J Istanbul Unıv Fac Dent 2015;49(1):19-26.
http://dx.doi.org/10.17096/jiufd.70199

BACTERICIDAL EFFECTS OF VARIOUS IRRIGATION SOLUTIONS AGAINST STAPHYLOCOCCUS AUREUS IN HUMAN ROOT CANAL

Çeşitli İrrigasyon Solüsyonlarının İnsan Kök Kanalındaki Staphylococcus aureus'a Karşı Antibakteriyel Etkileri

Recai ZAN[1], Gizem KUTLU[1], İhsan HUBBEZOĞLU[2], Zeynep SÜMER[3], Tutku TUNÇ[3], Zuhal MUTLU[1]

Received: 25/11/2014
Accepted:08/01/2015

ABSTRACT

Purpose: *The aim of the present study was to evaluate and to compare the antibacterial effects of various irrigation solutions against Staphylococcus aureus (S. aureus) in human root canals.*
Materials and Methods: *120 single-root mandibular premolar teeth were selected. The teeth were prepared and sterilized. S. aureus was incubated in the root canals and kept at 37°C for 24h. The infected root canals were divided into one positive (saline) and one negative (sodium hypochlorite) control, and four experimental groups [Ethylene-diaminetetra-aceticacid, Chlorhexidine Gluconate, Super-oxidized water(SPO), Aqueous ozone] (n=20). Flow rate of irrigation was applied with 5 mL/min flow rate for 3 min to ensure standardization among all study groups. Following the irrigation, paper points were placed in the root canals and then transferred in sterile eppendorf. Remaining bacteria were counted on blood agar plates and the data were analyzed using one-way ANOVA and Tukey's test.*
Results: *Although there were statistically significant differences among SPO and other experimental groups (p<0.05), there was no statistically significant difference between SPO and NaOCl (p>0.05).*
Conclusion: *Super-oxidized water may be recommended as an alternative irrigation solution instead of NaOCl against S. aureus in root canals.*

Keywords: *Disinfection, irrigants, Staphylococcus aureus*

ÖZ

Amaç: *Bu çalışmanın amacı, insan kök kanallarında Staphylococcus aureus (S. aureus)'a karşı çeşitli irrigasyon solüsyonlarının antibakteriyel etkilerini değerlendirmek ve karşılaştırmaktır.*
Gereç ve Yöntem: *120 tek kök mandibular premolar dişler seçildi. Dişler genişletildi ve sterilize edildi. S. aureus kök kanalı içinde inkübe edildi ve 24 saat boyunca 37°C'de tutuldu. Enfekte kök kanalları bir pozitif (serum fizyolojik) ve bir negatif (sodyum hipoklorit) kontrol ve dört deney grubuna (Etilen diamin tetra asetik asit, Chlorhexidine Gluconate, Super-oxidized water, Aqueous ozone) ayrıldı (n=20). İrrigasyon akış hızı, tüm çalışma grupları arasında standardizasyonu sağlamak amacıyla 5 mL/dakika akış hızıyla 3 dakika boyunca uygulandı. İrrigasyonu takiben kağıt koniler kök kanalı içine yerleştirildi ve daha sonra steril eppendorf tüplerine aktarıldı. Kalan bakteriler kanlı agar plakaları üzerinde sayıldı ve veriler tek yönlü ANOVA ve Tukey testi kullanılarak analiz edildi.*
Bulgular: *Süper-okside su ile diğer deney grupları arasında istatistiksel olarak anlamlı fark olmasına rağmen (p<0,05), SPO ile NaOCl arasında istatistiksel olarak anlamlı bir fark bulunamamıştır (p>0,05).*
Sonuç: *Süper-okside su, kök kanallarındaki S. aureus'a karşı NaOCl'ye alternatif bir irrigasyon solüsyonu olarak tavsiye edilebilir.*

Anahtar kelimeler: *Dezenfeksiyon, irrigan, Staphylococcus aureus*

[1] Department of Endodontics Faculty of Dentistry Cumhuriyet University Sivas
[2] Department of Restorative Faculty of Dentistry Cumhuriyet University Sivas
[3] Department of Microbiology Faculty of Medicine Cumhuriyet University, Sivas

Introduction

In endodontics, one of the most important procedures in root canal treatment is chemo-mechanical preparation of the canal system. Irrigation is complementary to instrumentation in facilitating removal of bacteria, debris and therapeutic materials such as gutta-percha, sealer and medicaments from root canals. The effectiveness of irrigation relies on both the mechanical flushing action and the ability of irrigants to dissolve tissue. The ideal irrigant should be strongly antimicrobial but not toxic to the periapical tissues and if extruded through the apex, facilitate the ceasing of walls, clean inaccessible areas, prevent canal clogging by dentine chips and remove the debris in the root canal system (1).

