In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of Enterococcus faecalis

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Abstract

Aim The aim of this study was to assess, in vitro, the effectiveness of several concentrations of NaOCl (0.5%, 1%, 2.5%, 4% and 5.25%) and two forms of chlorhexidine gluconate (gel and liquid) in three concentrations (0.2%, 1% and 2%) in the elimination of E. faecalis.

Methodology A broth dilution test using 24-well cell culture plates was performed and the time taken for the irrigants to kill bacterial cells was recorded. Isolated 24 h colonies of pure cultures of E. faecalis grown on 10% sheep blood plus Brain Heart Infusion (BHI) agar plates were suspended in sterile 0.85% NaCl solution. The cell suspension was adjusted spectrophotometrically to match the turbidity of a McFarland 0.5 scale. One mL of each tested substance was placed on the bottom of wells of 24-well cell culture plates (Corning, NY), including the control group (sterile saline). Six wells were used for each time period and irrigant concentration. Two mL of the bacterial suspension were ultrasonically mixed for 10 s with the irrigants and placed in contact with them for 10, 30, and 45 s; 1, 3, 5, 10, 20, and 30 min; and 1 and 2 h. After each period of time, 1 mL from each well was transferred to tubes containing 2 mL of freshly prepared BHI + neutralizers in order to prevent a residual action of the irrigants. All tubes were incubated at 37 °C for 7 days. The tubes considered to have positive growth were those which presented medium turbidity during the incubation period. Data were analysed statistically by the Kruskal–Wallis test, with the level of significance set at P < 0.05.

Results All irrigants were effective in killing E. faecalis, but at different times. Chlorhexidine in the liquid form at all concentrations tested (0.2%, 1% and 2%) and NaOCl (5.25%) were the most effective irrigants. However, the time required by 0.2% chlorhexidine liquid and 2% chlorhexidine gel to promote negative cultures was only 30 s and 1 min, respectively.

Conclusions Even though all tested irrigants possessed antibacterial activity, the time required to eliminate E. faecalis depended on the concentration and type of irrigant used.

Keywords: antimicrobial activity, chlorhexidine, endodontics, sodium hypochlorite.

Introduction
Complete debridement and disinfection of the pulpal space are considered to be essential for predictable long-term success in endodontic treatment. Residual pulpal tissue, bacteria, and dentine debris may persist in the irregularities of root canal systems, even after meticulous mechanical preparation (Abou-Rass & Piccinino 1982). Therefore, several irrigant solutions have been recommended for use in combination with canal preparation. However, the efficacy of these procedures also depends upon the vulnerability of the involved species. Anaerobic bacteria, especially black-pigmented Gram-negatives, have been linked...
to the signs and symptoms of endodontic disease (Gomes et al. 1996a) but facultative bacteria, such as Enterococcus faecalis, have also been isolated from pathologically involved root canals, being considered one of the most resistant species in the oral cavity and a possible cause of failure of root canal treatment (Gomes et al. 1996b).

An irrigant serves to flush out debris from within the instrumented root canals, dissolve organic tissue remnants, disinfect the root canal space and provide lubrication during instrumentation, without causing irritation to biological tissues (Cheung & Stock 1993, Ingle et al. 1994). Sodium hypochlorite (NaOCl) has become the most popular agent for endodontic irrigation, even though its optimum working concentration has not been universally agreed (Cheung & Stock 1993). Chlorhexidine gluconate, a less malodorous and toxic agent, has been suggested as an irrigant based on its antibacterial effects, substantivity and lower cytotoxicity than NaOCl, whilst demonstrating efficient clinical performance (Leonardo et al. 1999).

The purpose of this study was to assess in vitro the effectiveness of NaOCl and two forms of chlorhexidine gluconate (liquid and gel) at different concentrations in the elimination of E. faecalis.

**Materials and methods**

The irrigants tested in the elimination of E. faecalis were several concentrations of NaOCl (0.5%, 1%, 2.5%, 4% and 5.25%) and two forms of chlorhexidine gluconate (gel and liquid) in three concentrations (0.2%, 1% and 2%). All irrigants were prepared by the same manufacturer (Drogal Farmácia de Manipulação Ltda., Piracicaba, Brazil). NaOCl and chlorhexidine liquid at different concentrations were diluted with sterile water without preservatives. Chlorhexidine gel consisted of gel base (1% natrosol) and chlorhexidine gluconate.

Isolated 24 h colonies of pure cultures of E. faecalis (ATCC 29212) grown on 10% sheep blood plus Brain Heart Infusion (BHI, Oxoid, Basingstoke, UK) agar plates were suspended in sterile 0.85% NaCl solution. The cell suspension was adjusted spectrophotometrically to match the turbidity of a McFarland 0.5 scale (1.5 × 10^8 cfu mL^{-1}).

