus sterile water (9:7)

The Ca(OH)₂ pastes were prepared using Ca(OH)₂ pro analysis (Labsynth Produtos para Laboratório LTDA, Diadema, SP, Brazil). The consistency of the pastes was similar to that of toothpaste, with a viscosity of 3501 cP at 0.1 rpm (Brookfield Digital Reometer, model DV-III-IV, São Paulo, SP, Brazil). The pH of each Ca(OH)₂ paste was determined with a pH meter (DIGIMED DM 21 V7c, São Paulo, SP, Brazil) and ranged from 12.5 to 13. The 2.0% CHX gel was manufactured (Proderma Farmácia de Manipulação Ltda., Piracicaba, SP, Brazil). Chlorhexidine gel consisted of a gel base (1% natrosol) and chlorhexidine gluconate at pH 7.0. Natrosol gel (hydroxyethyl cellulose) is a non-ionic, highly inert, and water-soluble agent. The 0.9% sterile saline and 1.0% natrosol were used as controls.

The species of microorganisms used in this experiment were two aerobes, one facultative and three strict anaerobic microorganisms commonly isolated from infected root canals, as follows: Candida albicans (ATCC 10556), Staphylococcus aureus (ATCC 25923), Enterococcus faecalis (ATCC 29212) were grown on Brain Heart Infusion Agar plates (BHI, Lab M, Bury, UK); and Porphyromonas endodontalis, Porphyromonas gingivalis and Prevotella intermedia grown on Fastidious Anaerobe Agar plates (FAA, Lab M, Bury, UK). In both media, 5% defibrinated sheep blood was added.

**Agar diffusion method**

The methodology used was adapted from Gomes et al. All microorganisms were previously subcultured in appropriate culture media and under gaseous conditions to confirm their purity.

The aerobes and the facultative anaerobic strains were individually inoculated into tubes containing 5 mL of sterile 0.9% saline solution. The suspension was adjusted spectrophotometrically at 800 nm (Optical Density 800) to match the turbidity of 1.5 × 10⁸ CFU mL⁻¹ (equivalent to 0.5 McFarland standard). Five hundred μL of each test microorganism suspension was used to inoculate glass bottles containing 50 mL of BHI Agar (Lab M, Bury, UK) at 46°C, mixed and poured onto 130 mm plates containing a previously set layer of Mueller Hinton agar (Oxoid, Unipath Ltd, Basingstoke, UK).

The isolated anaerobic microorganisms were suspended spectrophotometrically at 800 nm to match the turbidity of 3.0 × 10⁸ CFU mL⁻¹ (equivalent to 1 McFarland standard). Sterile swabs were dipped into the bacterial suspension and were used to inoculate pre-reduced 70 mm plates containing 5% sheep-blood-Fastidious Anaerobe Agar (FAA - Lab-M, Bury, UK).
The inoculum procedures used were appropriate to provide a semi-confluent growth of the tested microorganisms.

Sterilized stainless steel tubes of 8.0 × 1.0 × 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. The plates were maintained for 2 hours at room temperature in the appropriate gaseous conditions to allow the diffusion of the agents through the agar and then incubated at 37°C again under the appropriate gaseous conditions for an appropriate period of time: aerobes, 24 hours; facultative, 24-48 hours in a CO₂ incubator (Jouan, Saint Herblain, France), in an atmosphere of 10% CO₂ and anaerobes in the anaerobic workstation (Don Whitley Scientific, Bradford, UK) in an atmosphere of 10% H₂, 10% CO₂, 80% N₂ for 7 days.

Zones of inhibition of microbial growth around the cylinder containing the tested substances were measured and recorded after the incubation period. The inhibitory zone was considered to be the shortest distance (mm) between the outer margin of the cylinder and the initial point of the microbial growth. Six replicates were made for each microorganism. The Kruskal-Wallis test was used to determine the differences in susceptibility to intracanal medication between microbial species.

