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ORIGINAL ARTICLE

Calcium Hypochlorite Solutions – An In Vitro Evaluation of Antimicrobial Action and Pulp Dissolution

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ABSTRACT

Objective: To compare the antimicrobial activity and tissue dissolution capacity of calcium hypochlorite ($Ca(OCl)_2$) solution with sodium hypochlorite (NaOCl) solution at 0.5%, 1.0%, 2.5%, and 5.25% concentrations.

Methods: To determine the inhibition halos produced by the tested substances against Enterococcus faecalis, the agar diffusion method was employed. Additionally, the broth contact method was used to determine the time required for the inhibition of *E. faecalis*. Bovine pulp fragments were used to test the dissolution. Half of the pulps were freely deposited samples in cell culture wells, and the remaining samples were fixed on bovine dentine bases.

Results: For both $Ca(OCl)_2$ and NaOCl solutions, the greatest inhibition zones were observed at 5.25% concentration. However, the most significant inhibition zone was measured with 5.25% $Ca(OCl)_2$ solution (17.38 mm). Hypochlorite solutions at 2.5% and 5.25% concentrations required less time to inhibit *E. faecalis* than those at 0.5% and 1.0% concentrations ($P<0.05$). There was no difference in inhibition times between 2.5% and 5.25% hypochlorite solutions ($P>0.05$). The most effective hypochlorite solution concentrations were 5.25% and 2.5% for dissolving pulp fragments ($P<0.05$). Additionally, suspended pulp fragments were more susceptible to dissolution than fragments attached to dentine blocks ($P<0.05$), except for 0.5% $Ca(OCl)_2$.

Conclusion: $Ca(OCl)_2$ solutions showed antimicrobial activity against *E. faecalis* and can dissolve pulp tissues. Future studies are warranted to examine the suitability of $Ca(OCl)_2$ in the chemico–mechanical preparation of the root canal system.

Keywords: Antimicrobial activity, calcium hypochlorite, dental pulp, endodontics, sodium hypochlorite

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HIGHLIGHTS

- $Ca(OCl)_2$ showed comparable results to NaOCl in-terms of tissue dissolution and antimicrobial activity.
- $Ca(OCl)_2$ can be an alternative irrigant in the chemical–mechanical preparation of the root canal system.
- Pulp dissolution and antibacterial activity of $Ca(OCl)_2$ were improved with higher concentrations.

INTRODUCTION

The primary objectives of instrumentation procedures are the mechanical debridement of the root canal system and the creation of a space for the delivery of antimicrobial substances (1). The uses of chemical solutions enhances the flushing out of debris, increases the tissue dissolving effect, and helps in disinfecting the root canal system (1).

Sodium hypochlorite (NaOCl), which neutralizes and degrades fatty acids and amino acids, is the most commonly used irrigation solution (1). Hypochlorous acid releases chlorine and combines with the amine group of proteins to form chloramines, which modulate microbial cell metabolism. Chlorine is also an active oxidizer, allowing the irreversible inhibition of essential bacterial enzymes (2). The effectiveness of organic tissue dissolution by NaOCl is well known (3-9). However, NaOCl is chemically unstable, and external agents, such as temperature, light, and storage conditions, can influence the availability of chlorine ions and interfere with its effectiveness (1, 10). Therefore, other auxiliary chemical solutions should be investigated.

Calcium hypochlorite ($Ca(OCl)_2$) is used for industrial sterilization as well as for the whitening, treatment, and purification of water because it is easily dissolved in distilled water (11, 12). In con-trast to NaOCl, $Ca(OCl)_2$ is relatively more stable and has a higher available chlorine ion percentage (8, 13).

Dutta and Saunders (8) demonstrated the potential of $Ca(OCl)_2$ solutions to dissolve bovine muscle tissue. Taneja et al. (9) reported that $Ca(OCl)_2$ solutions can dissolve pulp tissue and their efficiency are gradually increased with the time of exposure and concentration. Previous studies evaluated the antimicrobial action of $Ca(OCl)_2$ by infecting extracted teeth with Enterococcus faecalis and observed a reduction in the microbial load by 2.5% $Ca(OCl)_2$, which was as active as 2.5% NaOCl (14, 15).

