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Original

Synergistic antibacterial activity of chlorhexidine and hydrogen peroxide against *Enterococcus faecalis*

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Abstract: The aim of the present study was to compare the *in vitro* antibacterial activity of common root canal irrigants with a combination technique against intratubular *Enterococcus faecalis*. Seventy-five human single-rooted teeth were selected and their crowns and root-ends were removed to obtain specimens 5 mm in length. The specimens were contaminated with *E. faecalis* and divided into five experimental groups (n = 15). These groups were irrigated with 2% chlorhexidine (CHX), 3% hydrogen peroxide (H_2O_2), 5.25% sodium hypochlorite (NaOCl), CHX/H_2O_2 and sterile saline (control). Surface and deep dentinal chips were collected for each sample. After incubation, the numbers of colony-forming units (CFUs) were counted. The Kruskal-Wallis and Mann-Whitney *U* tests were used for statistical analysis (α = 0.05). In the surface dentin, CHX, NaOCl, and CHX/H_2O_2 had significantly higher antibacterial activity than H_2O_2 ($P < 0.05$). In the deep dentin, NaOCl and CHX/H_2O_2 had significantly higher antibacterial activity than CHX and H_2O_2 ($P < 0.05$). CHX/H_2O_2 had similar antibacterial effectiveness to NaOCl in both surface and deep dentinal tubules. This combination can be considered a potentially useful irrigant for root canal treatment.
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Introduction

Removal of remaining pulp tissue and elimination of microorganisms are the main fundamentals of successful endodontic therapy. Microorganisms infecting the root canal may survive endodontic procedures due to anatomical complexity of the canal and limited access of instruments (1,2). Therefore, the use of an effective irrigant is necessary to eradicate microorganisms from areas that are not accessible to instruments. A variety of irrigants have been introduced in an attempt to reduce or eliminate the number of bacteria in the root canal system. An irrigant at the very least should have antibacterial activity and a capacity to dissolve tissues (3). Such solutions should also be compatible with periradicular tissues, and capable of maintaining their therapeutic effect for a long period of time.

Sodium hypochlorite (NaOCl) is currently the most commonly used irrigant during the mechanical instrumentation phase of endodontic therapy. Its tissue-dissolving and antibacterial properties have been well proven. However, it is not substantive and is highly irritating to periradicular tissues at higher concentrations (4). In addition, factors such as concentration (5), temperature (6), and pH (7) greatly affect its efficacy.

Substantivity is an important factor in the selection of a suitable irrigant. Chlorhexidine (CHX) is a broad-spectrum antimicrobial agent active against aerobic and anaerobic bacteria. It has been shown that CHX has antibacterial efficacy comparable to that of NaOCl (8).

CHX has strong binding affinity to the hydroxyapatite in dentin, enamel and cementum, and can be slowly released. This property gives CHX more longer-lasting bacteriostatic activity (9), and the antibacterial efficacy of CHX has been shown to continue for up to 72 h after instrumentation (10). On the other hand, CHX lacks the ability to dissolve organic matter, which negatively affects its cleaning capacity (11).

Hydrogen peroxide (H_2O_2) is another agent used for sterilization and disinfection purposes. H_2O_2 is an active agent with potential inhibitory effects on bacteria, viruses, fungi, yeast and spores (12). It produces hydroxyl free radicals, which attack the protein and DNA of microbes (12). It has been shown that H_2O_2 lacks antibacterial activity, and for this reason it is not used alone as an irrigant during endodontic treatment (3). In order to increase the antibacterial effectiveness of irrigating solutions, combination techniques have been suggested. It has been proposed that combining H_2O_2 with CHX would significantly increase the resulting antibacterial activity in comparison with either agent alone (3,13,14).

Many studies have investigated the effectiveness of different irrigants against *Enterococcus faecalis* (13,15,16). However, there is no conclusive data to indicate the efficacy of using combinations of irrigants for endodontic treatment. In addition, data on the depth of penetration of endodontic irrigants into dentinal tubules are limited. Therefore, the present study was designed to compare the *in vitro* effectiveness of 2% CHX, 3% H_2O_2, and 5.25% NaOCl with that of 2% CHX/3% H_2O_2 against *E. faecalis* in both surface and deep dentinal tubules.

Materials and Methods

Specimen preparation

This study was approved by the ethics committee of Isfahan University of Medical Sciences and Torabinejad Dental Research Center (#390550).