One mL of each tested substance was placed on the bottom of wells of 24-well cell culture plates (Corning, NY, USA, ref. no. 3524, well Vol. 3.2 mL), including the control group (sterile saline). Six wells were used for each time period and irrigant concentration (i.e. from each well, only one time period and irrigant were tested). Overall, 792 wells were used, comprising 726 for all the test irrigants and 66 for the control group. Two mL of the bacterial suspension were ultrasonically mixed for 10 s with the irrigants and placed in contact with them for 10, 30, and 45 s; 1, 3, 5, 10, 20, and 30 min; and 1 and 2 h. After each period of time, 1 mL from each well was transferred to tubes containing 2 mL of freshly prepared BHI + neutralizers in order to prevent continued action of the irrigants. The neutralizer for NaOCl was 0.6% sodium thiosulphate, whilst 0.5% Tween 80 + 0.07% lecithin was used for chlorhexidine (Siqueira et al. 1999b). All tubes were incubated at 37 °C for 7 days. The tubes considered to have positive bacterial growth were those which presented medium turbidity matching the turbidity of a McFarland 4 scale (12 × 10^8 cfu mL^{-1}) during the incubation period.

The purity of the positive cultures was confirmed by Gram staining, by colony morphology on BHI agar + blood and by the use of a biochemical identification kit (API 20 Strep – bioMéuriex SA, Marcy-l’Étoile, France). The results were analysed statistically by the Kruskal-Wallis test, with the level of significance set at P < 0.05.

**Results**

Table 1 shows the contact time required by each tested irrigant to produce 100% of inhibition growth of E. faecalis.

All irrigants were effective in killing the bacteria tested, but at different times. Chlorhexidine gluconate liquid (1% and 2%) and NaOCl (5.25%) took significantly less time (>30 s) to eliminate E. faecalis than the other irrigants tested. However, the time required by chlorhexidine liquid (0.2%) and chlorhexidine gel (2%) to produce negative cultures was only 30 s and 1 min, respectively. When used at 0.2% concentration, chlorhexidine gel destroyed bacterial cells after 2 h of contact, as opposed to only 15 min when used at 1.0% concentration.

**Table 1** Contact time required by each tested irrigant to produce negative cultures (i.e. 100% inhibition growth) of E. faecalis

<table>
<thead>
<tr>
<th>Irrigants</th>
<th>Contact time</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2% chlorhexidine gel</td>
<td>2 h</td>
</tr>
<tr>
<td>1.0% chlorhexidine gel</td>
<td>15 min</td>
</tr>
<tr>
<td>2.0% chlorhexidine gel</td>
<td>1 min</td>
</tr>
<tr>
<td>0.2% chlorhexidine liquid</td>
<td>30 s</td>
</tr>
<tr>
<td>1.0% chlorhexidine liquid</td>
<td>&lt; 30 s</td>
</tr>
<tr>
<td>0.5% sodium hypochlorite</td>
<td>30 min</td>
</tr>
<tr>
<td>1.0% sodium hypochlorite</td>
<td>20 min</td>
</tr>
<tr>
<td>2.5% sodium hypochlorite</td>
<td>10 min</td>
</tr>
<tr>
<td>4.0% sodium hypochlorite</td>
<td>5 min</td>
</tr>
<tr>
<td>5.25% sodium hypochlorite</td>
<td>&lt; 30 s</td>
</tr>
</tbody>
</table>

*Number of tests performed using the irrigants: 726; number of tests performed using sterile saline (control group): 66.

The antimicrobial activity of NaOCl was related to its concentration, i.e. higher concentrations took less time to inhibit bacterial growth than lower concentrations.

All specimens in the control group yielded positive cultures and E. faecalis was always recovered from all positive cultures.

Discussion

Laboratory tests of any kind are only the first steps in a study of the effectiveness of irrigants. Antimicrobial activity of an in vitro environment depends upon the pH of the substrates in plates or tubes, sensitivity of the drug, bacterial source (wild strains or collection species), the number of bacteria inoculated, incubation time, and the metabolic activity of the microorganisms. On the other hand, the duration of effectiveness of the drug, temperature, contamination and possible leakage of the agent into the mouth must be considered whilst working in vivo (Updegraff & Chang 1977, Ayhan et al. 1999).

