

Control of Microorganisms *In Vitro* by Endodontic Irrigants

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The aim of this study was to determine the minimum inhibitory concentration (MIC) and antimicrobial effectiveness by the direct exposure test of 4 endodontic irrigants [1% sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX), 1% calcium hydroxide (Ca(OH)₂, prepared with 1 g of Ca(OH)₂ and 100 mL of sterile distilled water), a solution of Ca(OH)₂ + detergent (HCT20)] for *S. aureus*, *E. faecalis*, *P. aeruginosa*, *B. subtilis*, *C. albicans* and a mixed culture. Microbial growth was analyzed by two methods: turbidity of the culture medium that was confirmed by Gram stain and subculture in a specific nutrient broth. In the dilution test, NaOCl solution showed MIC equal to 0.1% for *S. aureus*, *E. faecalis*, *P. aeruginosa* and *C. albicans* and equal to 1% for *B. subtilis* and the mixed culture. CHX (2%) presented MIC equal to 0.000002% for *S. aureus*, 0.02% for *E. faecalis*, *B. subtilis*, *C. albicans* and the mixed culture and 0.002% for *P. aeruginosa*. Ca(OH)₂ solution (1%) showed MIC greater than 1% for all the microorganisms except *P. aeruginosa* for which it was equal to 1%. Calcium hydroxide solution + detergent showed MIC equal to 4.5 mL for *S. aureus*, *P. aeruginosa*, *B. subtilis*, *C. albicans* and the mixed culture and greater than 4.5 mL for *E. faecalis*. In the direct exposure test, NaOCl had better antimicrobial effectiveness for all microorganisms at all times. CHX (2%) was effective for *S. aureus*, *E. faecalis* and *C. albicans* at all times, and ineffective for *P. aeruginosa*, *B. subtilis* and the mixed culture. The other solutions showed the worst results.

Key Words: endodontic irrigants, sodium hypochlorite, chlorhexidine digluconate, intracanal dressing.

INTRODUCTION

The process observed between endodontic infection and consequential host response has led to several therapeutic trends. The sanitization process of an infected root canal is the objective of endodontic therapy. Chemical and mechanical cleaning and shaping significantly reduce the number of microorganisms, but do not eliminate them (1).

Although, various irrigants have been indicated for the sanitization process in apical periodontitis and many of them have shown varied degrees of antimicrobial effectiveness (1-5), the choice of the ideal irrigant solution and its concentration is difficult. In a clinical study, Byström and Sundqvist (6) showed no antibacterial difference between 0.5% and 5% NaOCl solutions.

Heling and Chandler (7) analyzed the antimicrobial effect of irrigant combinations within dentinal tubules and concluded that 0.12% CHX and 1% NaOCl were similarly effective. Ohara et al. (2) compared six irrigants on selected anaerobic bacteria. 0.2% CHX was the most effective; 3% hydrogen peroxide, 5.25% NaOCl and 17% REDTA were less effective while the saturated solution of Ca(OH)₂ and saline proved to be totally ineffective. Ayhan et al. (4) reported the antimicrobial effects of various endodontic irrigants on selected microorganisms and observed that 5.25% NaOCl was superior and the reduced concentration of 0.5% resulted in significantly decreased antimicrobial effectiveness.

Considering the different research results, an important factor to analyze is the MIC of endodontic

irrigant solutions capable of controlling microorganisms and, then, to compare antimicrobial effectiveness. Thus, the purpose of this study was to compare *in vitro* the antimicrobial effectiveness of 1% NaOCl, 2% CHX, 1% Ca(OH)₂ solution and Ca(OH)₂ solution with a detergent and determine their MIC using *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans* and a mixed culture of these microorganisms as biological indicators.

MATERIAL AND METHODS

Test Organisms

Four reference bacterial strains – *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), and *Bacillus subtilis* (ATCC 6633), obtained from the American Type Culture Collection, and one strain of yeast *Candida albicans* (ICB/USP – 562), isolated at

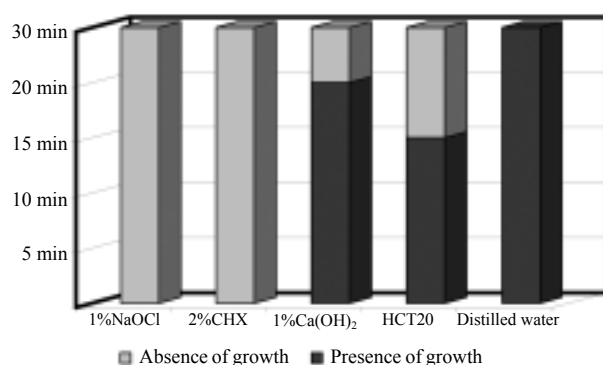


Figure 1. Antimicrobial effect of irrigant solutions for *S. aureus*.

