ORIGINAL RESEARCH

Antimicrobial Efficacy of Calcium and Sodium Hypochlorite at Different Concentrations on a Biofilm of *Enterococcus faecalis* and *Candida albicans*: An *In Vitro* Comparative Study

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Abstract

Aim: To compare the antimicrobial efficacy of sodium hypochlorite (NaClO at 2.5% and 5.25%) and calcium hypochlorite [$Ca(ClO)_2$ at 2.5%] on a biofilm of *Enterococcus faecalis* ATCC 29212™ and *Candida albicans* ATCC 10231™.

Materials and methods: We performed an experimental *in vitro* study. Strains of *C. albicans* and *E. faecalis*, which had previously been reactivated were used. Then the colonies to be used were standardized in a turbidity standard to guarantee a quantity of 108 (CFU/mL) using the McFarland scale (0.5). Subsequently, the biofilm formed in brain-heart infusion agar was seeded into 42 sterile disks previously embedded with the experimental substances. Both 2.5% NaClO and $Ca(ClO)_2$ solutions were placed in each Petri dish. They were then incubated at 37°C for 24 hours and the inhibition halos were measured using the Kirby-Bauer technique.

Results: The means between the halos corresponding to NaClO and $Ca(ClO)_2$ at 2.5% were 13.38 ± 0.64 mm and 13.42 ± 0.62 mm, respectively. According to the Tukey test, no statistically significant differences were found between the hypochlorite groups evaluated ($p = 0.989$).

Conclusion: Both $Ca(ClO)_2$ and NaClO have a similar antimicrobial efficacy with biofilm based on *E. faecalis* and *C. albicans*, with no statistically significant differences between the two.

Clinical significance: This study demonstrates the effectiveness of $Ca(ClO)_2$ and NaClO as endodontic irrigators to combat the most frequent microorganisms of the root canal.

Keywords: Antibacterial activity, Antifungal activity, Calcium hypochlorite, Sodium hypochlorite.

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Introduction

Endodontic treatment involves the total elimination of microorganisms found in both primary and refractory infectious processes of the root canal system, and this is achieved by a series of factors to, one of the main factors being irrigation.[1–4]

Persistent root canal infections are related to the retention of microorganisms in the dentinal tissue before, during, or after endodontic treatment. The most frequently involved microorganisms are *E. faecalis* and *C. albicans*. Instrumentation alone cannot eliminate all the pulp tissues and microorganisms because of the complexity of the root canals. Therefore, it is essential to accompany the instrumentation process using a solution that complies with most the characteristics of an ideal irrigator.[5–7]

Irrigation during endodontic treatment is one of the determining factors of successful treatment, being a complement to mechanical maneuver or instrumentation in the different root canals since it helps with the removal of pulp tissues and serves as a lubricant, in addition to having antibacterial action against the different aerobic and anaerobic microorganisms present in the canals as well as bacterial toxins in the duct system.[8–12]

For these reasons, sodium hypochlorite (NaClO) has become the main irrigator used during endodontic treatment. At its different concentrations, this compound complies with most of the previously mentioned characteristics such as lubricant, disinfectant, and excellent organic tissue solvent. However, it also has cytotoxic characteristics, which led to the search for new alternatives for solutions, such as calcium hypochlorite $Ca(ClO)_2$, which are primarily considered to be effective against different microorganisms, especially those mentioned above.[4,12–14] Many solutions have been studied in the attempt to replace NaClO because of its toxicity. Among these solutions, $Ca(ClO)_2$ is a stable chemical routinely used in health sciences. According to scientific literature, this solution shows antimicrobial properties and has the potential to dissolve organic tissues.[4,13,14]

Therefore, the aim of this study was to compare the antimicrobial efficacy of NaClO (at 2.5% and 5.25%) and Ca(ClO) (at 2.5%) in a biofilm of *E. faecalis* ATCC 29212™ and *C. albicans* ATCC 10231™.

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Materials and Methods

The present *in vitro* experimental study was conducted at the Faculty of Dentistry of the Universidad Nacional Mayor de San Marcos. The sample consisted of 42 Whatman paper disks embedded with the experimental substances together with *E. faecalis* ATCC 29212™ and *C. albicans* ATCC 10231™. This sample size was calculated based on the data obtained in a previous pilot study, with an $\alpha = 0.05$ and a $\beta = 0.8$ using the Stata®15 statistical software to make up the following groups:

Group I: NaClO at 5.25% vs a biofilm of *C. albicans* and *E. faecalis* ($n = 14$).