In this *in vitro* experimental study, 75 extracted human single-rooted teeth were cleaned and placed in 2.5% NaOCl for 24 h. After surface disinfection, the teeth were stored in normal saline. The experimental method used in this study was a modification of the model described by Haapasalo and Orstavik (17). The tooth crowns and root-ends were removed to obtain uniform specimens with a root length of 5 mm. The canals were enlarged using a Gates Glidden (GG) #3 bur (Maillefer, Ballaigues, Switzerland) 0.9 mm in diameter to standardize the internal diameters. As a result, cylindrical roots 5 mm long with a 0.9-mm inner diameter were obtained. The outer surfaces of the specimens were covered with an epoxy resin (3M Dental Products, Bracknell, UK). The canals were treated with 17% EDTA for 5 min followed by 5.25% NaOCl for another 5 min to remove the smear layer. Then, the canals were irrigated with normal saline and the roots were sterilized by autoclaving for 30 min at 121°C.

Root canal contamination

Pure *E. faecalis* cultures (ATCC 29212) were cultivated in brain heart infusion (BHI, HiMedia, Mumbai, India) broth medium and then suspended in 4.0 mL of BHI. The cell suspension was adjusted spectrophotometrically to match the turbidity of *E. faecalis* at 6.3×10^8 colony-forming units (CFU)/mL (equivalent to ≈2.0 McFarland standards). One end of each root cylinder was sealed with temporary cement (Cimpat, Septodont, Saint-Maur-des-Fossés, France) and 3 µL of the suspension was placed into the root canal using an automatic pipette. The other end of the cylinder was sealed with temporary cement. The specimens were placed into petri dishes covered with humid sterile gauze and incubated for 21 days at 37°C. On 7th and 14th days, the temporary cement was removed and fresh inocula were added to the canals to ensure bacterial viability.

Experimental groups

The contaminated roots were randomly assigned to 5 groups (n = 15) according to the irrigant used for canal disinfection as follows: Group 1, 2% CHX (FGM Dental Products, Joinville, SC, Brazil); Group 2, 3% H_2O_2 (Merck, Darmstadt, Germany); Group 3, 5.25% NaOCl (Merck); Group 4, 2% CHX/3% H_2O_2 (1:1 mixing ratio); Group 5, Control (sterile saline).

The canals were irrigated using 5 mL of each solution for 10 min on the basis of a previous study (18). A 27-gauge syringe was used to irrigate the canals and

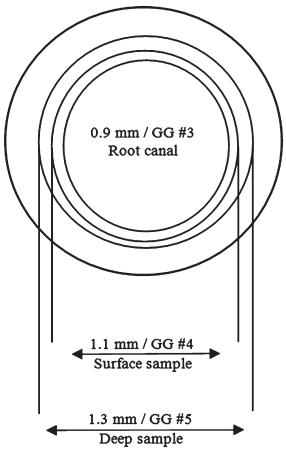

Fig. 1 Sequential removal of dentinal chips from the surface (GG #4) and deep (GG #5) dentin.

a #30 file (Mani Inc., Utsunomiya, Japan) was used to agitate the irrigants inside the canals for 1 min. After the irrigation procedure, the root canals were irrigated with 5 mL of sterile saline and dried with sterile paper points.

Dentin samples

Canals in the root cylinders were enlarged using GG #4 (1.1 mm diameter) and #5 (1.3 mm diameter) burs to collect surface and deep dentin samples, respectively (Fig. 1). Each bur was used three times throughout the entire extent of the root canal. The dentinal chips were collected in a tube containing 1 mL of BHI medium. The samples were mixed for 1 min and using serial dilution 25 µL of each sample was poured onto blood agar culture medium (HiMedia, Mumbai, India) and incubated at 37°C for 24 h. The growing CFUs were counted by a blinded microbiologist. Bacterial identity was confirmed by colony morphology and gram staining.

Data analysis

The Kruskal-Wallis test was used to analyze differences in CFU among the experimental groups. Paired comparisons were done using the Mann-Whitney *U* test. SPSS 15.0 Software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, and statistical significance was set at a confidence level of 95%.

