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RESEARCH ARTICLE

Efficacy of Calcium Hydroxide Alone or with Clorhexidine against Staphylococcus Aureus and Enterococcus Faecalis

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Abstract

The primary goal of endodontic therapy is cleaning and shaping effectively the root canal system in order to reduce microbiota in infected teeth. Calcium hydroxide ($Ca(OH)_2$) and chlorhexidine (CHX) has been widely used in root canal treatment. The aim of this study was to evaluate in vitro the efficacy of $Ca(OH)_2$ with or without CHX against *S. aureus* and *E. faecalis*. Different experiments were used to evaluate the inhibitory activity of $Ca(OH)_2$ alone or in combination with CHX. The percentage of dead bacteria is calculated relatively to the growth control by determining the number of living bacteria (CFU/mL) of each tube using the agar plate count method. Experiment one: $Ca(OH)_2$ in combination with CHX (0.06%) killed more than 6-log in all of the concentrations above the solubility of $Ca(OH)_2$ studied against *S. aureus* and *E. faecalis*, respectively. Therefore, $Ca(OH)_2$ in H_2O killed all the bacteria but the last concentration in case of *S. aureus*. Experiment two: different concentrations of $Ca(OH)_2$ below their solubility in H_2O that proceeded from an initial solution, the highest concentration used (0.6g/L) killed only 4-log and 2-log against *E. faecalis* and *S. aureus*. Experiment three: The growth of *S. aureus* was completely inhibited by CHX ranged from 0.06% to 0.0018%. In the case of *E. faecalis*, all the bacteria were killed by CHX ranged from 0.06% to 0.00094%. The combinations at 0.0018% and 0.0075% of CHX at a 0.3g/L concentration of $Ca(OH)_2$ exhibits the most efficient antimicrobial activity against *S. aureus* and *E. faecalis*, respectively.

Keywords: Calcium hydroxide; Chlorhexidine, S aureus, E Faecalis, Time-Kill Test, Endodontic Infection.

Introduction

Bacterial invasion of the root canal system is essential for the onset and maintenance of periapical diseases; thus, it is necessary an endodontic treatment to reduce bacteria in the root canal system [1–4].

E. faecalis is a Gram-positive bacterium [1] that can resist bactericidal substances commonly used in endodontic procedures [1], and play an important role in the aetiology of persistently infected root canal [1,5–7]. Its prevalence in endodontic infections is 40% and varies from 24% to 77% in endodontic reinfections [8,9].

The primary goal of endodontic therapy is cleaning and shaping effectively the root canal system in order to reduce microbiota in infected teeth [10,11], although it has been shown that it is impossible to obtain the elimination of bacteria in all cases [1,12]. Calcium hydroxide ($Ca(OH)_2$) has been widely used in root canal treatment [5,11,13–15]. $Ca(OH)_2$ has been commonly used as an intracanal medication because of it antimicrobial capacity and ability to dissolve tissue and induce mineralization. Its antimicrobial property is associated with its dissociation into calcium and hydroxyl ions, which procedures an alkaline pH, causing a destructive effect on the bacterial membrane [1,14,16–18].

The chlorhexidine (CHX) also is a substance that issued as an intracanal medication due to its antimicrobial activity. This is bactericidal and effective against Gram positive and Gram negative bacteria [1,18–20]. At low concentrations it is bacteriostatic, while at high concentrations it is bactericidal, resulting in coagulation and precipitations of cytoplasm [17,21].

Whether a combination of $Ca(OH)_2$ and CHX is more effective than $Ca(OH)_2$ alone against *E. faecalis* is a matter of controversy [14]. Many studies have attempted to compare antibacterial effect of $Ca(OH)_2$ alone or in combination with CHX [14]. Some studies have shown an increased antibacterial effect when CHX is added to $Ca(OH)_2$ [1,13,22–25], while other studies have failed to show any benefits in incorporating CHX to $Ca(OH)_2$ [6,26–28].

$Ca(OH)_2$ is the "gold standard" in endodontic practice, although some authors also recommend the use of CHX, and others the combined use of both. Because there is an important debate in this regard, since different studies present contradictory results, the objective of this article is was to evaluate in vitro the efficacy of $Ca(OH)_2$ with or without CHX against *S. aureus* and *E. faecalis*.