Enterococcus faecalis, a facultatively anaerobic Gram-positive coccus, has been implicated in persistent root canal infections (Engström 1964, Cavallero et al. 1989, Gomes et al. 1999b, Molander et al. 1998) and has been used in several previous studies on the efficacy of endodontic irrigants (Shih et al. 1970, Parson et al. 1980, Vahdaty et al. 1993, Siqueira et al. 1997, Heling & Chandler 1998, Siqueira et al. 1998a, Ayhan et al. 1999), especially for its high level of resistance to a wide range of antimicrobial agents (Heath et al. 1996). In the present study we used ATCC strain because it was also utilized in previous in vitro studies investigating the antibacterial effects of endodontic irrigants (Siqueira et al. 1997, 1998a, Gomes et al. 1999, Ferraz et al., in press).

Sodium hypochlorite solution is, to date, the most commonly employed root canal irrigant. However, no general agreement exists regarding its optimal concentration (Baumgartner & Cuenin 1992, Heling & Chandler 1998, Ayhan et al. 1999). For instance, 0.5% NaOCl took 3 min to destroy bacterial cells, as demonstrated in the present work. On the other hand, the use of NaOCl at high concentrations is undesirable because it is an irritant to periapical tissues (Spångberg et al. 1973), even though its antimicrobial action is proportional to its concentration.

The present results showed that 5.25% was the most efficient concentration of NaOCl assessed, killing the bacterial cells in less than 30 s, in agreement with previous studies (Senia et al. 1975).

Chlorhexidine gluconate is a cationic bisguanide that seems to act by adsorbing onto the cell wall of the microorganism and causing leakage of intracellular components. At low chlorhexidine concentrations, small molecular weight substances will leak out, especially potassium and phosphorous, resulting in a bacteriostatic effect. At higher concentrations, chlorhexidine has a bactericidal effect due to precipitation and/or coagulation of the cytoplasm, probably caused by protein cross-linking (Fardal & Turnbull 1986).

Chlorhexidine gluconate has been used in endodontics as an irrigant solution, but always in a liquid presentation. Chlorhexidine gel was evaluated as an intracanal medication, demonstrating good performance (Siqueira & Uzedra 1997). The natrosol gel (hydroxyethyl cellulose, pH 5.5) used as a base for chlorhexidine gluconate is soluble in water and widely used to thicken shampoos, gels and soaps. The gel formulation may keep the active
principle' of chlorhexidine gluconate in contact with the microorganisms longer, inhibiting their growth.

In a previous study (Gomes et al. 1999) that tested the antimicrobial activity of several irrigants, including chlorhexidine gluconate (in gel and solution) and different concentrations of NaOCl against selected endodontic microorganisms, by means of the agar diffusion method, chlorhexidine gel was more efficient than the liquid presentation at equivalent concentrations, although no significant difference was detected. In addition, the growth inhibition haloes produced by both forms of 2% chlorhexidine were significantly larger than those created by all concentrations of NaOCl, including 5.25%. All microbial species tested in that study were sensitive to chlorhexidine gluconate, either in gel or in solution, at both tested concentrations. Chlorhexidine demonstrated a broad spectrum of antimicrobial action, in agreement with the results of previous studies (Delany et al. 1982, Ringel et al. 1982, Jeansonne & White 1994).

The results of the present study confirm those obtained by Ohara et al. (1993), although these investigators only used chlorhexidine in a liquid formulation. In both experiments, the antimicrobial activity of chlorhexidine gluconate was superior to that of NaOCl, except for 0.2% chlorhexidine gluconate gel, which took almost 2 h to produce negative cultures. The present investigation tested the antimicrobial activity of chlorhexidine and NaOCl through direct contact with E. faecalis suspension and showed that chlorhexidine liquid killed the bacterial cells more rapidly than chlorhexidine gel; this is in contrast to our previous work (Gomes et al. 1999). This fact can be explained by the methodologies used; in this study, chlorhexidine liquid 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 chlorhexidine, thus requiring a longer time to act against the microorganisms. In the agar diffusion method, the gel formulation kept the active agent in contact with the inoculated media for a longer time, producing the largest inhibition zones. However, during root canal preparation the antimicrobial irrigant used should also act as lubricant, remove the smear layer, be water-soluble, be biocompatible with periapical tissues, and have contact with the microorganisms. A gel formulation has all these advantages (Ferraz et al., in press).

Conclusions

The time required to eliminate E. faecalis depended on the concentration and type of irrigant used. The present study confirmed the antimicrobial activity of chlorhexidine and sodium hypochlorite, and also provided new data on the properties of chlorhexidine gel as an endodontic irrigant. Studies using chlorhexidine gel are indicated, especially with respect to its mechanical properties. Further studies involving wild and collection strains, not only of E. faecalis but also of other endodontically related bacteria, might be informative. It should be emphasized that, as with most in vitro studies, the present findings remain to be confirmed clinically.

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