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In Vitro Activity of Superoxide Water on Viability of *Enterococcus faecalis* Biofilm on Root Canal Wall

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ABSTRACT

Introduction: The aim of this study was to compare the effect of root canal irrigation with superoxidized water and sodium hypochlorite on elimination of *Enterococcus faecalis* biofilm from the root canal walls. **Methods and Materials:** In this experimental study, a total of 32 extracted human central incisors were used. The crowns of all teeth were cut to length of 16 mm. After cleaning and shaping, then the specimens were sterilized in autoclave and then divided into four groups (n=8) as following: group 1 (positive control, root canal irrigation with normal saline), group 2 (negative control without biofilm), group 3 (root canal irrigation with sodium hypochlorite) and group 4 (root canal irrigation with superoxidized water). The bacterial suspension was inserted to root canals of teeth except for negative control group in order to form a microbial biofilm in incubator for 2 weeks. Then all the samples received root canal irrigation for 5 min based on their allocation. At the end, colony forming unit (CFU) was evaluated and biofilm formation and thickness was detected with scanning electron microscopy. The Kruskal Wallis and Dunn's tests were done for biofilm thickness and CFU, respectively with the level of significance set at 0.05. **Results:** In negative control group no biofilm formation and CFU was present. The CFU counts and biofilm thickness were significantly different between the experimental groups (P=0.001) and both parameters were less in samples with hypochlorite irrigation compared to positive control (52.56±5.82 µm for biofilm thickness and 1.2×10^7 CFU) and samples irrigated with superoxidized water (2.92±1.76 µm for biofilm thickness and 5.4×10^4 CFU). **Conclusion:** Based on this *in vitro* study reduction in biofilm thickness and CFU/mL was 100% for sodium hypochlorite and for superoxidized water was 98% and 90% for reduction in biofilm thickness and CFU/mL, respectively.

Keywords: Canal Irrigation; *Enterococcus faecalis*; Sodium Hypochlorite; Superoxidized Water

Introduction

Eradication of microorganisms from the root canal system is still a major concern in root canal treatment [1, 2]. Although chemomechanical preparation can effectively reduce the number of microorganisms, it cannot completely eliminate the bacteria harboring inside the root canal walls [3, 4]. Since reduction of microorganisms to the lowest possible level is essential for successful root canal treatment, irrigation with intra canal disinfecting agents is recommended for further success in combating bacteria in the root canal system [5].

Enterococcus faecalis (*E. faecalis*) is a facultative anaerobic gram positive cocci which is the most common microorganism found in root canal system of endodontically-treated teeth with apical periodontitis [6-8]. When in contact with antibacterial agents, *E. faecalis* can form biofilm as a resistance strategy that makes it thousand times more resistant than the state planktonic phase [9, 10]. Different irrigation solutions such as 5.25% sodium hypochlorite and chlorhexidine [11] (with different concentrations) are used in root canal treatment today [3, 12] but toxicity of sodium hypochlorite to periapical tissues, tooth discoloration as well as allergic reactions caused by chlorhexidine are worrying drawbacks of these solutions [9, 12, 13].

One of the research priorities is to find a solution that has fewer adverse effects while having antimicrobial activity. Superoxidized water is a neutral, colorless solution with a high oxidation-reduction potential. This solution is prepared with a small amount of salt along with some ordinary water in an electrolysis machine [14, 15]. The prepared solution has a pH of 7.5 to 6.5 with 30 ppm insoluble chlorine and its oxidation-reduction is 1000-1100 mV. During electrolysis, reactive forms of chlorine and oxygen molecules are formed, which can degrade nucleic acid, protein, and lipids. Previous studies have demonstrated the antimicrobial effect of superoxidized water on biofilm *in vitro* [15]. But still its antimicrobial effect as an intra-canal irrigation on *E. faecalis* biofilm is not reviewed. The aim of the present study was to evaluate and compare the antibacterial efficacy of superoxidized water and sodium hypochlorite on *E. faecalis* biofilm in extracted human root canals.

Materials and Methods

Sample preparation

For this experimental study, a total number of 32 single rooted human maxillary central incisors that were extracted for periodontal reasons, were chosen. The teeth had no internal/external resorption, calcification or root surface caries and were kept in normal saline until preparation. The teeth were first cleaned with soft tissue debris scaling curves and then the crowns were cut from the cervical area using a high speed handpiece. Each group had 8 samples (n=8) determined according to the results of the study by Zan *et al.* [16] and using the Minitab software considering α=0.05 and β=0.2.