Materials and Methods

Facultative anaerobes tested were *E. faecalis* (American Type Culture Collection [ATCC] 29212), *S. aureus* (ATCC 29213) and three replicates were made for each microorganism and experiment. The density of the inoculum in water was adjusted to the turbidity of 0.5 McFarland standard (1.5×10^8 bacteria/mL). All experiments were performed with a growth control of the initial inoculum of each bacterium.

Different experiments were used to evaluate the inhibitory activity of $Ca(OH)_2$ alone (Dentaflux) or in combination with CHX (0.12% Chlorhexidine, Kingingival. Kin).

The selection of concentrations was made based on the solubility of $Ca(OH)_2$ (1.2g/L) and commercial concentration of the CHX.

A.- Plating the entire suspension of different concentrations of $Ca(OH)_2$ in combination with H_2O or CHX: The inhibitory activity of different concentrations prepared individually of $Ca(OH)_2$ mixed with H_2O or CHX (0.06%) against *S. aureus* and *E. faecalis* were tested. All concentrations studied in this experiment were above the solubility of $Ca(OH)_2$ (1.2g/L). The final concentrations used were ranged from 300g/L to 0.78g/L of $Ca(OH)_2$ against *S. aureus* and from 300g/L to 1.56g/L of $Ca(OH)_2$ against *E. faecalis*. It is performed using several tubes containing a final volume that contained equal parts of bacterial suspen-

sion and the substance tested at different concentrations. The tubes were incubated at 35°C for half an hour. After that, the suspension was plated in Columbia blood agar (MAIM) ranged from one to six Petri dishes.

B.- Plating the suspension of different concentrations of $Ca(OH)_2$ in H_2O that proceeded from an initial solution: The inhibitory activity of different dilutions that proceeded from a solution at an initial concentration of $Ca(OH)_2$ mixed with H_2O were tested against *S. aureus* and *E. faecalis*, respectively. The final concentrations used were ranged from 0.6g/L to 0.0094g/L of $Ca(OH)_2$ mixed with H_2O. All concentrations studied in this experiment were below the solubility of $Ca(OH)_2$ (1.2g/L). It is performed using several tubes containing a final volume of 100µL that contained equal parts of bacterial suspension and the substance tested at different concentrations. The tubes were incubated at 35°C for half an hour. After that, the mixed suspension was plated in Columbia blood agar (MAIM).

C.- Comparison of two specific concentrations of CHX to which different concentrations of $Ca(OH)_2$ were added: The inhibitory activity of different final concentrations ranged from 0.06% to 0.00023% of CHX alone against *S. aureus* and from 0.06% to 0.00047% of CHX against *E. faecalis* were tested. In addition, two CHX concentrations that killed 6-log in the two bacteria were selected to add different concentrations of $Ca(OH)_2$ below the solubility of $Ca(OH)_2$ (1.2g/L) . The final concentrations used were ranged from 0.3g/L to 0.0047g/L of $Ca(OH)_2$. All the initial solutions were prepared in a final volume of 50mL. It is performed using several tubes containing a final volume of 100µL with a bacterial suspension (50µL) and the substance tested at different concentrations (50µL). The tubes were incubated at 35°C for half an hour. After that, the suspension was plated in Columbia blood agar (MAIM).

In all experiments, the plates were incubated at 35°C for 24 hours. Then, the percentage of dead bacteria is calculated relatively to the growth control by determining the number of living bacteria (CFU/mL) of each tube using the agar plate count method. Generally, the bactericidal effect is obtained with a lethality of 6-log for 24 hours.

A statistical analysis of the results was performed by using an independent sample t test to compare CHX to CHX+$Ca(OH)_2$ for each concentration.

Results

A.- Plating the entire suspension of different concentrations of $Ca(OH)_2$ in combination with H_2O or CHX: The antimicrobial activity of $Ca(OH)_2$ with H_2O or CHX was evaluated by counting the CFUs. Our results showed that $Ca(OH)_2$ in combination with CHX (0.06%) above the solubility of $Ca(OH)_2$ killed more than 6-log in all of the concentrations studied against *S. aureus*. Therefore, $Ca(OH)_2$ in H_2O not killed all the bacteria in the last concentration (0.78g/L $Ca(OH)_2$+CHX) against *S. aureus* (Figure 1). There was significant difference at 0.78g/L of $Ca(OH)_2$ between the H_2O and CHX ($p < 0.05$).