Sodium hypochlorite (NaOCl) has been widely used as irrigation solution used from 0.5 to 5.25 concentrations for the chemo-mechanical treatment of root canals (2). Concentration and toxicity of NaOCl are directly proportional (3). At low concentrations of NaOCl, reducing cytotoxic and irritating properties benefit treatment but decrease antibacterial effects (4).

Chlorhexidine gluconate (CHX) is another irrigantadvocated as an effective medication in endodontic treatment. CHX may bind to hydroxyapatite and soft tissues, changing their electrical field to compete with bacterial binding. It has lower toxicity than NaOCl but lacks tissue dissolving property. Moreover, antimicrobial effect of irrigant combinations within dentinal tubules has been suggested in endodontics (5).

Ethylene-diaminetetra-aceticacid (EDTA) demineralizes and removes the inorganic components and debris of the smear layer produced during instrumentation, leaving an organic fibrous component on the canal walls. Moreover, it may destabilize cells, causing lipopolysaccharide release (6). Furthermore, it is also highly effective in dentine tubule patency for effective root canal disinfection in vitro. EDTA is also considered active against anaerobic bacteria whilst having only a minute effect against aerobes (7).

In recent times, ozone has gained popularity in clinical research. Ozone is a powerful oxidizing agent that may eliminate microorganisms in root canals (8). It can be administered in either gaseous or aqueous form obtained from ozone generators. Both of the ozone types may oxidize the cell walls and cytoplasmatic membranes of microorganisms (9-11). Consequently, ozone types have been preferred as bactericidal, antiviral, and antifungal agents (12-14). Some research emphasized the antimicrobial efficacy of aqueous ozone. The low concentration of aqueous ozone was found to have insufficient disinfectant effect against pathogenic microorganisms in dental plaque (13), root canals (15), and acrylic resin plates (16). Moreover, one of the other properties is no toxicity to oral cells as in vitro (13). The most important disadvantage of aqueous ozone is unstability. For this reason, it should be used as soon as possible after being mixed. It is very difficult to keep this mixture at the same concentration for a long time (17). Due to these properties, ozone may be used as a new disinfectant agent in root canals.

Most of the endodontic disinfectants have been investigated against various microorganisms. Recently, new brand super-oxidized water has gained popularity in endodontic researches. SPO is a common form of oxygen that includes the highly reactive superoxide ion O_2^-. It is created when molecular oxygen gains a single electron. Moreover, superoxide radicals can attack susceptible biological targets, including proteins, lipids and nucleic acids (18). The lower toxicity is the other superiority of SPO according to NaOCl (19) and hydrogen peroxide (20). A few researchers emphasized the effect of SPO as an irrigation solution in root canals (21, 22).

S. aureus is one of the important resistant microorganism frequently isolated from recurrent root canal treatments.It plays a major role in the etiology of primary endodontic infections and persistent infections among break sessions during root canal therapy when the root canal was left open, (13, 14).

The research on new disinfectant agents has ensured the necessity to find new irrigants instead of NaOCl. Therefore, we evaluated and compared the antibacterial effects of different irrigation solutions as final irrigan against *S. aureus* inoculated in the root canals.

Materials and Methods

In present study, 120 single-root single-canal human mandibular permanent premolar teeth that were freshly extracted for orthodontic or periodontal reasons and that had no caries or restorations were used. Digital radiographs of teeth taken in buccal and approximate directions were used to determine the number and morphology of canals. Informed consent was obtained from the patients before the study and the study was approved by the Local Ethics Committee on Human Research of Cumhuriyet University (2014-04/04).