Blattes et al. (16) compared the cytotoxicity of $Ca(OCl)_2$ and NaOCl both in vitro over 3T3 fibroblasts and *in vivo* through the inflammatory response in rats. $Ca(OCl)_2$ showed favourable results for in vitro cell viability and induced a minimal inflammatory response. Swelling was observed immediately after injections of 2.5% NaOCl in some sites, which occurred transiently. $Ca(OCl)_2$ induced only a low-level inflammatory response. This is a clinically relevant finding because the extrusion of irrigants beyond apical constriction may occur, resulting in direct contact with the periapical tissue.

In endodontics, a lack of consistent information exists regarding the properties of $Ca(OCl)_2$ solutions as irrigants for the root canal system. Thus, the aim of the present study was to evaluate the tissue dissolution and antimicrobial ability of $Ca(OCl)_2$ solutions and to compare them with NaOCl solutions. The null hypotheses were that there are no difference in the antimicrobial activity and pulp dissolution between NaOCl and $Ca(OCl)_2$.

MATERIALS AND METHODS

Preparation of solutions

Solutions were prepared immediately before the experiments, as previously described by Leonardo et al. (13) and Blattes et al. (16). A 12% NaOCl solution (Farmaquímica S.A. Produtos Químicos, Porto Alegre, Brazil) was diluted using sterilized and distilled water to reach the four target concen-trations of 0.5%, 1%, 2.5%, and 5.25%.

$Ca(OCl)_2$ powder (Farmaquímica S.A. Produtos Químicos) with 65% purity was weighed on a precision balance (Sartorius AG, Gottingen, Germany) and mixed with distilled and sterilized water. After total dissolution, solutions were filtered (Filter Paper; Macherey-Nagel, Duren, Germany) twice to remove debris and stored in blinded, randomly numbered bottles.

Agar diffusion method

The agar diffusion method was adapted from EUCAST (17). Briefly, *E. faecalis* ATCC 29212 was subcultured on Muller–Hinton agar plates (MHA; HiMedia Laboratories Limited, Ghatkopar West, Mumbai, India). After growth in a solid medium, isolated colonies were suspended in tubes containing 5 mL of BHI broth culture medium (BHI; HiMedia Labora-tories Limited). The inoculum suspension was prepared by selecting morphologically similar colonies and suspending them in sterile saline (0.85%) to the density of a McFarland 0.5 standard (absorbance: 0.036). MHA (HiMedia Laboratories Limited) was prepared according to the manufacturer's instructions and dispensed in Petri dishes (depth: 4.0 mm). MHA has been inoculated with the *E. faecalis* suspension. Thereafter, a sterile cotton swab was dipped into the inoculum suspension, and excess fluid was removed by turning the swab against the inside of the tube. The inoculum was spread over the entire surface of the agar plate (Flow Chamber; Alba Johnson Equipamentos, Rio de Janeiro, Brazil). Two microgram ampicillin disks (Cefar Diagnostica, São Paulo, Brazil) were used as a positive control. Sterile paper filters (Filter Paper; Macherey-Nagel) with a 5 mm diameter were held with sterilized pliers and soaked with 20 µL of each hypochlorite solution. Ten disks were prepared for each solution. Within 15 min of application, plates were incubated (35°C±1°C for 24 h in air), and inhibition zone diameters were measured twice using a caliper (Mitutoyo Sul Americana Ltda, São Paulo, Brazil). Finally, the average diameter was determined for each sample.