In the case of *E. faecalis*, the different concentrations of $Ca(OH)_2$ in H_2O or in CHX (0.06%) killed more than 6-log in all of the concentrations studied (Figure 2). There was no significant difference between $Ca(OH)_2$ in H_2O or in CHX ($p > 0.05$).

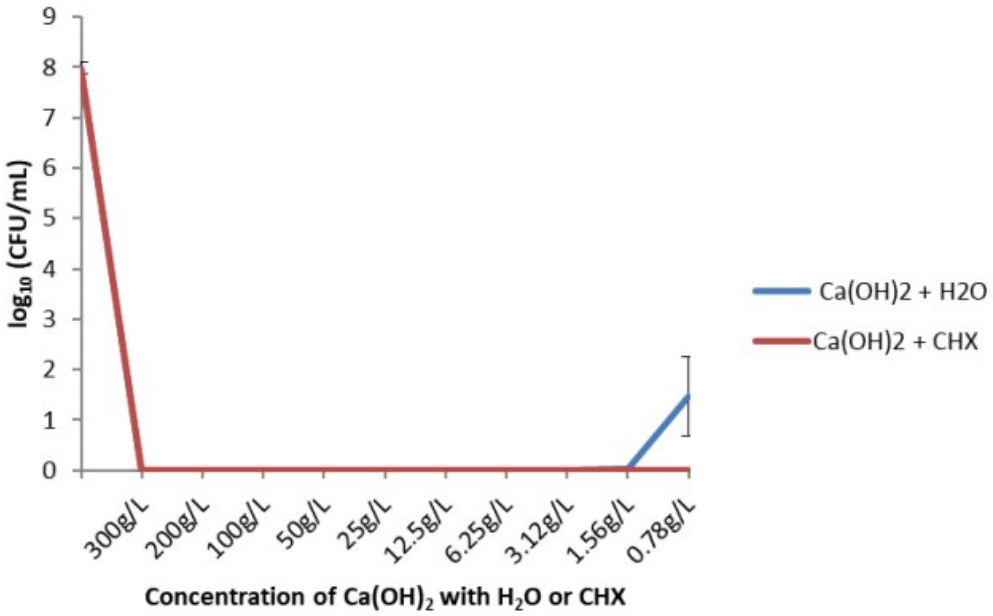

Figure 1: Antimicrobial activity of $Ca(OH)_2$+H_2O and $Ca(OH)_2$+CHX (0.06%) against S. aureus plating the entire suspension. The lines show the number of CFUs of S. aureus recovered after $Ca(OH)_2$+H_2O and Ca(OH)2+CHX treatment. Results represent the mean ± standard deviation of the experiments.