Results

In the surface and deep dentin samples, the minimum and maximum *E. faecalis* CFUs were counted in the CHX/H_2O_2 and control groups, respectively (Tables 1 and 2). The use of various irrigants significantly reduced the number of bacterial CFUs recovered from the surface and deep dentin samples in comparison with the control treatment ($P < 0.05$).

Surface dentin samples

CHX, NaOCl, and CHX/H_2O_2 were significantly more effective in reducing the number of bacterial CFUs in comparison with H_2O_2 ($P < 0.05$). There was no significant difference between the CHX, NaOCl, and CHX/H_2O_2 groups ($P > 0.05$).

Deep dentin samples

NaOCl and CHX/H_2O_2 were significantly more effective in reducing bacterial CFUs than CHX and H_2O_2 ($P < 0.05$). There was no significant difference between the NaOCl and CHX/H_2O_2 groups ($P > 0.05$). CHX was significantly more effective than H_2O_2 in reducing bacterial CFUs ($P < 0.05$).

Discussion

Since bacteria are the main cause of pulpal infections, an appropriate treatment protocol can eradicate microorganisms from the root canal system and ensure the success of root canal therapy. The use of irrigants as chemical adjuncts has been recommended because complete cleaning and disinfection of the root canal system cannot be guaranteed by mechanical means alone (19). It has been shown that the use of irrigants during root canal treatment can dramatically enhance the effectiveness of mechanical debridement (5). An ideal irrigant must be capable of adhering to the dentinal walls and applying its antibacterial effects directly (20). CHX, NaOCl, and H_2O_2 are canal irrigants with well-known advantages and drawbacks. Since there are conflicting results regarding

Table 1 *E. faecalis* CFUs using selected irrigants in the surface dentin

Irrigation solution	Mean	Standard deviation	Median	Minimum	Maximum
2% CHX	748.0	92.91	750.0	590.0	900.0
3% H_2O_2	1,591.33	68.44	1,600.0	1,490.0	1,710.0
5.25% NaOCl	707.3	92.08	700.0	540.0	850.0
2%CHX + 3%H_2O_2	688.0	94.51	710.0	520.0	840.0
Control	2,913.3	381.23	2,750.0	2,450.0	3,800.0

Table 2 *E. faecalis* CFUs using selected irrigants in the deep dentin

Irrigation solution	Mean	Standard deviation	Median	Minimum	Maximum
2% CHX	894.67	93.57	890.0	740.0	1,050.0
3% H_2O_2	1,292.67	88.76	1,300.0	1,100.0	1,440.0
5.25% NaOCl	737.33	92.62	740.0	570.0	880.0
2%CHX + 3%H_2O_2	696.67	105.4	660.0	530.0	880.0
Control	1,756.67	171.24	1,710.0	1,510.0	2,100.0

the antimicrobial efficacy of these solutions, in the present study, the antibacterial synergistic effect of a combination technique was compared with these established irrigants in the surface and deep dentinal tubules.

E. faecalis comprises a small proportion of the root canal flora in initial endodontic infections, but it is found in many secondary or persistent endodontic infections (21). Moreover, recent studies using molecular biological methods have shown that *E. faecalis* is rarely one of the most dominant species in retreatment cases (22,23). However, this bacterium is highly resistant to various harsh environmental conditions such as alkalinity, bile salts, starvation and many antibacterial agents (21,24). Therefore, it is very difficult to eradicate this strain from infected root canals. Since this bacterium is highly resistant to different antibacterial agents, the antimicrobial efficacy of endodontic irrigants against this bacterium has been assessed in various studies (5-7,14,16,25). Because of its high resistance and ease of handling in microbiological sampling, this bacterium was selected for assessment of the antibacterial activity of selected irrigants in the present study.

CHX/H_2O_2 showed antibacterial effectiveness comparable to that of NaOCl in both the surface and deep dentin. Furthermore, all of the irrigants used were significantly more effective than sterile saline in reducing bacterial CFUs (control group). The results of this investigation indicate that: 1) irrigation, as a concept during root canal treatment, can be effective in reducing bacterial loads; 2) irrigants, even with a short exposure time, can exert antibacterial effects beyond the root canal lumen and at depth in dentinal tubules; 3) the penetrating ability, and thus the antibacterial effectiveness, of endodontic irrigants is reduced in deep dentin compared with surface dentin; and 4) the penetrating ability and thus the antibacterial effectiveness of CHX/H_2O_2 and NaOCl in deep areas of dentinal tubules were superior to those of the other irrigants used in this study.