Group II: NaClO at 2.5% vs a biofilm of *C. albicans* and *E. faecalis* ($n = 14$).

Group III: $Ca(ClO)_2$ at 2.5% vs a biofilm of *C. albicans* and *E. faecalis* ($n = 14$).

Strain Reactivation

The first step was to reactivate the *E. faecalis* strain using Esculin bile agar as the culture medium incubated at 37°C for 72 hours under aerobic conditions. Likewise, the reactivation of *C. albicans* was carried out in 2% Sabouraud agar, with 48 hours of incubation at 37°C (Fig. 1A).

Sowing of Microorganisms

Colonies of the *E. faecalis* strain were extracted with a seeding loop and inoculated in 5 mL of physiological serum to prepare standardized saline suspensions of *C. albicans* and *E. faecalis* by means of a spectrophotometric technique ($\lambda = 530$ nm, OD = 1.258, and $\lambda = 760$ nm, OD = 1.258, respectively) adjusted to the McFarland scale (0.5) to ensure an amount of 108 colony forming units (CFU)/mL. Then 100 µL of each was taken with sterile tips and with an automatic pipette the suspension was placed on each respective plate and then spread with a swab all over brain heart agar in the Petri dishes. The entire procedure was performed under the required sterile conditions. Four presterilized Whatman no. 40 paper disks with a diameter of 5 mm previously embedded in 15 mL of the different irrigating solutions for *C. albicans* and *E. faecalis* were placed on each plate. Each disk included NaClO and $Ca(ClO)_2$ solutions and the plates were then placed in an incubator at 37°C for 24 hours waiting for the inhibition of bacterial growth (Fig. 1B).

Measurement of Inhibition Halos

Bacterial inhibition halos expressed in millimeter in diameter were read with a precision digital King's Foot rule (Mitutoyo), taking into account the mean of the diameters formed around each substance evaluated at 24 hours. The results were recorded in a data collection table using the Kirby–Bauer technique. The diameter of this zone of inhibition was directly proportional to the antibacterial activity of the solutions on *E. faecalis* and *C. albicans*. A value of 5 mm was defined as the absence of bacterial inhibition since it corresponds to the diameter of the Whatman disks no. 40 (Fig. 2A and B).

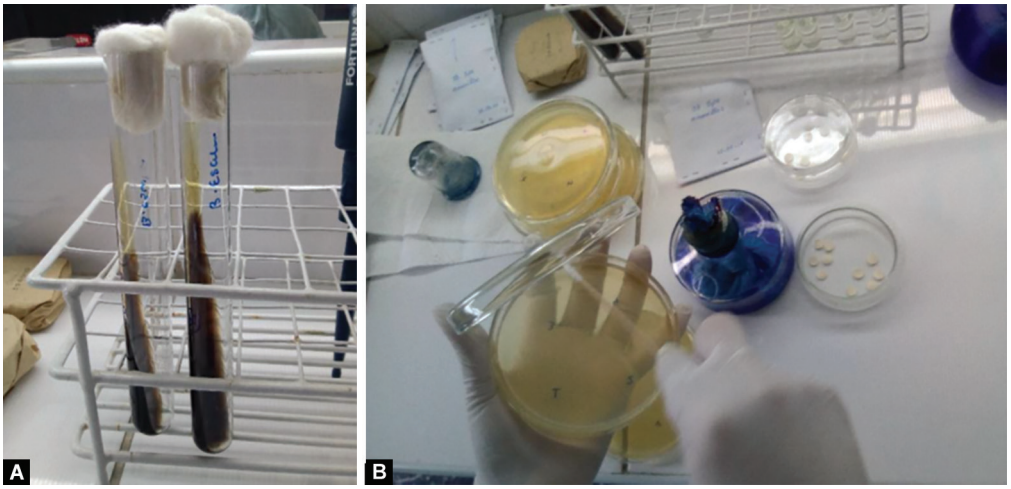
Statistical Analysis

The data were stored in the Microsoft Excel office 365 program and then transferred to the StataCorp Stata® 15.1 statistical software. Descriptive statistics were performed to obtain the means, standard deviations, and maximum and minimum values of the numerical variables. Normality was then determined by the Shapiro Wilk test. Finally, analysis of variance (ANOVA) parametric test was used and the Tukey test was used to perform the *post hoc* analysis. A p value <0.05 was considered as significant.