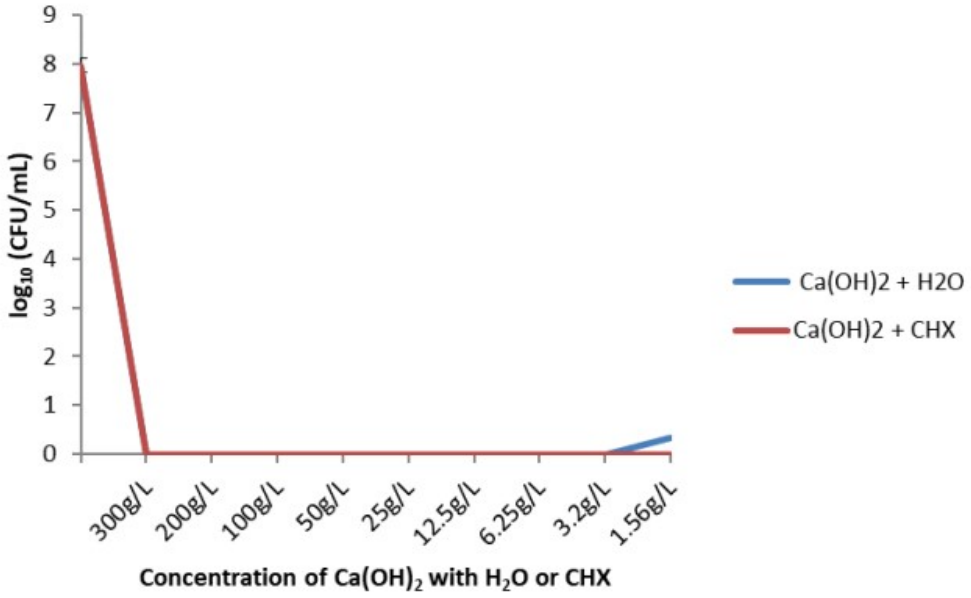

Figure 2: Antimicrobial activity of $Ca(OH)_2$+H_2O and $Ca(OH)_2$+CHX (0.06%) against *E. faecalis* plating the entire suspension. The lines show the number of CFUs of *E. faecalis* recovered after $Ca(OH)_2$+H_2O and $Ca(OH)_2$+CHX treatment. Results represent the mean ± standard deviation of the experiments.

B.- Plating the suspension of different concentrations of $Ca(OH)_2$ in H_2O that proceeded from an initial solution: The results for the different concentrations of $Ca(OH)_2$ in H_2O below the solubility of $Ca(OH)_2$ that proceeded from an initial solution against *S. aureus* and *E. faecalis* are shown in Figure 3 and 4, respectively. Figure 3 shows that the most effective concentration studied was 0.6g/L of $Ca(OH)_2$+H_2O, killing 2-log of *S. aureus*. Figure 4 shows that $Ca(OH)_2$ in H_2O could be more effective against *E. faecalis* because in concentration 0.6g/L and 0.3g/L of $Ca(OH)_2$+H_2O kill 4-log and 2-log of bacteria, respectively.

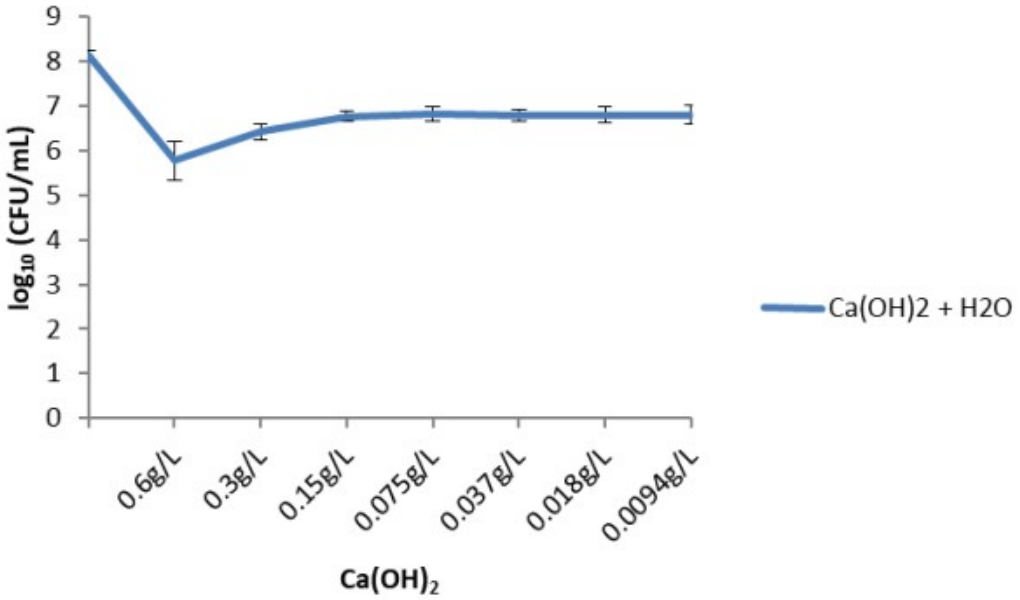

Figure 3: Antimicrobial activity of $Ca(OH)_2$ in H_2O below the solubility of $Ca(OH)_2$ against *S. aureus*. The line shows the number of CFUs of *S. aureus* recovered after $Ca(OH)_2$+H_2O treatment. Results represent the mean ± standard deviation of the experiments.

