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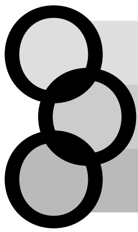

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In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite

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The aim of this study was to investigate in vitro the antimicrobial activity of 0.2%, 1%, and 2% chlorhexidine gluconate (CHX gel and CHX liquid), against endodontic pathogens and compare the results with the ones achieved by 0.5%, 1%, 2.5%, 4%, and 5.25% sodium hypochlorite (NaOCl). A broth dilution test was performed, and the timing for irrigants to kill microbial cells was recorded and statistically analyzed. Both 2.0% gel and liquid formulations eliminated *Staphylococcus aureus* and *Candida albicans* in 15 seconds, whereas the gel formulation killed *Enterococcus faecalis* in 1 minute. All tested irrigants eliminated *Porphyromonas endodontalis, Porphyromonas gingivalis,* and *Prevotella intermedia* in 15 seconds. The timing required for 1.0% and 2.0% CHX liquid to eliminate all microorganisms was the same required for 5.25% NaOCl. The antimicrobial action is related to type, concentration, and presentation form of the irrigants as well as the microbial susceptibility. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;97:79-84)**

One of the primary objectives of the endodontic therapy is the microbial reduction, which in turn promotes the normal healing process of the periodontal tissues.[1] Anaerobic bacteria, especially black-pigmented gram-negatives, have been linked to the signs and symptoms of pulpless teeth. However, facultative microorganisms such as *Enterococcus faecalis*, *Staphylococcus aureus*, and even *Candida albicans* are considered by many to be the most resistant species in the oral cavity, and one possible cause of root canal treatment failure.[2]

It is important not only to decrease the number of microorganisms[3] but also to reduce the debris, which is higher in canals prepared without irrigating solutions.[4] An endodontic irrigant should ideally exhibit powerful antimicrobial activity, dissolve organic tissue remnants, disinfect the root canal space, flush out debris from the instrumented root canals, provide lubrication, and have no cytotoxic effects on the periradicular tissues, among other properties.[5]

Sodium hypochlorite (NaOCl), the most used endodontic irrigant nowadays[6,7] has many of these properties,[6,8-13] but it has a cytotoxic effect when injected into the periapical tissues,[6,14-16] a foul smell and taste, a tendency to bleach clothes, and corrosive potential.[17,18] It is also known to produce allergic reactions.[19] Therefore, an equally effective but safer irrigant is desirable.[20]

Chlorhexidine (CHX) is widely used as a mouth rinse in the prevention and treatment of periodontal diseases and dental caries,[21] and has been suggested as an irrigating solution[7] or intracanal dressing[22,23] in endodontic therapy. The antimicrobial property of both irrigating solutions have been tested against *E faeca-*

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lis.[24] However, their antimicrobial activity against microorganisms such *S aureus* and *C albicans*, which are also considered to be resistant to endodontic therapy, has not been investigated yet. Furthermore, the time needed to eliminate microorganisms such as *Porphyromonas gingivalis*, *P endodontalis*, and *Prevotella intermedia*, which are related to endodontic symptomatology, has not been established.

Therefore, the purpose of this study was to investigate in vitro the antimicrobial activity of 0.2%, 1%, and 2% chlorhexidine gluconate in gel and liquid formulations against several endodontic pathogens and to compare the results with the ones achieved with 0.5%, 1%, 2.5%, 4%, and 5.25% NaOCl.

MATERIAL AND METHODS

The methodology used was adapted from Gomes et al.[24]

The intracanal irrigants tested were 2 presentation forms of CHX gluconate (gel and liquid) in 3 concentrations (0.2%, 1.0%, 2.0%), and NaOCl (0.5%, 1%, 2.5%, 4%, 5.25%). The same manufacturer prepared all irrigants (Proderma Farmácia de Manipulação Ltda, Piracicaba, SP, Brazil). The manufacturer diluted NaOCl and CHX liquid at different concentrations in sterile water without preservatives. The solutions were prepared 24 hours before the beginning of the experiment, always in small portions. CHX gel consisted of gel base (1% natrosol) and CHX gluconate. Sterile saline (0.89%) and natrosol (1%) were used as controls.