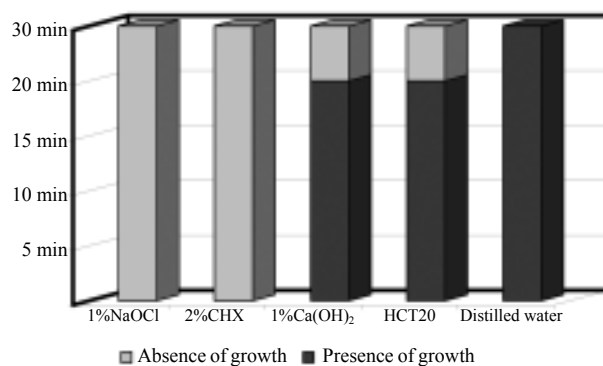


Figure 2. Antimicrobial effect of irrigant solutions for *E. faecalis*.

the Laboratory of Biological Science, University of São Paulo, Brazil, were included in this study.

The strains were inoculated in 7 mL of brain heart infusion (BHI; Difco Laboratories, Detroit, MI, USA) and incubated at 37°C for 24 h. To prepare the experimental suspensions, the biological indicators were cultivated on brain heart infusion agar (BHIA; Difco Laboratories), following the same incubation conditions. Microbial cells were resuspended in saline to give a final concentration of about 3×10^8 cells/mL, similar to that of tube No. 1 of the MacFarland scale. One mL of each of these pure suspensions was used to obtain a mixture of the test microorganisms.

Determining Minimum Inhibitory Concentration

The following irrigant solutions were used: 1% NaOCl (Halex Istar, Goiânia, GO, Brazil); 2% chlorhexidine digluconate (F.G.M., Joinville, SC, Brazil); 1% Ca(OH)₂ solution (prepared with 1 g of calcium hydroxide (Quimis, Maillinkoet Inc., St. Louis, MO, USA) and 100 mL of sterile distilled water); a solution of Ca(OH)₂ + detergent – lauryl diethyleneglycol ether sodium sulfate (Tergentol; Searle of Brazil, Rio de Janeiro, RJ, Brazil) (80 mL of 0.2% calcium hydroxide solution + 20 mL of Tergentol).

To determine the MIC of the irrigant solutions, ten-fold dilutions were made in 4.5 mL of BHI. After the dilution of the irrigant solutions, an inoculum of 0.1 mL obtained from the experimental suspensions was added, followed by incubation at 37°C for 48 h. Microbial growth was analyzed by two methods - turbidity of the culture medium and subculture in a specific nutrient broth - because some of the tested irrigants caused

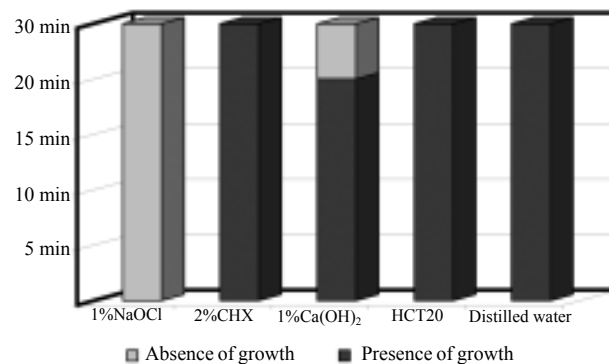


Figure 3. Antimicrobial effect of irrigant solutions for *P. aeruginosa*.

culture medium changes. In this particular method, an inoculum of 0.1 mL obtained from the BHI + irrigant + microorganisms was transferred to 7 mL of Lethen broth (LB; Difco Laboratories, Detroit, MI, USA), a medium containing or added with neutralizers: lecithin, Tween 80 and sodium thiosulfate (Art Laboratories, Campinas, SP, Brazil) in appropriate concentrations, and subsequently incubated under adequate atmosphere. After 48 h, 0.1 mL of these culture was transferred to 7 mL of BHI followed by identical incubation conditions. Finally, microbial growth was macroscopically evaluated by culture medium turbidity and when necessary microscopically confirmed by Gram stain. All assays were carried out in duplicate under aseptic techniques.