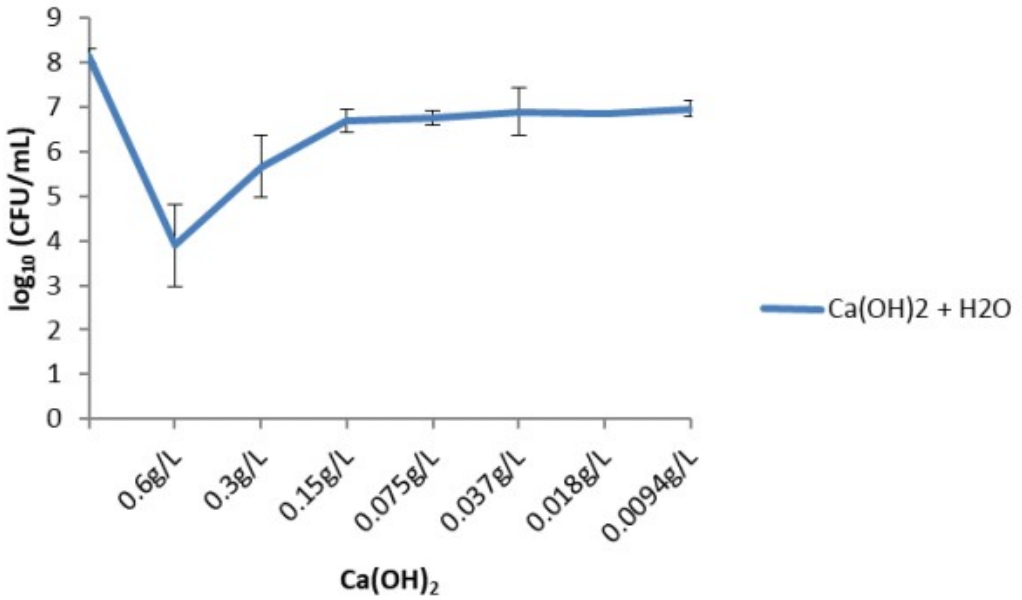

Figure 4: Antimicrobial activity of $Ca(OH)_2$ in H_2O below the solubility of $Ca(OH)_2$ against *E. faecalis*. The line shows the number of CFUs of *E. faecalis* recovered after $Ca(OH)_2$+H_2O treatment. Results represent the mean ± standard deviation of the experiments.

C.- Comparison of two specific concentrations of CHX to which different concentrations of $Ca(OH)_2$ were added: The results of CHX alone against *S. aureus* and *E. faecalis* are presented in Figures 5 and 7, and CHX in combination with $Ca(OH)_2$ in Figures 6 and 8. The growth of *S. aureus* was completely inhibited by CHX ranged from 0.06% to 0.0018% (Figure 5). Figure 6 shows that in most $Ca(OH)_2$ concentration CHX at 0.0018% is more effective than CHX at 0.0037%. There was significant difference at 0.15g/L and 0.075g/L of $Ca(OH)_2$ between CHX at 0.0037% and 0.0018% ($p < 0.05$).

In the case of *E. faecalis*, all the bacteria were killed by CHX ranged from 0.06% to 0.00094% (Figure 7). Figure 8 shows that 0.3g/L of $Ca(OH)_2$ and from 0.075g/L to 0.0047g/L of $Ca(OH)_2$, CHX at 0.0075% is more effective than CHX at 0.0037%. There was significant difference at 0.075g/L of $Ca(OH)_2$ between CHX at 0.0075% and 0.0037% ($p < 0.05$).