The species of microorganisms used in this experiment were (1) *E faecalis* ATCC 29212, (2) *C albicans* NTCC 3736, (3) *S aureus* ATCC 25923 (all of them grown on 5% sheep blood–Brain Heart Infusion [BHI] agar plates [Lab M, Bury, United Kingdom] for 48 hours at 37°C); and (4) *P gingivalis*, (5) *P endodontalis*, and (6) *P intermedia* (all of which were isolated from the root canal infections and identified by using conventional biochemical tests). For this research, the 3 strict anaerobic microorganisms were previously subcultured on 5% sheep blood–Fastidious Anaerobe Agar (FAA) plates (Lab M) for 48 hours in anaerobic gaseous conditions (10% CO_2, 10%H_2, and 80% N_2) at 37°C.

Aerobe strains (*C albicans* and *S aureus*) and the facultative strain (*E faecalis*) were individually inoculated into tubes containing 5 mL BHI sterile suspension. Such suspension was adjusted spectrophotometrically, according to Koo et al, who used the optimal density at 800 nm (optic density $[OD]_{800}$) to match the turbidity of 1.5×10^8 colony forming unit (CFU) mL^{-1} (equivalent to 0.5 McFarland standard). Strict anaerobic microorganisms were individually inoculated into tubes containing 5mL of Fastidious Anaerobe Broth (FAB, Lab M) sterile suspension, which were suspended spectrophotometrically at 800 nm (OD_{800}) to match the turbidity of 3.0×10^8 cfu mL^{-1} (equivalent to 1 McFarland standard).[25]

Six wells were used for each time period, irrigant, and microorganism, respectively. Overall, 5616 wells were used: 4752 for all the test irrigants and 864 for the control group.

One mL of each tested irrigant, as well as sterile saline or natrosol (control groups), was placed in 24-well cell culture plates (ref. no. 3524, vol 3.2 mL; Corning, NY). Two mL of the microbial suspension were added to the irrigants and to the control group solutions.

The well cell culture plates were placed onto an upside down 250-mL stainless steel griffin beaker (BK 1122; MGL Scientific, Elko, Nev) inside an ultrasonic cleaner (Bransonic Ultrasonics Corp, Dunbury, Conn) that had been previously filled with 1400 mL of distilled water up to the operating level. These plates were then ultrasonically mixed for 10 seconds and left to stand for different periods of time: for 15, 30, and 45 seconds; for 1, 3, 5, 10, 15, 20, and 30 minutes; and for 1 and 2 hours.

After each period of time, 1 mL from each well was transferred to tubes containing 3 mL of fresh broth media (BHI for aerobes and facultative strains; FAB for strict anaerobic microorganisms), which contained neutralizers in order to prevent continued action of the irrigants. The neutralizer for CHX was Tween 80 plus 0.07% lecithin, while 0.6% sodium thiosulfate was used for NaOCl .[24] All tubes were left at 37°C for 7 days in appropriate gaseous conditions for microbial growth. After this period, 10 μL of each tube was inoculated on agar plates and left at 37°C for 24 to 48 days in appropriate gaseous condition to investigate all possible bacterial growth. 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, API C AUX, API 20 Staph (BioMérieux SA, Marcy-l'Etoile, France), and the RapID ANA II System (Remel Inc, Lenexa, Kan). The time needed for each irrigant to produce total microbial inhibition growth was recorded, transformed into seconds, and analyzed statistically by the Kruskal-Wallis test, with significance level set at $P < .05$.

RESULTS

Samples adherence and normality were tested by using the GMC program (USP, Ribeirão Preto, SP, Brazil), demonstrating that the data were nonparametric. Then the samples were compared by using Kruskal-Wallis test (BioEstat program, CNpQ, 2000; Brasília, DF, Brazil), with significance level at $P < .05$. The data were retransformed into seconds, minutes, and hours to make comparisons of results easier.