Direct contact method

E. faecalis ATCC 29212 was subcultured on MHA plates (HiMedia Laboratories Limited) and incubated for 18–24 h at 37°C in a microbiological oven. After growth on solid medium, isolated colonies were suspended in tubes containing 5 mL BHI broth medium (HiMedia Laboratories Limited). After mechanical agitation, the suspension was adjusted in a spectrophotometer with 540 nm wavelength at an absorbance value of 0.036 to reach the concentration equivalent to 0.5 of the McFarland scale (1.5×108 bacteria/mL). The tests were conducted in triplicate. In six-well cell culture plates (Corning Inc., Corning, USA), the first well of each row harbored 1 mL of the microbial suspension. From the same row, each following well had 1 mL of BHI broth (HiMedia Laboratories Limited) supplemented with 5% sodium thiosulfate as the inhibitor for the NaOCl and $Ca(OCl)_2$ solutions. The tested solution (1 mL) was inserted in the first well containing BHI with the inoculum. Plates were placed in the orbital shaker, and then 200 µL of the mixture was removed and put in wells containing BHI broth+neutralizer at the following time points: 15 s, 30 s, 1 min, 5 min, and 10 min. Thereafter, plates were incubated (37°C±1°C for 24 h in air). The absence of microbial growth corresponded to the absence of turbidity in the broth. The time required to produce the total microbial growth inhibition was recorded for each plate, and the median time was determined. From each well broth, 25 µL was plated onto MHA, and within 15 min of application, plates were incubated (37°C±1°C for 24 h in air). The absence of *E. faecalis* colonies determined the bactericidal effect of the solution.

Pulp dissolution test

Bovine pulp tissue was used for the experiment. Standard-ized fragments with a 5 mm length and 0.03–0.04 g weight were made. All fragments were weighed on a precision bal-ance (M1203; BEL Engineering, Monza, Italy). Samples were distributed into 18 groups, as shown in Table 1, and saline solution was used as a control.

Dentine blocks were formed from the bovine incisors roots sectioned with a diamond disk (Isomet; Buehler, Lake Bluff, IL, USA). Half of the samples were placed inside the root canal

TABLE 1. Test groups for pulp dissolution regarding the method, the solution, and its concentration (10 samples in each group)

Method	Solution	Concentration	Group
Suspended pulp (SP)	Saline	0.9%	SP-Saline
	NaOCl	0.5%	SP-Na-0.5%
		1%	SP-Na-1.0%
		2.5%	SP-Na-2.5%
		5.25%	SP-Na-5.25%
	$Ca(OCl)_2$	0.5%	SP-Ca-0.5%
		1%	SP-Ca-1.0%
		2.5%	SP-Ca-2.5%
		5.25%	SP-Ca-5.25%
Dentine apparatus (DA)	Saline	0.9%	DA-Saline
	NaOCl	0.5%	DA-Na-0.5%
		1%	DA-Na-1.0%
		2.5%	DA-Na-2.5%
		5.25%	DA-Na-5.25%
	$Ca(OCl)_2$	0.5%	DA-Ca-0.5%
		1%	DA-Ca-1.0%
		2.5%	DA-Ca-2.5%
		5.25%	DA-Ca-5.25%

and fixed with a metallic needle (dentine apparatus), and the other half were individually placed inside the cell culture plate wells (suspended pulp).

Each sample was irrigated with 10 mL of the solution for 10 min. One microliter of the solution was replaced every minute. Samples were removed from wells and dentine blocks and left on sterile gauze for 1 min to remove the excess solution. Each sample was then weighed to determine its final weight and the percentage of weight loss following experimentation.

Statistical analysis

Statistical analysis was conducted using a statistical software package (SPSS v20.0 for Windows; IBM® SPSS Statistics, NY, USA). For all inferential statistics, the significance level was set at 0.05. The Shapiro-Wilk test was employed to determine data distribution. The Kruskal-Wallis test followed by Dunn's multiple-comparison test was employed to (1) compare inhibition halos produced by solutions, (2) compare the time for each irrigant to produce total microbial inhibition growth (in s), and (3) determine the difference in weight reduction between the groups with fragments suspended and those with fragments attached to dentine blocks. Mann-Whitney test was employed to determine changes in weight loss per-centage between suspended pulp fragments and fragments attached to dentine.

RESULTS

The diameter of the inhibition zones is shown in Fig. 1. A statistically significant difference was observed between different concentrations of the same chemical agent ($P<0.05$). However, there was no difference between NaOCl and $Ca(OCl)_2$ solutions with the same concentration ($P>0.05$). In addition, at 2.5% and 5.25% concentrations, $Ca(OCl)_2$ solutions were not different from the positive control solution ($P>0.05$).

