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

Carmen Gómez1, Doris Salcedo-Moncada2, Gerardo Ayala3, Romel Watanabe4, Martha Pineda5, Daniel Alvítez-Temoche6, Frank Mayta-Tovalino7

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 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.

The Journal of Contemporary Dental Practice (2020): 10.5005/jp-journals-10024-2771

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 of 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, disinfector, 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.11,12–14 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)2 (at 2.5%) in a biofilm of E. faecalis ATCC 29212™ and C. albicans ATCC 10231™.
**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 α = 0.05 and β = 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 Esulin 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 (λ = 530 nm, OD = 1.258, and λ = 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 of 0.989. However, 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
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*. 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. Therefore, this study seeks to verify that Ca(ClO)$_2$, which is a more stable chemical compound, contains a higher concentration of chlorine at 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.

**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 (mm) | SD (mm) | Min (mm) | Max (mm) | 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

**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 |
| NaClO 2.5%            | Ca(ClO)$_2$ 2.5% | 2.4 - 4.09 | <0.001 |
| NaClO 5.25%           | NaClO 5.25% | -4.14 - 2.45 | <0.001 |
| Ca(ClO)$_2$ 2.5%      | Ca(ClO)$_2$ 2.5% | -0.89 - 0.79 | 0.989 |
| NaClO 2.5%            | NaClO 2.5% | -4.09 - 0.79 | <0.001 |

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*. 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. 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. 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. 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. used the same technique to evaluate the antibacterial activity of chlorhexidine against *E. faecalis*. In addition, Fidalgo et al. 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. that evaluated the antimicrobial activity of various concentrations of NaClO in *Actinomyces naeslundii, Actinomyces israelii, 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. evaluated the antimicrobial activity of different solutions of chemical irrigants 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. 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., 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. 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. 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. 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. and also a high antimicrobial activity similar to NaClO found by Dumani et al. Fidalgo et al. also 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. demonstrated a high antimicrobial capacity of Ca(ClO)₂ over *E. faecalis*. Valera et al. 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., Fidalgo et al., and Radcliffe et al. 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. demonstrated that Ca(ClO)₂ has a high antimicrobial capacity over *E. faecalis*. Lastly, in a study performed in 2013, Valera et al. 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**

We thank the Faculty of Dentistry of the Universidad Nacional Mayor de San Marcos for providing the facilities to carry out the experimental part of this study. We also wish to thank the Universidad Científica del Sur for its constant unconditional support in the preparation and drafting of this scientific manuscript.
REFERENCES

1. Dumani A, Tanrisever D, Sihay D, et al. Efficacy of calcium hypochlorite with and without Er:Cr: yttrium, scandium, gallium, garnet laser activation on Enterococcus faecalis in experimentally infected root canals. Niger J Clin Pract 2019;22(2):215–220.

2. Pramodh NR, Kumar CV, Pradeep MR, et al. Comparative evaluation of tensile strength in die stone incorporated with sodium and calcium hypochlorite as disinfectants: an in vitro study. J Contemp Dent Pract 2017;18(12):1185–1189. DOI: 10.5005/j-p-journals-10024-2197.

3. Soligo LT, Lodi E, Farina AP, et al. Antibacterial efficacy of synthetic and natural-derived novel endodontic irrigant solutions. Braz Dent J 2018;29(5):459–464. DOI: 10.1590/0103-6440201802172.

4. Tyan K, Kang J, Jin K, et al. Evaluation of the antimicrobial efficacy and skin safety of a novel color additive in combination with chlorine disinfectants. Am J Infect Control 2018;46(11):1254–1261. DOI: 10.1016/j.ajic.2018.04.223.

5. Sedigh-Shams M, Gholami A, Abbaszadegan A, et al. Antimicrobial efficacy and cytocompatibility of calcium hypochlorite solution as a root canal irrigant: an in vitro investigation. Iran Endod J 2016;11(3):169–174.

6. Dumani A, Guvenmez HK, Yilmaz S, et al. Antibacterial efficacy of calcium hypochlorite with vibirge sonic irrigation system on Enterococcus faecalis: an in vitro study. Biomed Res Int 2016;2016:8076131. DOI: 10.1155/2016/8076131.

7. Liao CH. Acidified sodium chloride as an alternative to chlorine for elimination of salmonella on alfalfa seeds. J Food Sci 2009;74(4):M159–M164. DOI: 10.1111/j.1750-3841.2009.01125.x.