Testing Antimicrobial Action after Different Exposure Times

Three hundred and sixty size 50 sterile absorbent paper points (Tanari, Tanariman Indústria, Ltda., Manacaru, AM, Brazil) were immersed in the experi-

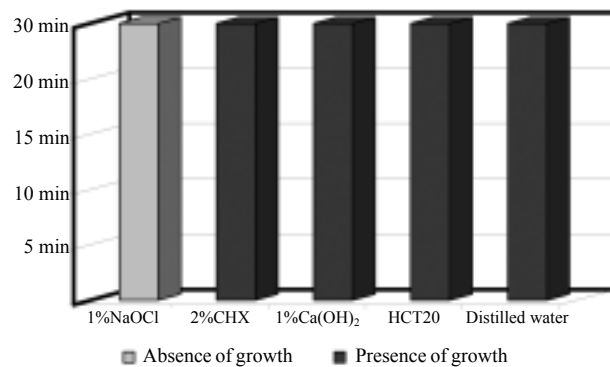


Figure 4. Antimicrobial effect of irrigant solutions for *B. subtilis*.

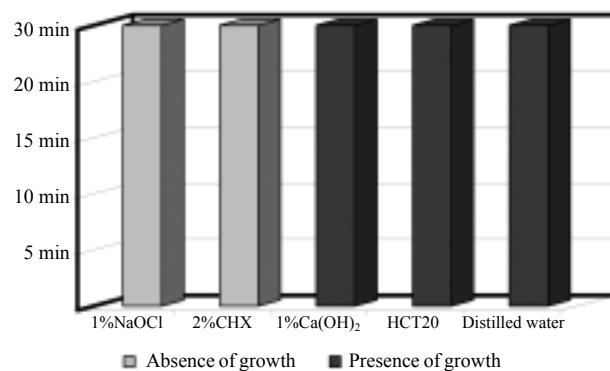


Figure 5. Antimicrobial effect of irrigant solutions for *C. albicans*.

mental microbial suspension for 5 min. The paper points were then placed on Petri dishes and covered with 10 mL of the irrigant solutions. Two controls were used for testing LB sterility and for checking microorganism viability throughout the experiment.

At intervals of 5, 10, 15, 20 and 30 min, 72 absorbent paper points were removed from contact with the irrigant solutions and individually transported and immersed in 7 mL of LB followed by incubation at 37°C for 48 h. Microbial growth was evaluated by the previously described methods. After evaluating LB changes, an inoculum of 0.1 mL was obtained from medium and transferred to 7 mL of BHI, and incubated under identical conditions. Microbial growth was verified by turbidity of the culture medium and in some cases by Gram stain. All assays were carried out in duplicate under aseptic conditions.

RESULTS

In the dilution test, NaOCl solution showed MIC equal to 0.1% for *S. aureus*, *E. faecalis*, *P. aeruginosa* and *C. albicans* and equal to 1% for *B. subtilis* and the mixed culture. CHX (2%) presented MIC equal to 0.000002% for *S. aureus*, 0.02% for *E. faecalis*, *B. subtilis*, *C. albicans* and the mixed culture and 0.002% for *P. aeruginosa*. Ca(OH)₂ solution (1%) showed MIC greater than 1% for all the microorganisms except *P. aeruginosa* for which it was equal to 1%. Ca(OH)₂ solution + detergent showed MIC equal to 4.5 mL for *S. aureus*, *P. aeruginosa*, *B. subtilis*, *C. albicans* and the mixed culture and greater than 4.5 mL for *E. faecalis*.