Results

Table 1 shows that the endodontic irrigator presenting the highest antimicrobial efficacy was 5.25% NaClO followed by $Ca(ClO)_2$ at 2.5% and NaClO at 2.5%, with values of 16.68 ± 0.97, 13.42 ± 0.62, and 13.38 ± 0.64 mm, respectively (Fig. 3). On the contrary, when determining the normal distribution, it was found that the three irrigators had normality with a p value >0.05 in all groups. Finally, when carrying out the comparative analysis of antibacterial efficacy by means of ANOVA test, statistically significant differences were found among the effects of the three endodontic irrigators with a p value <0.001.

Table 2 shows that in the *post-hoc* analysis (Tukey), no statistically significant differences were found between the antibacterial effect of NaClO at 2.5% vs $Ca(ClO)_2$ at 2.5% and $Ca(ClO)_2$ at 2.5% vs NaClO at 2.5% with a p value of 0.989. However, significant differences were found in the other groups with a p value <0.001.

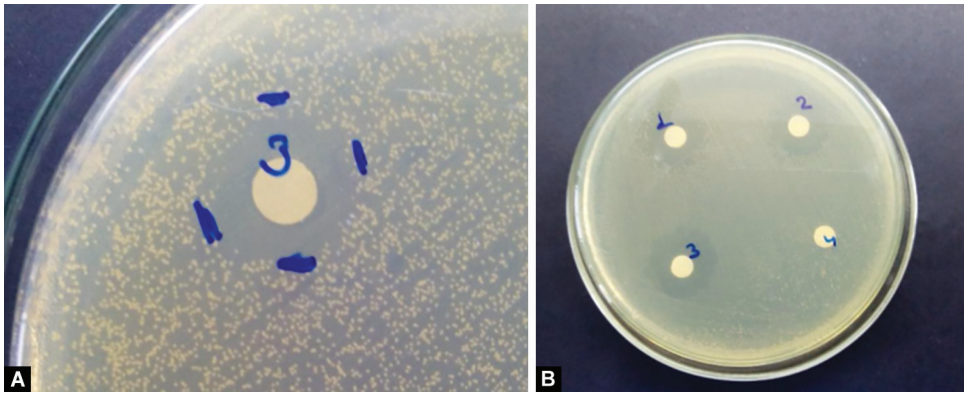


Figs 1A and B: (A) Reactivation of *Enterococcus faecalis* strain; (B) Seeding of *Enterococcus faecalis* and *Candida albicans* in Petri dishes

Table 1: *In vitro* comparison of the antimicrobial effectiveness of different endodontic irrigators in the biofilm of *E. faecalis* and *C. albicans*

Endodontic irrigators	*Mean*	*SD*	*Min*	*Max*	*p**	*p***
NaClO 5.25%	16.68	0.97	15.41	18.52	0.850	<0.001
NaClO 2.5%	13.38	0.64	12.52	14.41	0.558	
$Ca(ClO)_2$ 2.5%	13.42	0.62	12.63	14.36	0.363	
Distilled water	0	0	0	0		

All values were recorded in millimeter (mm). The distilled water group was the negative control; and since it did not present any effect, it was excluded from all the statistical analyses
*Shapiro Wilk test
**ANOVA test
Level of significance ($p < 0.05$)
NaClO, sodium hypochlorite; $Ca(ClO)_2$, calcium hypochlorite



Figs 2A and B: (A) Measurement of inhibition halos; (B) Confrontation of experimental groups

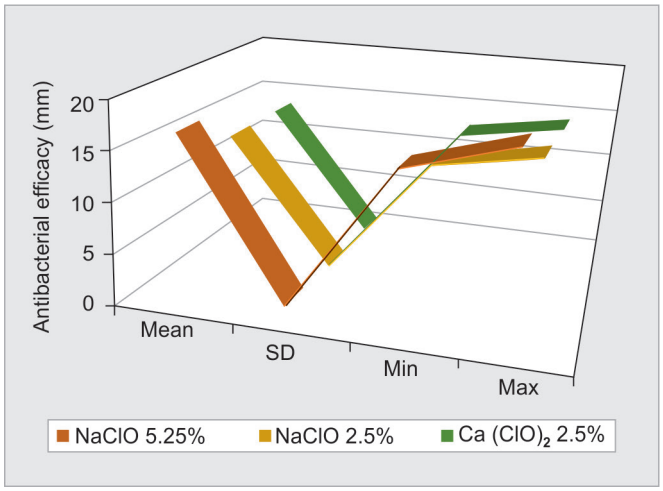


Fig. 3: *In vitro* comparison of sodium and calcium hypochlorite at different concentrations in biofilm of *E. faecalis* and *C. albicans*