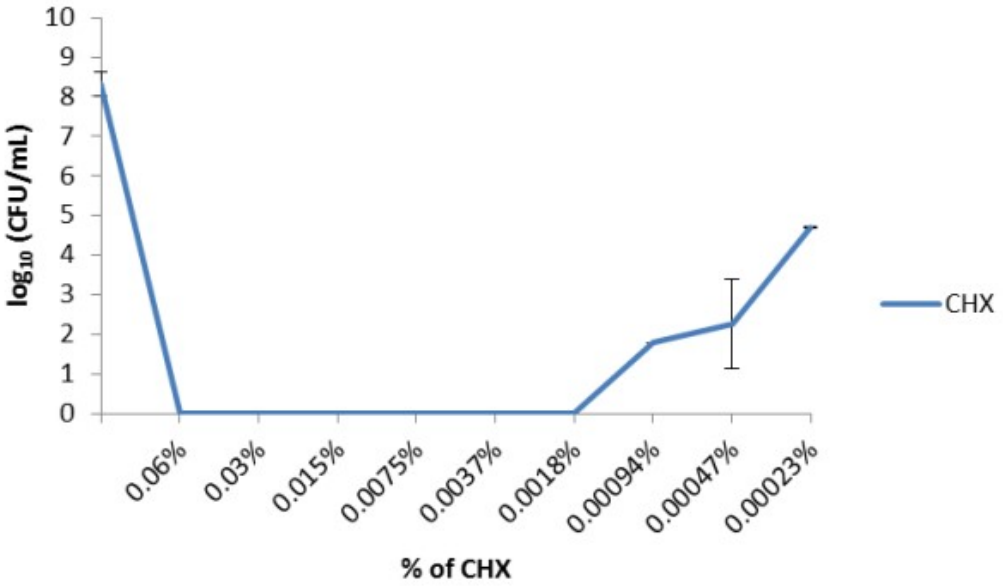

Figure 5: Antimicrobial activity of CHX alone against *S. aureus*. The line shows the number of CFUs of *S. aureus* recovered after CHX treatment. Results represent the mean ± standard deviation of the experiments.

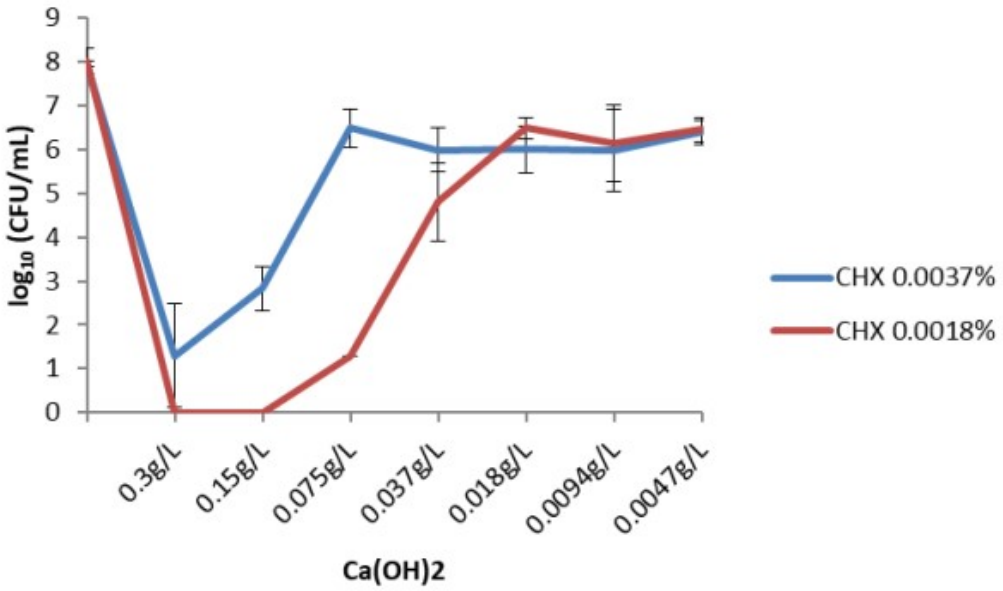

Figure 6: Antimicrobial activity of different concentrations of $Ca(OH)_2$ in 0.0037% and 0.0018% of CHX against *S. aureus*. The lines show the number of CFUs of *S. aureus* recovered after $Ca(OH)_2$+CHX treatment. Results represent the mean ± standard deviation of the experiments.