In the deep dentin, NaOCl showed a significantly higher antibacterial effect than CHX. This significant difference may highlight the higher penetrability of NaOCl into dentinal tubules relative to CHX. However, when CHX was combined with H_2O_2, its antibacterial effect was enhanced. This improvement may be due to better penetration into dentinal tubules or a synergistic additive effect of the two irrigants. NaOCl solution is the most commonly used root canal irrigant in endodontic treatment (3). In addition to antibacterial effectiveness and low toxicity at lower concentrations, NaOCl has a good solvent action on necrotic and organic tissue (26). The major shortcoming of NaOCl is its inadequate effect against some microorganisms at lower concentrations. Furthermore, few conclusive studies have recommended optimal concentrations for root canal irrigation (3). Various studies have investigated the antibacterial efficacy of NaOCl and CHX against *E. faecalis* and have demonstrated no significant difference between these two agents (8,16,27,28). Similarly, the results of the present study indicated no significant difference between NaOCl and CHX in surface dentinal tubules.

In the present study, CHX demonstrated higher antibacterial activity than H_2O_2 against *E. faecalis* in surface and deep dentin. CHX has a broad antibacterial spectrum and a very low toxicity even at high concentrations (29,30). It has been proposed that the antibacterial effect of CHX stems from its ability to denature the bacterial cell wall while forming irreversible defects in the membrane (13). Since H_2O_2 exerts its antimicrobial action against intracellular proteins and DNA, the use of other agents in combination with H_2O_2 has been recommended (3). It has been postulated that the efficacy of H_2O_2 can be increased by enhancing its permeability through the cell wall (13). The results of this investigation are in accordance with the findings of Shahriari et al. (14), as H_2O_2 alone showed significantly lower antibacterial activity than CHX against *E. faecalis*.

The use of synergism between two active agents seems to be a logical approach for maximizing their effectiveness while reducing their side effects. The antibacterial effect of CHX and H_2O_2 has been proved in different studies; however, these two agents have some shortcomings when used alone. CHX has a bitter taste and can stain teeth (31), whereas H_2O_2 can cause mucosal irritation (32), induce pathologic alterations (33), and be cytotoxic to pulp (34). Considering the oxidative properties of H_2O_2, Steinberg et al. (13) postulated that this combination may reduce the tooth-staining side effect of CHX. In the present study, the combination of CHX and H_2O_2 was used to test the hypothesis that these two agents in combination can enhance the overall antibacterial effect of each alone. It was shown that this combination had significantly higher antibacterial activity than H_2O_2 alone in the surface dentin. In addition, this combination had significantly higher antibacterial effectiveness than either CHX or H_2O_2 alone in the deep dentin. This increase of antibacterial activity can be explained by the fact that CHX increases the permeability of the bacterial cell membrane by denaturing the cell wall, thus facilitating penetration of H_2O_2 through the cell membrane (13). Using methodology similar to that in the present study, Heling and Chandler (18) reported the synergistic activity of CHX and H_2O_2 at certain concentrations. Steinberg et

al. (13) indicated that the antibacterial effectiveness of CHX and H_2O_2 in combination was higher than that of either of the irrigants alone. Shahriari et al. (14) showed that the number of *E. faecalis* CFUs fell to a minimum following irrigation with CHX and H_2O_2 in combination, thus concurring with the present findings.

There were some limitations to this study. First, it used an *in vitro* model, whereas *in vivo*, the nature of endodontic infections is significantly different. Second, a single microorganism was used to infect the root canals, whereas endodontic infections are usually polymicrobial and show different dynamics. *In vivo*, other microorganisms may interact with *E. faecalis* and thus alter its behavior. Third, combinations of various concentrations of CHX and H_2O_2 may exert different degrees of antibacterial effectiveness. Fourth, under *in vivo* conditions, any remaining tissues and fluids in the root canal system may reduce the efficiency of irrigants.

In conclusion, within the present study limitations, we have demonstrated that CHX and H_2O_2 in combination have antibacterial activity similar to that of NaOCl both in the surface and deep dentinal tubules. Even though this *in vitro* study does not reflect the clinical situation, this combination of agents can be considered potentially useful for irrigation during root canal treatment. Further research should focus on the biocompatibility and *in vivo* clinical efficiency of this combination.

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