**Direct contact method**

All microorganisms were subcultured onto appropriate culture media under gaseous conditions for 48 hours. Facultative strains were individually inoculated into tubes containing 5mL BHI medium (Lab M), which were adjusted spectrophotometrically to 800 nm to match the turbidity of 1.5 × 10⁸ CFU mL⁻¹ (equivalent to 0.5 McFarland standard). Strict anaerobic microorganisms were individually inoculated into tubes containing 5mL Fastidious Anaerobe Broth ([FAB], LabM, Bury, UK) sterile suspension, which was adjusted spectrophotometrically to 800 nm to match the turbidity of 3.0 × 10⁸ CFU mL⁻¹ (equivalent to 1 McFarland standard).

Six wells were used for each time period, microorganism, and Ca(OH)₂ paste, respectively. Overall, 510 wells were used, comprising 306 for all the tested pastes, and 204 for control groups (i.e., 102 for each medicament or each control group).

One mL of each tested substance was placed at the bottom of each well of 24-well cell culture plates (Corning, NY, USA, ref. No. 3524, well Vol. 3.2 mL), including the control groups (sterile saline and natrosol). Two mL of the microbial suspension were ultrasonically mixed for 10 seconds with the medicaments and left to stand for 15 seconds and 30 seconds; for 1, 3, 5, 10, 15 and 30 minutes; for 1, 2, 4, 6, 8, 12, 24, and 48 hours; and for 7 days. After each period of time, 1 mL from each well was transferred to tubes containing 3 mL of freshly prepared broth medium to which the neutralizer was added in order to avoid the medicaments producing a residual action. 0.5% citric acid was used to neutralize the calcium hydroxide. 0.5% Tween 80 plus 0.07% lecithin was used to neutralize the chlorhexidine and both medicaments combined. All tubes were left at 37°C for 7 days in the appropriate gaseous condition. Agar plates were inoculated with 10 μL from each tube and the plates were incubated at 24-48 hours in the appropriate gaseous conditions. The purity of the positive cultures was confirmed by Gram staining, by colony morphology on blood agar plates, and by the use of biochemical identification kits (API 20 Strep BioMérieux SA, Marcy-l’Etoile, France; API C AUX BioMérieux SA, Marcy-l’Etoile; API 20 Staph BioMérieux SA, Marcy-l’Etoile; Rapid ID 32 BioMérieux SA, Marcy-l’Etoile; Rapid ANA II System, Remel INC., Lenexa, KS, USA). Mini-iapi software (BioMérieux SA) was used to automatically read Rapid ID 32 tests and visually read API range tests from BioMérieux.

The time required for each medicament to produce total microbial growth inhibition was recorded, transformed into seconds, and analyzed statistically using the Kruskal-Wallis test, with significance level set at p < 0.05.

**RESULTS**

**Agar diffusion method**

Mean values of microbial growth inhibition (in mm) produced by the combination of Ca(OH)₂ plus CHX gel paste, CHX gel, and Ca(OH)₂ plus sterile water are shown in Table 1.

The association of Ca(OH)₂ plus 2% CHX gel affected the microbial growth, producing inhibitory zones ranging from 2.84 to 6.5 mm against all microorganisms tested. However, 2% CHX gel alone demonstrated the strongest antimicrobial action, showing the largest inhibitory growth zones, which ranged from 4.33 to 21.67 mm. On the other hand, Ca(OH)₂ manipulated with sterile water did not produce inhibitory zones (0 mm) against any of the tested microorganisms, inhibiting the microorganisms only in direct contact.

Strict anaerobes (*Porphyromonas gingivalis, Prevotella intermedia* and *Porphyromonas endodontalis*) were the most susceptible microorganisms, showing the largest inhibition zones, which ranged from 0 to 21.67 mm. The aerobes (*Candida albicans*, *Staphylococcus aureus*) and facultative microorganisms (*Enterococcus faecalis*) were more resistant to all medicaments used, producing inhibition zones ranging from 0 to 9.67 mm.
Table 2 shows the mean contact time required for Ca(OH)\textsubscript{2} plus 2\% CHX gel, 2\% CHX gel alone, and Ca(OH)\textsubscript{2} plus sterile water to produce 100\% microbial growth inhibition.