After having been cleaned of residues, the fresh-extracted human teeth were kept at +4°C in a 0.9% saline solution. The coronal portions of teeth were cut below the level of enamel-cementum junction using sterile diamond discs under the cooling water to obtain the 14-16 mm length of each root. The root canals were then entered with #15 K-File (Mani Inc, Tochigi, Japan) hand tools and the path of the canal was determined. The tip of the file was transmitted to measure the length of each canal until it became visible in the apical foramen. Then it was withdrawn 1 mm from measured length. The root canals were shaped with ProTaper (Dentsply, Tulsa, OK, USA) rotary Ni-Ti instruments using the crown-down method by the electric motor (Denta ports DP-ZX, J. Morita MFG Corp., Kyoto, Japan). First, the coronal third of roots were expanded with SX files. The median third of roots were then reached with S1 and S2 files. The F1, F2, and F3 files were, respectively, applied to shape the apical third of the canals. Canals were irrigated with 1 ml of 5.25% solution of NaOCl after the variation of each file. The roots were irrigated with 17% EDTA, 5.25% NaOCl, and distilled water for 10 min each to remove the smear layer, which was formed during root canal preparation and then dried with paper point. Bottles were placed in autoclave to ensure sterilization for 20 min each at 121°C (Melag, Euroklav 23V-S, Berlin, Germany). Then 3-fold nail polish (L'Oreal Jet-Set Diamond, Paris, France) was applied to the entire root surface of each tooth, including root tips. Finally, rubber caps which were embedded in the teeth were sterilized by Ethylene Oxide (EtO). Those caps were then placed in bottles (23).

Microbiologic procedures

S. aureus (ATCC 29212) strains were revived in the liquid nutrient media (Brain-heart infusion broth, Acumedia Manufactures Inc., Lansing, Michigan, USA) and were incubated at 37°C for 24 h. Prior to the each experiment, 0.5 McFarland turbidity was set with a kristalspecTM device, and McFarland standard number 0.5 was used to improve blood agar plates in order to obtain the bacterial growth in 1.5 X 10^8 colony-forming units (CFU/ml). The value of 10µl of bacterial culture was transferred to the mechanically expanded lumen of the root canal using a sterile micropipette and then kept at 37°C for 24 h. In order to control bacterial growth, the sterile paper points (Dentsply Maillefer, Ballaigues, Switzerland) were placed in the root canals inoculated with bacteria. Paper points were left in place for five minutes and soaked with the broth. Paper points were then placed in sterile Eppendorf tubes containing 0.5 ml brain-heart infusion broth (Merck 1.13825). After 15 minutes, 50 ml of liquid medium was taken with a steril micropipette from eppendorf with mixed vortex and was smear-planted to solid media (blood agar plates), which split before and after the applications of disinfection.

Experimental groups

Group 1, Saline (positive control) group. Infected root canals were irrigated for a duration of 3 min with 0.9% saline solution.

Group 2, NaOCl (negative control) group. Infected root canals were irrigated for a duration of 3 min with 5.25% NaOCl.

Group 3, Aqueous ozone group. Aqueous ozone was obtained with a custom-made ozone generator (TeknO^{3}zone, Izmir, Turkey) from TeknO^{3}zone company. The amount of aqueous ozone was measured with the help of the probe, which was in the reaction tank connected to the generator. The ozone density of the distilled water in the reactor tank was shown by the digital indicator on the generator. Power was controlled automatically by means of the automatic balancing system. Infected root canals were irrigated for a duration of 3 min with 8 ppm aqueous ozone (TeknO^{3}zone, Izmir, Turkey)

Group 4, Chlorhexidine Gluconate group. Infected root canals were irrigated with 2% CHX. The irrigation flow rate was 5 mL/min for 3 min.

Group 5, Super oxidized group. Infected root canals were irrigated with Super-oxidized water (Medilox; O-M Medical Dental Textile, Ankara, Turkey) that consists of a mixture of oxidizing substances including hypochlorous acid (HOCl) at a concentration of 50-80 mg/L, with a pH of 5.5 and a redox potential > 850- 1000 mV. The irrigation flow rate was 5 mL/min for 3 min.

Group 6, EDTA group. Infected root canals were irrigated with EDTA solution. The irrigation flow rate was 5 mL/min for 3 min.

Bacterial evaluation

Root canals were contaminated and followed by a 24 h incubation period. Paper points were placed in root canals for five min before irrigation for the control of the growth of microorganisms. Bacteria counting ensured standardization; examples with colony forming units (CFU) values under 1.5 x 10^8 CFU/ml were excluded. After irrigation, CFU counts of the breeding colonies of microorganisms were performed in blood agar plates. Then log CFU were calculated.