Table 2: *Post-hoc* test of the *in vitro* comparison of the different endodontic irrigators on biofilm of *E. faecalis* and *C. albicans*

Endodontic irrigators	*Groups*	*Confidence intervals 95%*	*p**	
NaClO 5.25%	NaClO 2.5%	2.45	4.14	<0.001
	$Ca(ClO)_2$ 2.5%	2.4	4.09	<0.001
NaClO 2.5%	NaClO 5.25%	−4.14	−2.45	<0.001
	$Ca(ClO)_2$ 2.5%	−0.89	0.79	0.989
$Ca(ClO)_2$ 2.5%	NaClO 5.25%	−4.09	−2.4	<0.001
	NaClO 2.5%	−0.79	0.89	0.989

All values were recorded in millimeter (mm)
*Tukey test
Level of significance ($p < 0.05$)
NaClO, sodium hypochlorite; $Ca(ClO)_2$, calcium hypochlorite

Discussion

Sodium hypochlorite is still the most commonly used irrigator in endodontics due to its antibacterial, lubricant, and tissue-solvent properties. The benefits it provides as an irrigator during endodontic therapy include effective elimination of vital and nonvital tissue, with broad antibacterial effects for destroying bacteria, fungi, spores, and viruses, among which the most predominant are *E. faecalis* and *C. albicans*.[3–6] These microorganisms have a high survival rate and both show tolerance to the conditions of adverse environments, making eradication difficult, in addition to being an excellent lubricant, favoring the action of the instruments, it has a low surface tension, half-life of prolonged storage but also presents cytotoxic characteristics.[8,10,11] Therefore, this study seeks to verify that $Ca(ClO)_2$ which is a more stable chemical compound, contains a higher concentration of chlorine in concentrations of 2.5% and 5.5% compared to NaClO under the same conditions, making eradication difficult. In addition to being an excellent lubricant, NaClO favors the action of the instruments, has a low surface tension and a prolonged half-life for storage, and also presents cytotoxic characteristics.[8,10,11] This study aimed to verify whether $Ca(ClO)_2$, which is a more stable chemical compound and contains higher concentrations of chlorine at concentrations of 2.5% and 5.5%, is more effective compared to NaClO under the same conditions.

Several laboratory methods can be used to determine the susceptibility of bacteria to antimicrobial agents *in vitro*, one of these being the agar diffusion technique. The study by Ayhan et al.[15] used this technique to evaluate the antimicrobial effect of several endodontic irrigators on microorganisms including *E. faecalis* and *C. albicans*, using 5.25% NaClO. Likewise, Lin et al.[16] used the same technique to evaluate the antibacterial activity of chlorhexidine against *E. faecalis*. In addition, Fidalgo et al.[17] also used this technique with three irrigating solutions including citric acid, ethylenediaminetetraacetic acid (EDTA), and NaClO at different concentrations in *E. faecalis*, *C. albicans*, and *Staphylococcus aureus*, which is why we worked with this standardized method of agar diffusion described by the National Committee for Clinical Laboratory Standards in our study.

Another clear example is the study by Radcliffe et al.[18] that evaluated the antimicrobial activity of various concentrations of NaClO in *Actinomyces naeslundii*, *Actinomyces isarelii*, *C. albicans*, and *E. faecalis*, considering the genus *Actinomyces* as the main microorganism present in endodontic infections. However, *E. faecalis* and *C. albicans* presented resistance to antimicrobial agents, and they were commonly found in persistent endodontic infections. It was also of note that Valera et al.[19] evaluated the antimicrobial activity of different solutions of chemical irrigators in *E. faecalis* and *C. albicans*, which were found to be primarily responsible for refractory endodontic processes. We consider based on previous research the cultivation of *E. faecalis* and *C. albicans* as the main microorganisms in this investigation. Wang et al.[20] found an association of *E. faecalis* present in saliva and root canals with apical periodontitis. Therefore, taking the above results into account in addition to those of Wang et al.,[20] who found an association between the presence of *E. faecalis* in saliva and root canals and the development of apical periodontitis, it is understood that *E. faecalis* and *C. albicans* are the main microorganisms involved in endodontic infections.