8. Coaguila-Llerena H, Rodrigues EM, Tanomaru-Filho M, et al. Effects of calcium hypochlorite and ochtenidine hydrochloride on L929 and human periodontal ligament cells. Braz Dent J 2019;30(3):213–219. DOI: 10.1590/0103-6440201902280.

9. Iglesias JE, Pinheiro LS, Weibel DE, et al. Influence of surfactants addition on the properties of calcium hypochlorite solutions. J Appl Oral Sci 2019;27:e20180157. DOI: 10.1590/1678-7757-2018-0157.

10. Cardoso LR, Balduasso FER, Delai D, et al. Effect of EDTA, sodium, and calcium hypochlorite on the inorganic component of root canal dentin: A SEM analysis. Microsc Res Tech 2019;82(2):128–133. DOI: 10.1002/jemt.23151.

11. Dal Bello Y, Mezzalira GI, Jaguszewski LA, et al. Effectiveness of calcium and sodium hypochlorite in association with reciprocating instrumentation on decontamination of root canals infected with Enterococcus faecalis. Aust Endod J 2019;45(1):92–97. DOI: 10.1111/aej.12289.

12. Souza MA, Tumelero Dias C, Zandoná J, et al. Antimicrobial activity of hypochlorite solutions and reciprocating instrumentation associated with photodynamic therapy on root canals infected with Enterococcus faecalis—an in vitro study. Photodiagnosis Photodyn Ther 2018;23:347–352. DOI: 10.1016/j.pdpdt.2018.07.015.

13. Blattes GB, Mestieri LB, Böttcher DE, et al. Cell migration, viability and tissue reaction of calcium hypochlorite based-solutions irrigants: An in vitro and in vivo study. Arch Oral Biol 2017;73:34–39. DOI: 10.1016/j.archoralbio.2016.08.037.

14. Leonardo NG, Carlotto IB, Luisi SB, et al. Calcium hypochlorite solutions: evaluation of surface tension and effect of different storage conditions and time periods over ph and available chlorine content. J Endod 2016;42(4):641–645. DOI: 10.1016/j.joen.2016.01.006.

15. Ayhan H, Sultan N, Çirak M, et al. Antimicrobial effects of various endodontic irrigants on selected microorganisms. Int Endod J 1999;32(2):99–102. DOI: 10.1046/j.1365-2591.1999.00196.x.

16. Lin YH, Mickel AK, Chogle S. Effectiveness of selected materials against Enterococcus faecalis: part 3. The antibacterial effect of calcium hydroxide and chlorhexidine on Enterococcus faecalis. J Endod 2003;29(9):565–566. DOI: 10.1097/00004770-200309000-00006.

17. Fidalgo T, Barcelos R, Portela M, et al. Inhibitory activity of root canal irrigants against Candida albicans, Enterococcus faecalis and Staphylococcus aureus. Braz Oral Res 2010;24(4):406–412. DOI: 10.1590/S1806-83242010000400006.

18. Radcliffe C, Potouridou L, Qureshi R, et al. Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms Actinomyces israelii, A. naeslundii, Candida albicans and Enterococcus faecalis. Int Endod J 2004;37(7):438–446. DOI: 10.1111/j.1365-2591.2004.00752.x.

19. Valera MC, Maekawa LE, Oliveira L, et al. In vitro antimicrobial activity of auxiliary chemical substances and natural extracts on Candida albicans and Enterococcus faecalis in root canals. J Appl Oral Sci 2013;21(2):118–123. DOI: 10.1590/1678-7757201302135.

20. Wang Q, Zhang C, Chu C, et al. Prevalence of Enterococcus faecalis in saliva and filled root canals of teeth associated with apical periodontitis. International Journal of Oral Science 2012;4(1):19–23. DOI: 10.1038/ijos.2012.17.

21. Dutta A, Saunders W. Comparative evaluation of calcium hypochlorite and sodium hypochlorite on soft-tissue dissolution. J Endod 2012;38(10):1395–1398. DOI: 10.1016/j.joen.2012.06.020.

22. Tanomaru-Filho M, Leonardo MR, Silva LA, et al. Inflammatory response to different endodontic irrigating solutions. Int Endod J 2002;35:735–739. DOI: 10.1046/j.1365-2591.2002.00544.x.