Statistical analysis

The study variables were analyzed using SPSS statistical software for Windows (22.0 version, IBM SPSS Inc.,Chicago, IL, USA). Since the distribution of the data was consistent with the assumptions of normal distribution, one-way analysis of variance (ANOVA) test was used for multiple group comparisons. Post-hoc pairwise comparisons were performed with Tukey's test. Confidence interval was set to 95 % and p values less than 0.05 were considered statistically significant.

Results

CFU countings for study groups and statistical comparisons that were obtained from all groups are given in Table 1.

Although there were statistically significant differences among super-oxidized water and other experimental groups ($p<0.05$), there was no statistically significant difference when compared with NaOCl ($p>0.05$). As a result of pairwise comparisons performed among experimental groups, differences were found statistically significant ($p<0.05$). Moreover, there were statistically significant differences among saline (negative control) and other all groups ($p<0.05$).

Table 1. *Colony forming unit (CFU), log CFU, median, minimum and maximum values for groups and statistical comparison (SD: standard deviation).*

Groups *n= 20*	**Before irrigation (Sx) (CFU mL^{-1})**	**After irrigation (Sx ± SD) (Log CFU mL^{-1})**	**Median**	**Minimum**	**Maximum**
Group 1 *Saline (Negative control)*	1.5 X 10^8	6.07 ± 0.76	6.035	6.00	6.17
Group 2 *Adueous Ozone*	1.5 X 10^8	0.90 ± 0.78	1.000	0.00	2.00
Group 3 *EDTA*	1.5 X 10^8	4.04 ± 0.19	4.000	3.69	4.30
Group 4 **CHX**	1.5 X 10^8	3.32 ± 0.35	3.210	3.00	4.00
Group 5 *Super-oxidized water*	1.5 X 10^8	0.20 ± 0.41*	0.000	0.00	1.00
Group 6 *NaOCl (Positive control)*	1.5 X 10^8	0.00 ± 0.00*	0.000	0.00	0.00

F= 751.542, P=0.000, The groups indicated by the same symbol (*) showed no difference (p>0.05).
There were significantly statistical differences among all other groups (p<0.05).

Discussion

In endodontics various new disinfectant agents have been researched since bacterial invasion leads to dangerous pulpal and periapical diseases. Recently, toxicity of NaOCl has gained more importance in terms of undesirable complications throughout the treatment period. In the lights of these problems, alternative irrigation solutions have been tried and compared with conventional agents. Today, the most commonly used irrigation solution used in the endodontic treatments is sodium hypochlorite NaOCl (1). Some studies have shown that NaOCl in different percentages indicates the high antibacterial effect against *S. aureus* (2, 24, 25). In the present study, we also used 5.25% NaOCl in root canals and similar results were found with the above-mentioned studies (2, 24, 25). Moreover, unlike NaOCl, the saline was showed the lowest antibacterial effect ($p<0.05$).In recent times, aqueous ozone gained popularity as irrigation solution for root canal disinfection. There are only a few studies that examined this issue in literature. In a study, the researchers investigated the antimicrobial effect of aqueous ozone against *Enterococcus faecalis* (*E. faecalis*) in root canals. The significant reduction of microorganism was observed by Cardoso *et al* (16). Another research have emphasized bactericidal effect of aqueous ozone applied with different application techniques in bovine teeth for 10 min. As a result, the bacterial elimination effect of aqueous ozone was demonstrated by Nagayoshi *et al.* (8). In another scientific clinical investigation, Bialoszewski *et al*. (26) investigated bactericidal activity of ozonated water against *S. aureus* on polystyrene titration plates for 30 seconds. César *et al*. (27) evaluated the antimicrobial effects of ozonated water on the diamond dental burs were experimentally contaminated with *S. aureus*. The ozonated water was found effective in reducing the CFU of S. aureus for the sanitization of dental instruments for 10 and 30 min. Consequently, ozonated water was found to be an effective bactericidal agent against *S. aureus* (26, 27). In the present study, 8 ppm density of the aqueous ozone was applied for 3 min into the root canals infected by *S. aureus*. As a result, aqueous ozone exhibited significant antibacterial effects. Although there were some important dissimilarities such as irrigation time (16, 26, 27), the experimental samples (8,26,27) and concentration (8, 16, 26), the results of the present study indicated similar and stronger outcomes compared to the above mentioned studies (8, 16, 26, 27).