Table I shows the contact time required for CHX

Table I. Contact time required for chlorhexidine gluconate at various concentrations, in liquid and gel formulations, to produce negative cultures (ie, 100% inhibition of growth) for the tested microorganisms

Microorganisms	*S aureus*	*E faecalis*	*C albicans*	*P endodontalis*	*P gingivalis*	*P intermedia*	*Maximum time for each irrigant to produce negative cultures*
0.2% chlorhexidine gel	10 min (a, A)*	2 h (a, A)	10 min (a, A)	15 s (a, B)	15 s (a, B)	15 s (a, B)	2 h
1.0% chlorhexidine gel	30 s (a, A)	15 min (a, b, A)	15 s (b, B)	15 s (a, B)	15 s (a, B)	15 s (a, B)	15 min
2.0% chlorhexidine gel	15 s (b, B)	1 min (b, c, A)	15 s (b, B)	15 s (a, B)	15 s (a, B)	15 s (a, B)	1 min
0.2% chlorhexidine liquid	15 s (b, B)	30 s (c, d, A)	15 s (b, B)	15 s (a, B)	15 s (a, B)	15 s (a, B)	30 s
1.0% chlorhexidine liquid	15 s (b, A)	15 s (d, A)	15 s (b, B)	15 s (a, A)	15 s (a, A)	15 s (a, A)	15 s
2.0% chlorhexidine liquid	15 s (b, A)	15 s (d, A)	15 s (b, B)	15 s (a, A)	15 s (a, A)	15 s (a, A)	15 s

*Different letters (from *a* to *d,* and *A* and *B*) indicate significant difference (Kruskal-Wallis $P < .05$). Capital letters indicate differences in horizontal direction. Lower-case letters indicate differences in vertical direction.

Table II. Contact time required for sodium hypochlorite at various concentrations to produce negative cultures (ie, 100% inhibition of growth) for the tested microorganisms

Microorganisms	*S aureus*	*E faecalis*	*C albicans*	*P endodontalis*	*P gingivalis*	*P intermedia*	*Maximum time for each irrigant to produce negative cultures*
0.5% sodium hypochlorite	30 min (a, A)*	30 min (a, A)	30 min (a, A)	15 s (a, B)	15s (a, B)	15 s (a, B)	30 min
1.0% sodium hypochlorite	20 min (a, b, A)	20 min (a, b, A)	20 min (a, b, A)	15 s (a, B)	15s (a, B)	15 s (a, B)	20 min
2.5% sodium hypochlorite	10 min (b, c, A)	10 min (b, c, A)	10 min (b, c, A)	15 s (a, B)	15s (a, B)	15 s (a, B)	10 min
4.0% sodium hypochlorite	5 min (c, d, A)	5 min (c, d, A)	5 min (c, d, A)	15 s (a, B)	15s (a, B)	15 s (a, B)	5 min
5.25% sodium hypochlorite	15 s (d, A)	15 s (d, A)	15 s (d, A)	15 s (a, A)	15 s (a, A)	15 s (a, A)	15 s

*Different letters (from *a* to *d,* and *A* and *B*) indicate significant difference (Kruskal-Wallis $P < .05$). Capital letters indicate differences in horizontal direction. Lower-case letters indicate differences in vertical direction.

gluconate gel and liquid concentrations to produce negative cultures for all tested microorganisms. CHX liquid in all concentrations and the 2.0% CHX gel eliminated the facultative microorganism (*E faecalis*) and the aerobic microorganisms (*S aureus* and *C albicans*) in 1 minute or less. Only 15 seconds were needed for all tested CHX solutions to kill the gram-negative strictly-anaerobic microorganisms (*P gingivalis*, *P endodontalis*, and *P intermedia*).

CHX liquid, in all concentrations, killed all microorganisms in 30 seconds or less, whereas CHX gel took from 22 seconds (2% CHX gel) to 2 hours (0.2% CHX gel).