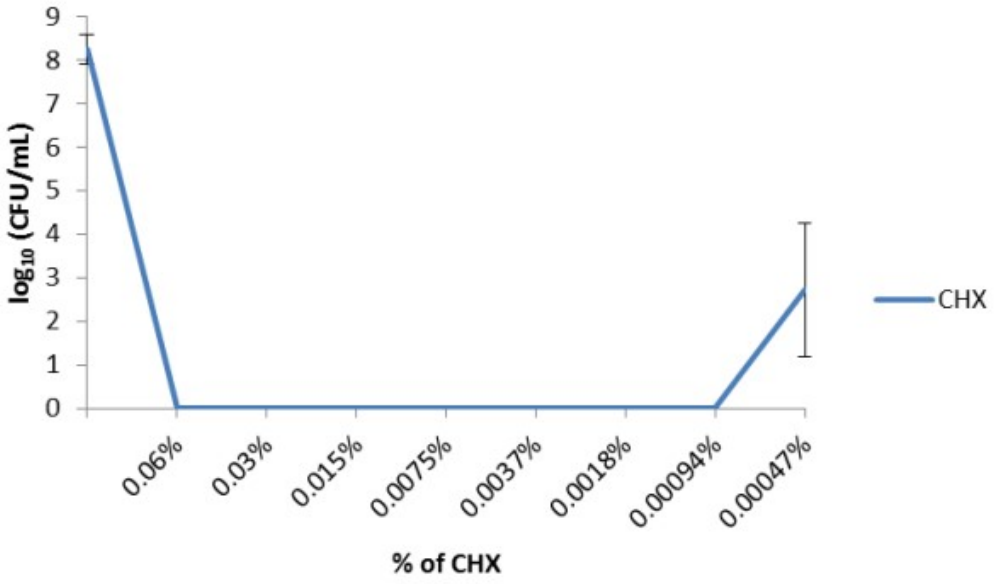

Figure 7: Antimicrobial activity of CHX alone against *E. faecalis*. The line shows the number of CFUs of *E. faecalis* recovered after CHX treatment. Results represent the mean ± standard deviation of the experiments.

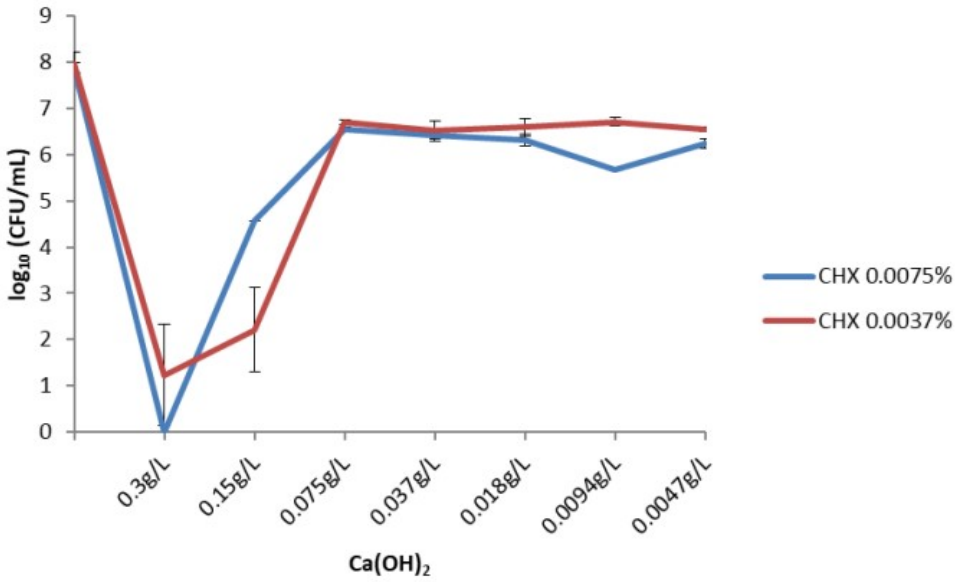

Figure 8: Antimicrobial activity of different concentrations of $Ca(OH)_2$ in 0.0075% and 0.0037% of CHX against *E. faecalis*. The lines show the number of CFUs of *E. faecalis* recovered after $Ca(OH)_2$+CHX treatment. Results represent the mean ± standard deviation of the experiments.

Discussion

Microorganisms play an important role in periapical diseases and their elimination during endodontic treatment is crucial for success of treatment [18]. $Ca(OH)_2$ is the most common intracanal medication [11,29,30]; and CHX is currently considered the gold standard of oral antiseptics [30]. At low concentrations, CHX has a bacteriostatic effect, however, at higher concentrations, CHX has a bactericidal effect [20]. The most commonly isolated species from root canals of teeth with failed endodontic treatment is *E. faecalis* [21].

A great variety of laboratory methods can be used to evaluate the *in vitro* antimicrobial activity of an extract or a pure compound. The agar diffusion test has frequently been used to test the efficacy of various antimicrobials [31]; however, the out-

come measure (i.e. the zone of inhibition) is dependent on the ability of the test antimicrobials to diffuse in the agar, the molecular size, solubility, diffusion of the materials through the aqueous agar medium, the sensitivity of the drug, bacterial source, 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 [6,7,22,32]. Besides, the bacterial growth inhibition does not mean the bacterial death, thus, this method cannot distinguish bactericidal and bacteriostatic effects [31]. On the other hand, Time-kill test is the most appropriate method for determining the bactericidal effect. It is a strong tool for obtaining information about concentration-dependent antimicrobial effect [31] but only a few studies have been used this method to evaluate antimicrobial activity [13,33,34].