Different irrigating solutions have been used in endodontic therapy such as chlorhexidine, EDTA, hydrogen peroxide, and NaClO and these are commonly used to achieve adequate decontamination of the root canal system. The latter is known for its high antimicrobial activity and its ability to promote the dissolution of organic matter as mentioned by Dutta et al.,[21] when they compared the capacity of NaClO and $Ca(ClO)_2$ as organic tissue solvents, in which no significant difference was observed; however, Tanomaru-Filho et al.,[22] compared the inflammatory response of NaClO and chlorhexidine and found a high cytotoxicity in NaClO, which was considered the greatest disadvantage relative to its use. Although some studies demonstrated the high antimicrobial activity of NaClO and its ability to promote the dissolution of organic matter in a study comparing the capacity of NaClO and $Ca(ClO)_2$ as organic tissue solvents, in which no significant difference was observed between both solutions. However, Tanomaru-Filho et al.,[22] compared the inflammatory response of NaClO and chlorhexidine and found a high cytotoxicity with NaClO, being the greatest disadvantage related to its use.

This research considers $Ca(ClO)_2$ as a new irrigator alternative, since it demonstrated the ability to dissolve organic tissue as mentioned by Dutta et al.,[21] and also a high antimicrobial activity similar to NaClO found by Dumani et al.,[6] Fidalgo et al.,[17] Radcliffe et al.,[18] who demonstrated that the antimicrobial activity of NaClO is directly proportional to the concentration used. In both cases, they evaluated NaClO at a concentration of 0.5%, 1%, 2.5%, and 5.25% before the biofilm was formed by the microorganism mainly *E. faecalis* present in refractory root infections, which showed susceptibility to said solution. In the same way, Dumani et al.,[6] demonstrated a high antimicrobial capacity of $Ca(ClO)_2$ over *E. faecalis*. Valera et al.,[19] in their research in 2013 demonstrated a high antifungal activity of NaClO on *C. albicans*. It shows that $Ca(ClO)_2$ may be a new irrigator alternative. It is able to dissolve organic tissue and also a high antimicrobial activity similar to NaClO. This has also been shown by Dumani et al.,[6] Fidalgo et al.,[17] and Radcliffe et al.,[18] who demonstrated that the antimicrobial activity of NaClO is directly proportional to the concentration used. In both cases, these authors evaluated NaClO at a concentration of 0.5%, 1%, 2.5%, and 5.25% against a biofilm formed by microorganisms mainly *E. faecalis* present in refractory root infections, among which showed susceptibility to this solution. Likewise, Dumani et al.[6] demonstrated that $Ca(ClO)_2$ has a high antimicrobial capacity over *E. faecalis*. Lastly, in a study performed in 2013, Valera et al.[19] demonstrated a high antifungal activity of NaClO in *C. albicans*.

The main limitations of this study were achieving and maintaining both NaClO and $Ca(ClO)_2$ at the different concentrations. Another important limitation lies in the scarce literature found about $Ca(ClO)_2$ as an irrigator during endodontic treatment and its potential use in dentistry. However, this research was carried out to contrast the efficacy of $Ca(ClO)_2$ and NaClO at the same concentration as antimicrobials to obtain new endodontic irrigation options against microorganisms present in chronic apical periodontitis such as *E. faecalis* whose presence ranges from 32% to 70% in the infected root canals, specifically in chronic processes. It is also commonly found in refractory processes and *C. albicans*, whose presence ranges between 1% and 17% in root canal infections, is also found in vast majority in endodontic refractory infections and in 8% primary infections, noting that this species of fungus is the most isolated in these pathologies. It should be noted that to date only few studies compare the antibacterial action of $Ca(ClO)_2$ as an endodontic irrigator against these microorganisms. According to the scientific literature and the results of this study, the authors suggest using $Ca(ClO)_2$ because it has the same antimicrobial effectiveness as NaClO. One of the main reasons for this choice is the low cytotoxicity that causes no harm to oral tissues.

Finally, future research is needed using a biofilm composed of a greater amount of microorganisms present in refractory periapical infections and comparing different concentrations of these endodontic solutions using passive and active irrigation techniques either with *in vitro* methodology or by evaluating long-term results through *in vivo* studies.

Conclusion

Both $Ca(ClO)_2$ and NaClO solutions at 2.5% demonstrate similar antibacterial action, with no statistically significant differences in terms of antimicrobial inhibition in *E. faecalis* and *C. albicans* biofilm.

Acknowledgments

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