Table II shows the contact time required for sodium hypochlorite in different concentrations to produce negative cultures for all tested microorganisms. The 0.5% and 1.0% NaOCl took a longer length of time than the other tested concentrations (30 minutes and 20 minutes, respectively) to eliminate the facultative and aerobic microorganisms. The 5.25% NaOCl showed the best performance, killing the same microorganisms in 15 seconds. The same timing, 15 seconds, was needed for all NaOCl solutions to kill the gram-negative strictly-anaerobic microorganisms.

The saline and natrosol control groups did not inhibit growth of any of the microorganisms tested.

The in vitro antimicrobial effects of the most effective irrigants were ranked from strongest to weakest as follows: 5.25% NaOCl, 2% CHX liquid, and 1% CHX liquid (all 3 in the same level), followed by 0.2% CHX liquid and 2% CHX gel. All of them took 1 minute or less to eliminate all tested microorganisms. The least effective irrigant was 0.2% CHX gel, with a maximum time of 2 hours to eliminate the microbial cells (Fig 1).

The microbial resistance to all solutions tested can be ranked from the strongest to the weakest as follows: *E faecalis* was the strongest, with *S aureus*, and *C albicans* next (at the same level), followed by *P endodontalis*, *P gingivalis*, and *P intermedia* (all 3 at the same level) (Fig 2).

DISCUSSION

NaOCl solution is, to date, the most commonly employed root canal irrigant, but no general agreement exists regarding its optimal concentration, which ranges from 0.5% to 5.25%.[4] Its antimicrobial activity is proportional to the drug concentration, as shown in the present work. To obtain acceptable cytotoxic levels,

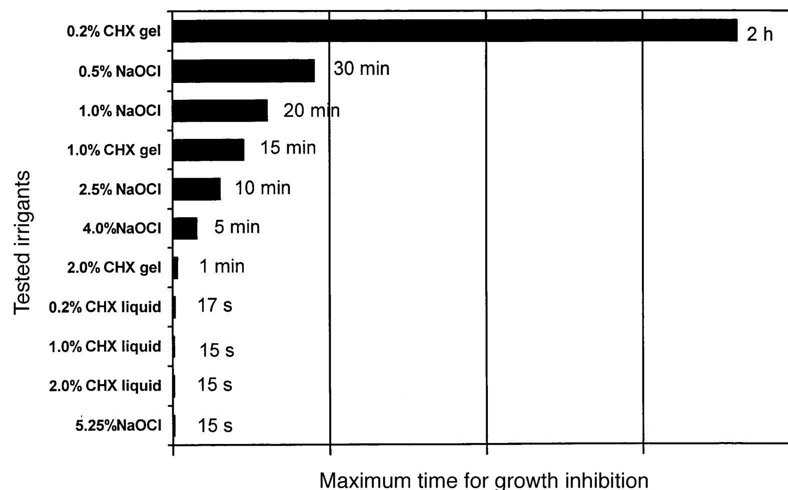

Fig 1. Maximum time (in seconds, minutes, or hours) spent by each tested irrigant to kill all microorganisms.

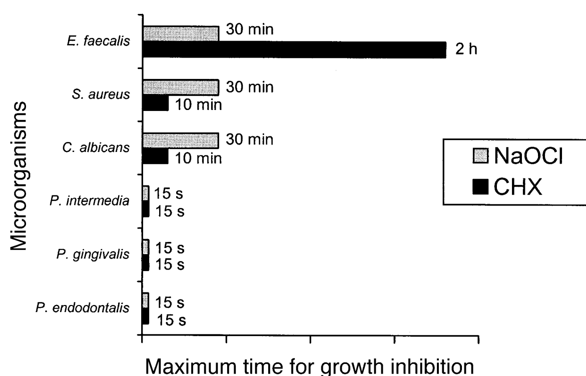

Fig 2. Resistance of each of the tested microorganisms to elimination. Maximum time (in seconds, minutes, or hours) spent by all tested irrigants to kill each microorganism.