The disc diffusion assay was a frequently used method to determine the antibacterial properties of CHX, $Ca(OH)_2$ [30]. But the benefit of mixing $Ca(OH)_2$ with CHX to improve the antibacterial property in elimination of *E. faecalis* remains a matter of controversy. The possible reasons for this controversy are the differences in the methods and materials used, including microbiological assessments (e.g. agar diffusion method, dentin block model etc.), concentrations and physical forms of CHX (e.g. gel, solution), time periods of experiments, strains and concentrations of *E. faecalis*, methods of bacterial inoculation, methods used for placing the medicaments, and depths of sampling [14].

Our results have shown that $Ca(OH)_2$ in H_2O has good efficacy against *E faecalis* and *S. aureus* in experiment "A" with concentrations above the solubility of $Ca(OH)_2$, but in experiment "B" with lower concentrations below the solubility of $Ca(OH)_2$ is worse, killing only 4-log and 2-log, respectively. Previous studies have shown $Ca(OH)_2$ alone to be a non-effective or relatively ineffective intracanal medicament against *E. faecalis* [21,22]. However, Zubizarreta et al. has proven that $Ca(OH)_2$ was effective against *E. faecalis*, regardless of the agent and solvent concentration employed [15]. In addition, another study suggests 10% $Ca(OH)_2$ alone is effective [27].

Regarding to CHX, in the present study, it was also observed that different concentrations of CHX showed inhibitory action against *E. faecalis* and *S. aureus*. Stuart et al. has been reported that CHX alone has been shown to provide as good or even better, antimicrobial action against *E. faecalis* than $Ca(OH)_2$+CHX combinations [8]. Similar results have been reported by Basrani et al., Lakhani et al., and Yadav et al. [18,22,35].

As far as intracanal medicament is concerned, CHX is generally more effective than $Ca(OH)_2$ against *E. faecalis* infection in dentinal tubules [20]. In fact, the antimicrobial activity of CHX is reduced when combined with $Ca(OH)_2$ [6,8,20]. The lower effectiveness of chlorhexidine in the $Ca(OH)_2$+CHX mixture is probably the result of CHX precipitation, which happens at a high pH [6,36]. Our results verified that CHX alone, in experiment "C", showed highest antimicrobial activity than $Ca(OH)_2$+CHX at concentrations below the solubility of $Ca(OH)_2$. Nevertheless, the experiment "A" carried out with concentrations above the solubility of $Ca(OH)_2$ have showed similar antimicrobial properties mixing $Ca(OH)_2$ with CHX or H_2O, these results could be due to the different methodology and concentrations used in the design of the experiment.

On the other hand, the antimicrobial activity of $Ca(OH)_2$ increases with the combination with CHX. Several Studies have exposed that antimicrobial activity of $Ca(OH)_2$ increases when mixed with CHX [6–8,20]. However, a review reported by Saatchi et al. concludes that it appears that mixing $Ca(OH)_2$ with CHX does not improve its *ex vivo* antibacterial property as an intracanal medicament against *E. faecalis* [14]. In the present study, the results in experiment "C" suggested that the efficacy of $Ca(OH)_2$+CHX is concentration-dependent against *E. faecalis* and *S. aureus*. Our study showed that the results obtained are different according to the design of the experiment and according to the concentrations used of each of the two substances studied. So that, further studies are required to corroborate these results suggestive of the beneficial nature of CHX and ($Ca(OH)_2$) for endodontic infection treatment.

Conclusion

On the basis of the results obtained and the experimental conditions used in this study, mixing $Ca(OH)_2$ with CHX does not improve CHX antibacterial property against *S. aureus* and *E. faecalis*. The combinations at 0.0018% and 0.0075% of CHX at a 0.3g/L concentration of $Ca(OH)_2$ exhibits the most efficient antimicrobial activity against *S. aureus* and *E. faecalis*, respectively.

Conflicts of Interest

The authors stated that there are no conflicts of interest regarding publication of this article

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