After working length determination by subtracting 1 mm from the length of a #15 K-file (Mani, Tochigi, Japan) and its emergence through the apical foramen, the root canals of all samples were prepared using ProTaper rotary system (Dentsply Maillefer, Ballaigues, Switzerland) up to F4 (40/0.06). Between each two files, the canals were irrigated with 5 mL of 1% NaOCl with 27 gauge needle. Then for smear layer removal the teeth were first washed with 17% EDTA (Asia Chemi Teb. Co., Tehran, Iran) for 1 min and then with 5.25% sodium hypochlorite for another one-min and finally with normal saline. The tooth samples were placed in a microtube containing normal saline and autoclaved at 121°C and 15 psi for 30 min.

Bacterial inoculation

Enterococcus faecalis strain (ATCC 29212) was obtained from Department of Bacteriology, Pasteur Institute, Tehran, Iran, and cultured in aerobic conditions at 37°C in BHI (brain-heart infusion) broth medium. After culturing and preparation of the bacterial suspension, bacterial concentration was confirmed by spectrophotometer (optical density (OD) 600 nm: 0.08-0.1). Then, under the hood and aseptic conditions 0.02 mL of sampler was added to the root canal of each group except for the negative control group and the samples were incubated for two weeks for microbial biofilm formation. They were incubated at 150 rpm at 37°C. During the time required for microbial biofilm formation the culture medium was refreshed every 48 h under aseptic conditions. After two weeks, in order to ensure mature biofilm formation, 2 samples were randomly selected and longitudinally sectioned and examined under scanning electron microscopy (SEM).

To remove bacteria in the planktonic phase and loosely attached bacteria, the specimens were washed with 5 mL of normal saline. Then the canals were dried using size 30 sterile paper points (Gapadent Co Ltd, Tianjin, China). Samples were divided into four groups of eight as following: group 1; positive control group with microbial biofilm irrigated with 5 mL of normal saline with 27-guage needle which was left inside the canal for 5 min, group 2; negative control group, without microbial biofilm irrigated with 5 mL of normal saline with 27-guage needle which was left inside the canal for 5 min, group 3; with microbial biofilm irrigated with 5 mL of superoxidized water with 27-guage needle which was left inside the canal for 5 min, group 4; microbial biofilm irrigated with 5 mL of 5% NaOCl with 27-guage needle which was left inside the canal for 5 min.

Then each sample was vertically cut in two halves using a chisel. For biofilm removal, half of each sample was placed in a test tube containing 10 cc of sterile saline to dilute the test tubes for 20 sec using a vortex sonicator. Sampler was extracted with 0.01 mL of vortex solution and cultured in BHI broth (BHI, Merck, Darmstadt, Germany). Samples were also taken from control groups and cultured. After 24 h colony counting was performed and colony forming units were calculated in each mL (colony forming unit/mL or CFU/mL). The second half of specimens were prepared and plated with a gold vacuum apparatus for SEM.

Statistical analysis

Data obtained from biofilm thickness and CFU/mL were analyzed using the Kruskal-Wallis software and Dunn's statistical comparison. The level of significance was set at 0.05.

Table 1. The mean (SD) of biofilm thickness and colony forming unit (CFU) per mL in study groups

	Biofilm thickness	Colony Forming Unit/mL	
Experimental and control groups	**Mean (SD)**	**Mean (SD) before irrigation**	**Mean (SD) post irrigation**
Control positive	52.56 (5.82)	12^{6} $(11)^{5}$	11^{6} $(11)^{5}$
Control negative	0	0	0
Sodium hypochlorite	0	11^{6} $(11)^{5}$	0
Super oxidized water	2.92 (1.76)	12^{6} $(11)^{5}$	5^{4} $(1)^{5}$

Results

There was a significant difference between biofilm thickness of experimental and positive control groups (P=0.001). Superoxidized water and NaOCl showed 90 and 100 percent reduction in biofilm thickness, respectively. The difference between biofilm thickness in NaOCl and superoxidized water groups was statistically significant (P=0.021) (Table 1).