The combination of Ca(OH)\textsubscript{2} plus 2\% CHX gel took from 30 seconds to 6 hours to eliminate all microorganisms, while 2.0 \% CHX gel alone took 1 minute or less, and Ca(OH)\textsubscript{2} combined with sterile water required from 30 seconds to 24 hours to kill the tested microorganisms.

The strict anaerobes were more susceptible than the aerobes or the facultative anaerobic microorganisms, however, without statistical significant differences. Porphyromonas gingivalis, Prevotella intermedia, and Porphyromonas endodontalis were eliminated in less than 1 minute.

Candida albicans (an aerobe microorganism) and Enterococcus faecalis (a facultative microorganism) showed similar susceptibility, being killed in 24 hours by Ca(OH)\textsubscript{2} pastes, in 1 hour by the combined medicaments (Ca(OH)\textsubscript{2} plus 2\% CHX gel), and 15 seconds by 2.0 \% CHX gel alone.

Staphylococcus aureus was eliminated in 8 hours by Ca(OH)\textsubscript{2} plus sterile water and in 6 hours by Ca(OH)\textsubscript{2} plus 2\% CHX gel.

The smallest zone of growth inhibition (in mm) of each medicament using the Agar diffusion method, and the maximum time (in seconds or hours) taken by each medicament to produce negative cultures using the Direct contact method are shown in Figure 1. As a whole, Ca(OH)\textsubscript{2} plus sterile water pastes required up to 24 hours to eliminate the tested microorganisms and did not produce inhibitory zones (0 mm). The 2\% CHX gel alone required up to 1 minute to eliminate the tested microorganisms and the smallest zone of inhibition measured 4.33 mm. Ca(OH)\textsubscript{2} plus 2\% CHX gel required up to 6 hours to eliminate the tested microorganisms and the smallest zone of inhibition growth measured 3.83 mm.

DISCUSSION

Despite the antimicrobial properties of the chemomechanical preparation and the intracanal dressings, the elimination of the microorganisms may not be uniform due to the varying vulnerabilities of the involved species. Furthermore, the anatomical complexities of many root canals and consequent limitations of access by instruments, irrigants, and intracanal medications are well-recognized factors.

Even though anaerobic bacteria, especially black-pigmented Gram-negatives, have been linked to signs and symptoms, facultative bacteria, such as Enterococcus faecalis have been considered one of the most
resistant species in the oral cavity and a possible cause of root canal treatment failure.34,35

Traditional methods have demonstrated that enterococci constitute a small percentage of the microbial species isolated from root canals of teeth with necrotic dental pulps. However, they are the most commonly isolated species from root canals of teeth with failed endodontic treatment, being recovered from approximately 50% of the canals.15,16,34 This percentage can be as high as 70% when root-filled teeth are associated with chronic apical periodontitis.37,38 This may be due to their increased resistance to instrumentation and to antiseptic agents.39,40 Moreover, they tolerate very high pH values, varying from 9 to 11.

E. faecalis has demonstrated the capacity to survive in an environment in which there are scant available nutrients and in which commensalism with other bacteria is minimal. It has been postulated that a virulence factor of E. faecalis in failed endodontically treated teeth may be related to the ability of E. faecalis cells to maintain the capability to invade dentinal tubules and adhere to collagen in the presence of human serum.41

The majority of the research on calcium hydroxide antimicrobial activity used the agar diffusion method, which only indicates the medicament’s potential to eliminate microorganisms.24 Moreover, the results of the agar diffusion method, as the other in vitro tests, depend upon the molecular size, solubility and diffusion of the materials through the aqueous agar medium, the sensitivity of the drug, bacterial source (wild strains or collection species), the number of bacteria inoculated, pH of the substrates in plates, agar viscosity, storage conditions of the agar plates, incubation time and the metabolic activity of the microorganisms. Therefore, the inhibition zones may be more related to the materials’ solubility and diffusibility in agar than to their actual efficacy against the microorganisms.13 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. Therefore, our study also used the direct contact method, which relies on direct and close contact between the test substance and test material and allows gel and paste formulations more similar diffusion conditions than the agar diffusion method.