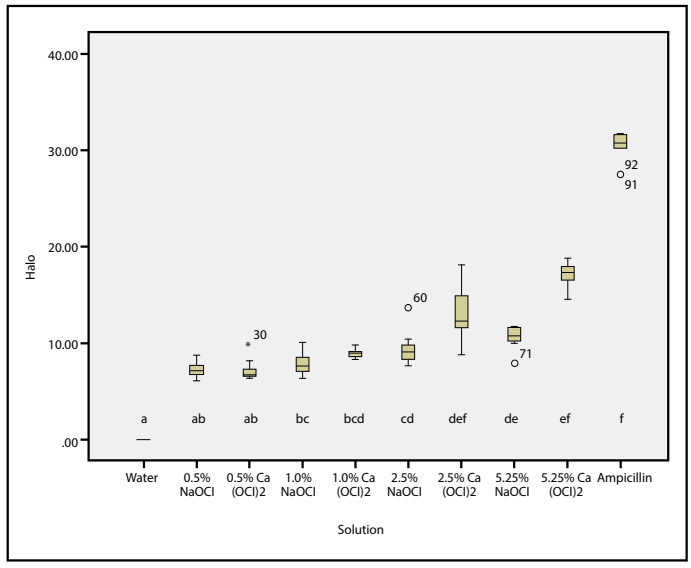

Figure 1. Areas of inhibition in mm of *E. faecalis* in the control and experimental groups.
The same letter indicates statistically significant similarity (Kruskal–Wallis, followed by Dunn's multiple-comparison test)

TABLE 2. The presence of *E. faecalis* growth when in contact of NaOCl or $Ca(OCl)_2$ solutions after different periods of exposure

Solution	Periods of exposure				
	15 sec	30 sec	1 min	5 min	10 min
0.5% NaOCl	+	+	+	+	+
1.0% NaOCl	+	+	-	-	-
2.5% NaOCl	-	-	-	-	-
5.25% NaOCl	-	-	-	-	-
0.5% $Ca(OCl)_2$	+	+	+	+	+
1.0% $Ca(OCl)_2$	+	-	-	-	-
2.5% $Ca(OCl)_2$	-	-	-	-	-
5.25% $Ca(OCl)_2$	-	-	-	-	-

The time required for the growth inhibition of *E. faecalis* produced by each substance is described in Table 2. These data suggest that, for both hypochlorite solutions, higher concentrations yielded shorter times for the growth inhibition of *E. faecalis*. In addition, no statistically significant differ-ence in inhibition times was observed for 2.5% NaOCl, 5.25% NaOCl, 2.5% $Ca(OCl)_2$, and 5.25% $Ca(OCl)_2$ solutions ($P>0.05$). The results of statistical comparisons among the solutions are shown in Table 3.

Table 4 presents the percentage of pulp dissolution after immersion in each solution. Suspended pulp fragments were more susceptible to becoming dissolved than fragments attached to dentine blocks, with the exception of 0.5% $Ca(OCl)_2$. For suspended pulp fragments, the highest concentrations of NaOCl and $Ca(OCl)_2$ (2.5% and 5.25%) were more effective in dissolving tissue than those of saline, 0.5% and 1.0% NaOCl, and 0.5% $Ca(OCl)_2$. When pulp fragments were attached to dentine, 5.25% NaOCl and $Ca(OCl)_2$ solutions were more effective than saline, 0.5% and 1% NaOCl, and 0.5% $Ca(OCl)_2$ in promoting tissue dissolution.

TABLE 3. Statistical comparison (P values) for the time needed for E. faecalis inhibition growth among experimental groups in the broth method

	NaOCl 0.5%	NaOCl 1%	NaOCl 2.5%	NaOCl 5.25%	$Ca(OCl)_2$ 0.5%	$Ca(OCl)_2$ 1%	$Ca(OCl)_2$ 2.5%	$Ca(OCl)_2$ 5.25%
NaOCl 0.5%		0.129	**0.004**	**0.004**	1.0	0.191	**0.045**	**0.014**
NaOCl 1%			0.162	0.162	0.129	0.832	0.627	0.346
NaOCl 2.5%				1.0	**0.004**	0.107	0.362	0.649
NaOCl 5.25%					**0.004**	0.107	0.362	0.649
$Ca(OCl)_2$ 0.5%						0.191	**0.045**	**0.014**
$Ca(OCl)_2$ 1%							0.485	0.248
$Ca(OCl)_2$ 2.5%								0.649
$Ca(OCl)_2$ 5.25%								