0.5% NaOCl is recommended,[10] but this concentration needs at least 30 minutes to inhibit the growth of facultative microorganisms. On the other hand, our study found that 5.25% NaOCl kills microorganisms in seconds, agreeing with the findings of Senia et al[8] and Gomes et al.[24]

It seems that the antimicrobial activity of NaOCl depends on the concentration of undissociated hypochlorous acid (HClO) in solution. HClO exerts its germicidal effect by an oxidative action on sulfydryl groups of bacterial enzymes. As essential enzymes are inhibited, important metabolic reactions are disrupted, resulting in the death of the bacterial cells.[18]

CHX has been recommended as an alternative irrigating solution to NaOCl, especially in cases of open apex[11] (owing to its biocompatibility) or in cases of related allergy to bleaching solutions. The antimicrobial effect of CHX is related to the cationic molecule binding to negatively charged bacterial cell walls, thereby altering bacterial osmotic equilibrium.[26]

Ohara et al[27] determined the antibacterial effects of various endodontic irrigants against selected anaerobic bacteria, showing that diluting NaOCl rapidly takes away its effect, while CHX in a liquid formulation is effective even in very low concentrations against a number of bacteria. Other authors[10,18,28] who evaluated the antimicrobial property of NaOCl showed that it rapidly reduces the bacterial counts, especially in higher concentrations, a finding that was duplicated in our study. In the present work the 2.0% CHX gluconate (in both presentation forms) and 5.25% NaOCl had similar antimicrobial performance against all tested microorganisms, agreeing with the studies of Jeansonne and White[11] and Gomes et al.[24] However, the 2.0% CHX is a less toxic and malodorous agent than 0.5% NaOCl.[16]

Although in the present research the residual effect of the CHX was neutralized by the addition of Tween 80 plus 0.07% lecithin in the media, clinically CHX's substantivity seems to be another advantage over NaOCl, sustaining the antimicrobial activity over a period of 48 hours[29] or 72 h[16] after treatment.

A disadvantage of CHX is that it does not dissolve organic tissues. However, CHX gel, a viscous formulation that makes instrumentation easier, thus increasing the mechanical removal of the organic tissues, compensates for its inability to dissolve them.[30] Furthermore, it also decreases the smear layer formation, which does not happen with the liquid formulation.[31] Therefore, even though our study demonstrates that the antimicrobial activity of CHX liquid is equal or superior to CHX gel when the direct contact method is used, the results of Ferraz et al,[31] Gomes et al,[24] and Vivac-

qua-Gomes et al[30] indicate that the gel formulation has more clinical advantages.

In the present investigation, CHX liquid (as well as NaOCl) mixed very well with the bacterial suspension, immediately exerting its antimicrobial action, whereas the gel formulation, which is more difficult to mix, prevented direct contact between bacterial cells and CHX, thus requiring a longer time to act against the microrganisms. Other studies[24] found that CHX in gel formulation produced larger inhibition zones than the liquid formulations (including NaOCl), perhaps because the gel kept the active agent in contact with the inoculated media for a longer time

It has been reported that CHX does not inactivate lipopolysaccharide (LPS), which is a structural component of the Gram-negative bacteria's outer cell envelope and can be either secreted in vesicles by growing organisms or released after the organism's death.[16,29] However, neither does NaOCl.[32] Despite this fact, if the teeth are symptom-free and the canals dried, it is possible to perform a successful treatment in a single endodontic visit.[33] With the reduced working time made possible by the advent of rotary techniques for root canal preparation,[28] the irrigant of choice should be one that exerts its antimicrobial activity quickly against the majority of microorganisms found in the root canal and dentinal tubules. For this reason, it is important to know both the time required by an irrigant to kill microorganisms and the irrigant's residual antimicrobial activity after canal preparation.

This study demonstrates that NaOCl and liquid and gel chlorhexidine gluconate at all tested concentrations can be used as irrigating solutions owing to their antimicrobial properties, except for the 0.2% CHX gel, whose time (2 hours) to eliminate *E faecalis* exceeds the average time usually spent (1 hour) for chemomechanical preparation.

All tested irrigants showed antimicrobial activity according to the irrigant type, concentration, and presentation form, as well as microbial susceptibility.

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