Using the direct exposure test, NaOCl had the best antimicrobial effectiveness for all microorganisms

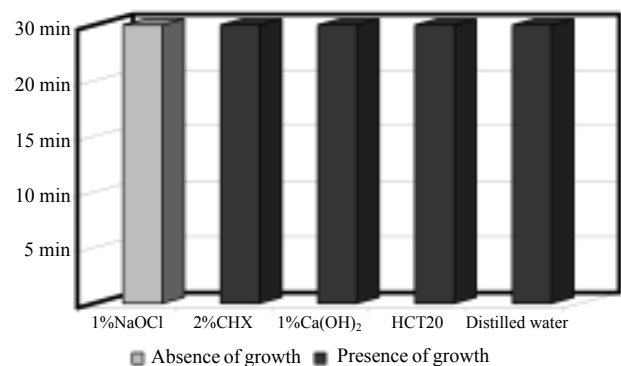


Figure 6. Antimicrobial effect of irrigant solutions for the mixed culture of microorganisms.

at all times. CHX was effective for *S. aureus*, *E. faecalis* and *C. albicans* at all times, and ineffective for *P. aeruginosa*, *B. subtilis* and the mixed culture of the microorganisms. The irrigant solutions containing calcium hydroxide showed the worst results. The results are shown in Figures 1-6.

DISCUSSION

Various results have been reported for irrigant antimicrobial effectiveness. Similar results have been reported for NaOCl and CHX (5, 7-8); however, other research has shown that NaOCl has better antimicrobial activity than CHX (4, 9-10), or, conversely, that CHX has better antimicrobial activity than NaOCl (2-3). These differences may have been caused by different experimental methods, concentration, type of irrigant solution, or the period of analysis.

Estrela et al. (11) discussed that the physico-chemical characteristics of NaOCl are essential for the explanation of its mechanism of action. Saponification, neutralization of amino acids and chloramination reactions that occur in the presence of microorganisms and organic tissue aid the antimicrobial and tissue dissolution processes. Antimicrobial activity is related to bacterial essential enzymatic sites promoting irreversible inactivation by hydroxyl ions and chloramination reaction. Sodium hypochlorite promotes biosynthetic alterations in cell metabolism, phospholipid destruction, and formation of chloramines that interfere in cell metabolism and oxidant action. The organic dissolution action can be observed in the saponification reaction when sodium hypochlorite destroys lipids and fatty acids resulting in soap and glycerol.

The MIC of 1% NaOCl was 0.1% for *S. aureus*, *E. faecalis*, *P. aeruginosa* and *C. albicans* and 1% for *B. subtilis* and the mixed culture. Thus, the use of the lower concentration, 1% NaOCl solution, can be recommended because of its antimicrobial activity, pulp tissue dissolution and acceptable tissue cytotoxicity. At higher concentrations, NaOCl is more aggressive while at lower concentrations (0.5% to 1%), it is biocompatible (12,13). For a substance to meet acceptable parameters of biocompatibility it must present only a discrete tissue reaction or none at all time periods and moderate or intense tissue reaction at 7 days that decreases in intensity with time, reaching a non-significant tissue reaction at 60 days (14). Other properties must also be

considered when choosing an irrigant solution, i.e., NaOCl provides expressive tissue solvent action.

It is important to emphasize that the experimental methods, biological indicators and exposure time can influence the magnitude of the antimicrobial effect of irrigant solutions (15). Estrela et al. (15) verified the antimicrobial action of 2% NaOCl and CHX by two different methods. The best performance of antimicrobial effectiveness of NaOCl was observed with the direct exposure test, and of CHX with the agar diffusion test.

The solvent effect of 0.1%, 1.0%, 2.5% and 5.0% NaOCl solutions on bovine pulp tissue, the level of residual chlorine, pH and surface tension before and after tissue dissolution were studied *in vitro* by Spanó et al. (16). Their results showed that all concentrations of NaOCl reduced the pH and the surface tension, and the higher concentrations had the least consumption of chlorine during tissue dissolution. The residual chlorine was directly proportional to the concentration in the process of pulp tissue dissolution and there was residual chlorine at all concentrations used in this study.

Other research has concluded that CHX presents antimicrobial action (2,3,5,15,17). In the present study, *S. aureus*, *E. faecalis* and *C. albicans* showed greater sensitivity to CHX. The MIC of CHX for *S. aureus* was 0.000002%. Emilson (17) observed lower MIC for *Staphylococcus sp.*, *S. mutans* and *S. salivarius*. Silva (3) determined, *in vivo*, the antimicrobial effectiveness of 1% NaOCl and 2% CHX as endodontic irrigants. Using 1% NaOCl as irrigant, 16.7% and 83.3% of the canals were positive in the microbiological test, immediately and 7 days after therapy, respectively. Using 2% CHX, the percentages of positive cultures were 8.3% and 41.7%, respectively, taking into account the immediate and residual effects. This indicates that both irrigants possess a similar effect immediately after biochemical treatment; however, irrigation with 2% chlorhexidine was more effective than 1% NaOCl after 7 days. Gomes et al. (5) investigated, *in vitro*, antimicrobial activity of several concentrations of NaOCl and CHX in the elimination of *E. faecalis*. CHX (1% and 2%) and 5.25% NaOCl required significantly less time to eliminate *E. faecalis*.