Even though the direct contact method used is more reliable than the agar diffusion tests, some limitations could also be found, such as: a) clinically, the effectiveness of the material is generally reduced by the buffering effect of dentin; b) the amount of medication placed inside the canal is usually smaller than that used in in vitro studies; c) the polymicrobial nature of endodontic infections is difficult to reproduce in vitro; d) and the presence of biofilms which might require more time and volume of medicaments to exert the same antimicrobial activity.

Even with the limitations of the in vitro tests, in this study it was possible to compare the antimicrobial activity and the time required by intracanal medicaments to eliminate endodontic pathogens.

In the present study Ca(OH)2 paste mixed with sterile water had antimicrobial action in all cases, but only by direct contact, which is in agreement with previous studies.32,42 Other studies have also reported the failure of Ca(OH)2 to eliminate enterococci effectively.43,39

In the contact test, the required time for Ca(OH)2 plus sterile water paste to kill all microorganisms was dramatically longer than for the other medicaments, ranging from 30 seconds to 24 hours depending on the microbial susceptibility. Even though it was able to eliminate microorganisms, it cannot be forgotten that there was a close contact between the microorganisms and the paste. Perhaps such contact does not occur in the total root canal system, where microorganisms can be located inside the dentinal tubules. Moreover, the low solubility and diffusibility of Ca(OH)2, as well as the dentine buffering ability, may make it difficult to attain an increased pH capable of eliminating bacteria located within dentinal tubules or enclosed in anatomical variations.44

Our results verified that chlorhexidine, in gel formulation at 2%, is a powerful antimicrobial agent, showing the largest inhibition zones, which ranged from 4.33 to 21.67 mm. Chlorhexidine gel quickly killed all micro-
organisms tested, in 1 minute or less. Enterococcus faecalis was eliminated in only one minute, agreeing with another previous work. Although chlorhexidine showed better antimicrobial activity, it does not act as a physical barrier, staying in the canal for a shorter period of time.

In our research, the pastes manipulated with Ca(OH)₂ plus 2% chlorhexidine gel were effective against all microorganisms, showing larger inhibition zones than those made with sterile water as a vehicle. The inhibition zones varied from 2.83 to 6.50 mm, according to the microbial susceptibility. In the contact test, the required time for Ca(OH)₂ + CHX to kill all microorganisms ranged from 30 seconds to 6 hours.

Research in vitro has proved that some associations, such as calcium, urea, and sulphate lauryl sodium reduce the antimicrobial activity of chlorhexidine. In the present study, it was observed that the antimicrobial property of chlorhexidine was also reduced when it was combined with calcium hydroxide. On the other hand, the antimicrobial activity of Ca(OH)₂ increased with this association. The lower effectiveness of chlorhexidine in the Ca(OH)₂/CHX mixture is probably the result of chlorhexidine precipitation, which happens at a high pH.

In the present study, the pastes manipulated with Ca(OH)₂ plus 2% chlorhexidine gel were effective against all tested microorganisms, including Enterococcus faecalis, showing larger inhibition zones and shorter times to eliminate them, than those made with sterile water as a vehicle. Furthermore, the presence of Ca(OH)₂ in the paste formulation acts as a physical barrier, which will stay in the root canal for longer, preventing root canal re-infection, interrupting the nutrient supply to the remaining bacteria, and thus delaying recontamination. Moreover, the presence of chlorhexidine adds substantivity to the formulation, due to its adsorption capacity and slow liberation of active molecules by dental tissues. Therefore, chlorhexidine could maintain the canal free of microorganisms, even after being removed from the canal. Chlorhexidine’s substantivity sustains the antimicrobial activity over a period of 48 hours, 72 hours, and up to 7 days after being removed from the root canal. The pH achieved in this combination was higher than the one obtained with chlorhexidine alone, which would help control external and internal inflammatory root resorption.

CONCLUSION
On the basis of the results obtained and the experimental conditions used in this study, 2% CHX gel + Ca(OH)₂ showed better antimicrobial activity than Ca(OH)₂ manipulated with sterile water. This research emphasizes the importance of the contact between medicament and the infecting microorganisms, in order to achieve a substantial antimicrobial effect. Further research in vivo is required to determine the effectiveness of the antimicrobial activity of chlorhexidine gel, Ca(OH)₂ paste, and both these intracanal medicaments combined.

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