*Bold numbers in the columns indicate statistical difference between substances

TABLE 4. Percentage of tissue dissolution expressed as medians and percentiles

Irrigant	Concentration	Suspended Pulp		Dentine apparatus	
Saline	-	4.74	(1.14/7.27)$^{c\ A}$	3.24	(2.035/8.62)$^{d\ A}$
NaOCl Solution	0.5%	23.06	(16.37/26.53)$^{bc\ A}$	13.76	(7.47/21.33)$^{cd\ B}$
	1.0%	27.95	(24.19/34.11)$^{bc\ A}$	9.66	(7.06/20.41)$^{cd\ B}$
	2.5%	84.87	(73.96/90.96)^{aA}	42.38	(37.63/48.25)$^{abc\ B}$
	5.25%	97.28	(94.89/100.0)^{aA}	63.66	(58.36/72.30)$^{a\ B}$
$Ca(OCl)_2$ Solution	0.5%	18.29	(13.89/24.86)$^{bc\ A}$	13.27	(8.88/23.45)$^{cd\ A}$
	1.0%	43.28	(39.44/49.54)$^{ab\ A}$	23.45	(18.82/30.26)$^{bcd\ B}$
	2.5%	86.09	(70.02/91.50)^{aA}	46.09	(41.29/56.37)$^{abc\ B}$
	5.25%	83.33	(72.19/95.17)^{aA}	55.47	(50.78/62.37)$^{ab\ B}$

Different small letters in the same column indicate significant statistical difference among groups (Kruskal–Wallis test, post hoc Dunn, α=5%). Different capital letters in the same row mean significant statistical difference between the groups (Mann–Whitney U test, α=5%)

DISCUSSION

Although the antimicrobial activity and capacity of pulp dissolution of NaOCl has been extensively studied (1, 2, 10), few data are available on properties of $Ca(OCl)_2$ solutions, particularly at concentrations commonly used in endodontics for NaOCl.

The inhibition zones of 2.5% and 5.25% $Ca(OC)_2$ were statistically similar to those of ampicillin solutions. $Ca(OCl)_2$ solutions are extremely alkaline and have more available chlorine content than NaOCl (13). Further studies on the effect of $Ca(OCl)_2$ on microbial biofilms are required. For the agar diffusion and direct contact methods, the most highly concentrated hypochlorite solutions had the most intense and fastest antimicrobial effect. Vianna et al. (18) observed that the lower the concentration, the more limited the antimicrobial effect of NaOCl solutions, which was also observed in our experiment using $Ca(OCl)_2$.

In vitro studies suggested that different NaOCl concentrations may result in similar microbial reduction in infected teeth (19, 20) because of the high volume and constant application of irrigant applied during the root canal preparation. Furthermore, the use of rotary instruments and single-file systems reduces the time to clean and shape the root canals (20, 21), thus minimizing the time that the irrigant remains inside the root canal system. The first possible explanation is that larger volumes of irrigants are used when the canal size is increased, and the second involves the period of time that the irrigant remains in the canal (22).

Hypochlorite solutions at 0.5% need at least 10 min to show their antimicrobial effect. Gomes et al. (23) also reported that 0.5% NaOCl has inhibitory effects over microbial cells after 30 min of exposure. Consistent with previous research (18, 23), the present study found that solutions of greater concentration inhibited bacterial growth after 15 s. While the inhibition zones for 0.5% and 1.0% $Ca(OCl)_2$ solutions were similar, the times required to inhibit *E. faecalis* growth using the direct contact method were 10 min and 30 s, respectively. For NaOCl and $Ca(OCl)_2$ solutions at 2.5% and 5.25% concentrations, similar inhibition zones and times were observed for the tested microorganism. It is important to consider the cytotoxicity of solutions because a solution of low concentration will likely have low toxicity. $Ca(OCl)_2$ solutions showed acceptable outcomes in terms of cell migration, viability, and level of inflammatory response (16). These results suggest that $Ca(OCl)_2$ can be considered as an irrigant solution in endodontic procedures (16).