The inhibitory effect of CHX on the proteolytic activity may have an additional effect that contributes to the arrest of caries. There is ample evidence that CHX is a potent antimicrobial agent with high specific-

ity against cariogenic bacteria, i.e. *S. mutans* (18).

Buck et al. (19) analyzed the detoxification of endotoxin by endodontic irrigants (CHX, NaOCl, CHX chloride, ethanol, EDTA, water) and $\text{Ca}(\text{OH})_2$ and reported that the biologically active portion of endotoxin, lipid A, is hydrolyzed by highly alkaline chemicals, namely $\text{Ca}(\text{OH})_2$ or a mixture of CHX, sodium hypochlorite and ethanol. EDTA, NaOCl, CHX, CHX chloride, ethanol and water (control) showed little or no detoxifying ability for lipid A.

Antimicrobial action by calcium hydroxide is determined by the liberation of hydroxyl ions, which requires an ideal length of time for effective microbial destruction. The liberation of hydroxyl ions to attain the high pH that may completely eliminate the microorganisms can be delayed. However, the irrigant solutions containing $\text{Ca}(\text{OH})_2$ compared with other solutions tested did not show significant effectiveness at the periods studied in this experiment.

Using a different experimental method, Barbosa et al. (20) evaluated the antimicrobial efficacy of calcium hydroxide solutions, pure and with detergent added, on various microorganisms. The saturated calcium hydroxide solution was effective against only four of the 11 microorganisms studied over a 60-min exposure time. The calcium hydroxide containing detergent killed all 11 test organisms over a 30-min exposure time.

When choosing an irrigant solution, a combination of various properties, such as antimicrobial action, pulp tissue dissolution, and acceptable biologic compatibility, should be considered. NaOCl in less concentrated solutions (1.0%) provides these important properties. Further research is required to verify the antimicrobial efficiency of irrigant solutions in infected root canals with apical periodontitis.

RESUMO

O objetivo deste estudo foi determinar a concentração inibitória mínima (MIC) e o efeito antimicrobiano, através do teste de exposição direta, de quatro soluções irrigantes [hipoclorito de sódio a 1%, clorexidina a 2%, solução de hidróxido de cálcio a 1% – preparada com 1g de $\text{Ca}(\text{OH})_2$ e 100 mL de água destilada esterilizada, solução de hidróxido de cálcio + detergente (HCT20)] sobre *S. aureus*, *E. faecalis*, *P. aeruginosa*, *B. subtilis*, *C. albicans* e uma cultura mista. O crescimento microbiano foi analisado por dois métodos: turvação do meio de cultura e confirmação pela coloração de Gram e subcultura em caldo nutriente específico. No teste de diluição, o hipoclorito de sódio

a 1% apresentou MIC igual a 0,1% para *S. aureus*, *E. faecalis*, *P. aeruginosa*, e *C. albicans* e igual a 1% para o *B. subtilis* e a cultura mista. A clorexidina a 2% mostrou MIC igual a 0,000002% para o *S. aureus*, 0,02% para *E. faecalis*, *B. subtilis*, *C. albicans* e a cultura mista e 0,002% para *P. aeruginosa*. A solução de hidróxido de cálcio a 1% apresentou MIC superior a 1% para todos os microrganismos testados, com exceção da *P. aeruginosa*, cuja MIC foi igual a 1%. A solução de hidróxido de cálcio + detergente mostrou MIC igual a 4,5 mL para *S. aureus*, *P. aeruginosa*, *B. subtilis*, *C. albicans* e a cultura mista e superior a 4,5 mL para o *E. faecalis*. No teste de exposição direta, o hipoclorito de sódio a 1% apresentou melhor efeito antimicrobiano para todos os microrganismos em todos os períodos experimentais. A clorexidina a 2% foi efetiva sobre *S. aureus*, *E. faecalis*, e *C. albicans* em todos os períodos, e inefetivo sobre *P. aeruginosa*, *B. subtilis* e sobre a cultura mista. As outras soluções mostraram os piores resultados.

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