Regarding reduction in CFU/mL, superoxidized water and sodium hypochlorite showed 98% and 100% reduction, respectively. There was a significant difference in the CFU/mL between experimental and positive control groups (P=0.001). However, the difference between the negative control and NaOCl groups was insignificant (P=1). There was a significant difference between negative control and superoxidized water group as well as superoxidized water and NaOCl group (P=0.001) (Table 1).

Discussion

The present sowed that root canal irrigation with superoxide water can eliminate bacterial biofilm from the root canal. The purpose of root canal treatment (RCT) is to eliminated as much bacteria as possible or prevention of their (re)entry to the root canal system. Thus bacteria play an important role in the pathogenesis and progression of pulpal and periapical diseases [2, 17, 18].

Enterococcus faecalis is the most common bacteria isolated from the root canals of teeth with previously failed RCT and persistent infection [19, 20]. The ability of *E. faecalis* to form biofilm is one of the reasons of its resistance to RCT and common irrigation methods and medicaments [1, 21, 22]. Other studies have evaluated the antibacterial activity of superoxide water on planktonic form of *E. faecalis* inside root canals [18]. However bacteria in its biofilm is much more resistant to antibacterial agents whether as irrigants or medicaments [20]. Therefore, the present study used the mature biofilm of *E. faecalis* in the root canal samples which was confirmed with SEM. In our study, biofilm thickness was measured in each group using SEM, which is highly capable of detecting structural changes in biofilms [14, 19], although Paromonova *et al.* [23] claimed that the method of confocal laser scanning microscopy (CSML) is more economical and powerful than SEM.

Agents used for intra-canal irrigation are used to further reduce microorganisms [4]. Thus they should be able to kill bacteria, reduce inflammation and stimulate hard tissue formation without any toxic effects on host body tissue [24-26]. As the most common root canal irrigant, sodium hypochlorite dissolves the collagen fibers easily and is the only intra-canal irrigant that is capable of dissolving living and necrotic pulp tissue [27, 28] and is also capable of completely destroying *E. faecalis* microorganism [29]. However, toxicity and bad taste have made it difficult to use [26]. Superoxidized water, is a colorless acidic solution [14, 16, 30] formed during electrolysis with saline solution and forms reactive radicals of chlorine and oxygen, which can destroy nucleic acid, proteins, and lipids and also offer high bactericidal activity [14]. Another characteristic of this substance is its low toxicity and biocompatibility [31]. Zan *et al.* [16] evaluated the antibacterial effect of superoxide water in root canal contaminated with *E. faecalis* and showed super oxidized water could be an effective irrigating agent for human root canal treatment [16, 32]. Although this study is different from the current paper regarding the irrigation time and the sampling method that was done by paper points without sonication which can potentially reduce the accuracy and may cause false negative data [15]. While sonication removes the biofilm bacteria the paper point sampling collects planktonic microorganisms.

Gunaydin *et al.* [33] investigated the antibacterial effect of superoxidized water on different strains of bacteria and fungi. They concluded that superoxidized water had an acceptable performance against all tested microorganisms. The findings of this study stated that a one-min exposure of superoxidized water, which was diluted by a ratio of 1/2 during (the lowest exposure time in this study) was quite effective in reducing *E. faecalis* bacteria [33].

Another important point in our study was the difference in the chlorine content of the two solutions (NaOCl and super oxidized water) so that the amount of chlorine ion in sodium hypochlorite was significantly higher than superoxide water.

The ultimate goal of endodontic treatment is only to disinfect the root canal, not to sterilize it, so the reduction in CFU and biofilm thickness caused by superoxide water (98% and 90% reduction in biofilm thickness and CFU, respectively) is acceptable for root canal disinfection during RCT, while meeting one of the top goals which is using a solution with the least side effects and toxicity. Hypochlorite causes allergic effect in some people, so super oxidized water can be a good alternative to NaOCl and can be considered as an effective irrigation solution.

Conclusion

Sodium hypochlorite caused 100% reduction in biofilm thickness and CFU/mL of *E. faecalis*, but superoxidized water showed 98% eduction in biofilm thickness and 90% reduction in CFU/mL of the microorganism. According to this *in vitro* study, superoxidized water is less effective in infected root canals compared to hypochlorite solution.

Conflict of Interest: 'None declared'.

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