Consistent with previous research (4, 5, 6), high NaOCl concentration solutions had superior tissue dissolution ability than low concentration solutions. Few studies evaluated the tissue dissolution of $Ca(OCl)_2$ (8, 9), and the same behaviour

was observed for $Ca(OCl)_2$ solutions tested in the present study. In the present study, the maximum dissolution time adapted was 10 min, and there was no significant difference between solutions of the same concentration. Dutta and Saunders (8) reported similar tissue dissolving effects for NaOCl and $Ca(OCl)_2$ solutions only after 5 min of exposure in bovine muscle fragments. Further, Clarkson et al. (24) evaluated the tissuedissolving ability of two concentrations of NaOCl on porcine incisor pulps, and found that greater concentrations provide more rapid tissue dissolution. The poor performance of Milton in this study would suggest that, in a clinical setting, less dissolution of the pulp can be expected using this material as an irrigant. Mohammadi (10) indicated that NaOCl is a strong proteolytic agent and exhibits the best tissue-dissolving ability as an endodontic irrigant. Hand et al. (25) examined the dissolution ability of diluted at various concentrations (0.5%, 1.0%, 2.5%, and 5.25%), over necrotic tissue compared with that of saline, distilled water, and hydrogen peroxide. Results showed that 5.25% NaOCl is significantly more effective as a necrotic tissue solvent than any other solution tested. They also reported that the increase in the dissolution ability of necrotic tissues was proportional to the increase in NaOCl concentrations.

The effect of auxiliary solutions within the root canal can be modulated by inorganic and organic tissue components, and there is a strong relationship between the surface area in contact with the solution and tissue dissolution (26). Slutzky-Goldberg et al. (7) reported that the presence of dentine reduces the dissolving capacity of NaOCl and $Ca(OCl)_2$. Although dentine is known to reduce the antimicrobial effect of NaOCl (7), no mechanism has been described to elucidate its influence over the tissue dissolution ability of hypochlorite solutions.

Protocols to evaluate tissue dissolution in the literature are diverse and depend on tissue sources, time of exposure, substance renewal, and substance agitation (8, 27). The present findings are in agreement with previous in vitro studies and reinforce that NaOCl has a marked tissue dissolving ability (1, 27). Disk diffusion is one of the most widely used methods in routine clinical microbiology laboratories because of its reproducibility (17). The characteristics of the tested solutions, such as solution diffusion ability through the agar, may introduce bias in the experiment and may not represent their actual antimicrobial potential (28). In the present study, NaOCl and $Ca(OCl)_2$ solutions were diluted in water to overcome this limitation. The direct contact method ensures direct exposure of the microorganism to the chemical substance and allows the determination of time required to inhibit microbial growth. However, less solution is inserted in the root canal with this method, and thus, it may not represent *in vivo* conditions. Additionally, the microbial distribution in biofilms and pellets may modulate the antimicrobial effect of the solutions (29).

In the present study, the antimicrobial effect of $Ca(OCl)_2$ solutions was assessed using in vitro tests against *E. faecalis*. Since *E. faecalis* is one of the most resistant species in the oral cavity which invades and remains viable inside dentinal tubules, it has been selected as the microorganism of choice to test chemical agents that might be employed during root canal treatment (30). In the present study, $Ca(OCl)_2$ solutions showed antimicrobial activity against *E. faecalis* and were successful in dissolving pulp tissues.

CONCLUSION

The present study demonstrated that pulp dissolution and antimicrobial effects of $Ca(OCl)_2$ solutions across various concentrations were similar to those of NaOCl solutions. Additional clinical studies are needed to evaluate the use of this substance as an alternative irrigant in chemico-mechanical preparation of the root canal.

Disclosures

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethics Committee Approval: This study was approved by the Institutional Review Board of the Federal University of Rio Grande do Sul (numbers CAAE 26676514.7.0000.5347; 08758212.1.0000.5347; 36800214.0.0000.5347).

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