One of the conventional irrigation solutions used and researched for a long time is CHXgluconate. CHX is one of the main antimicrobial agent that may strongly eliminate *E. faecalis* that has been implicated in treatment failures (28). Moreover, Chlorhexidine may exhibit particularly the long-term antimicrobial properties with its unique ability to bind to hydroxyapatite. These properties were investigated in a few studies, for instance the anti-microbial efficacy of 2.5% sodium hypochlorite and 0.2% CHX were compared with an experimental irrigant in root canals. As a result, it showed a certain degree of antibacterial effect (29). In another research, Aslantas *et al*. (30) aimed to evaluate the effects of root canal irrigants on the microhardness of root canal dentin in the presence and absence of surface-modifying agents for 5 min. Consequently, EDTA, REDTA, NaOCl, and CHX significantly decreased the microhardness of root dentin ($p<0.05$). Moreover, another endodontic research compared the antibacterial efficacy of Chlorhexidine with its combinations. Microbial inhibition potential of CHX was observed. Additionally the mixture of CHX and a few antibiotics showed greater bactericidal effect (31). Furthermore, Dutta *et al*. (32) analyzed the antimicrobial effect of five irrigants and compared with 0.2% CHX through an agar diffusion test. Consequently, CHX indicated antifungal and antibacterial effect. The present study also aimed to investigate the antibacterial effect of CHX on *S. aureus* in human root canal. Although there were some differences such as irrigation time, concentration, experimental sample, the results showed certain similarities with those of the studies mentioned above (28-32).

In endodontics, several conventional irrigants have been used against various microorganisms. One of the leading irrigants preferred for a long time is EDTA. It may show the strong antibacterial effect on Gram-positive (*Streptococcus mutans* and *E. faecalis*) organisms in human root canal. It was also found effective against aerobe and anaerobe bacteria only for a minute (33). Morgental *et al*. aimed to examine the antibacterial effect of a conventional irrigation solution like EDTA in bovine incisors against *Enterococcus faecalis* for 1 min. As a result, its antibacterial effect was found too low to eliminate bacteria (34). Moreover, Aranda *et al*. (35) searched the antibacterial effectiveness of the NaOCl in combination with EDTA against *E. faecalis* contaminated root canals for 3 min. Consequently, NaOCl combinated with EDTA was found effective on *E. faecalis*. Furthermore, Heling *et al*. (36) investigated the antimicrobial effect of NaOCl with and without EDTA in varying concentrations as endodontic irrigants. Six standardized bovine incisor root specimens infected with *E. faecalis* were exposed to each solution. As a result, EDTA eliminated a small amount of bacteria. Few researches examined the bactericidal effect of EDTA on *S. aureus*. Mattigatti *et al*. (37) evaluated antimicrobial effect of various root canal medicaments against *S. aureus* on brain heart infusion agar for 1 min. EDTA was found to be effective on *S. aureus*. In light of these researches, we examined the effect of EDTA on *S. aureus* in root canal. As a result of the present study, EDTA was found little effective against *S. aureus*. Although there are some differences such as irrigation time (33, 34, 36, 37), sample type (34, 36) and kind of bacteria (33-36), this result showed similarity with that of above mentioned studies (33, 35-37). The super-oxidized water has attracted the attention of endodontists in recent times because of its antimicrobial effect. The antimicrobial activity of super-oxidized water has been demonstrated against bacteria, mycobacteria, viruses, fungi, and spores (19, 22, 38-40) in medical literature. By way of example, the microbicidal activity of super-oxidized water was found to be highly active against several micro-organisms when membrane filters were used on endoscopes after 2 min (19, 39). Furthermore, it was nontoxic for biological tissues (19). However, there are only few researches that emphasized the antimicrobial efficacy of SPO in literature. For instance, the antimicrobial action of NaOCl and super-oxidized water was investigated against *E. faecalis* in bovine root canals for 3 min. Although SPO could not destroy all bacteria like NaOCl, it demonstrated strong antibacterial effect in terms of killing *E. faecalis* (22). In another research, the antibacterial activity of super-oxidized water was examined by Yamada *et al*. (41) against cultured planktonic cells of cariogenic and periodontopathic bacteria on dental equipment for 15 seconds to 24 hours. The results emphasized the antibacterial effect of super-oxidized water on cariogenic and periodontopathic bacteria. On the other hand, there has been no published study to date on the examination of antibacterial effect of super-oxidized water against *S. aureus* in human root canal.Due to lack of literature on this subject, we investigated the bactericidal efficacy of super-oxidized water on *S. aureus* in human root canal for 3 min. Consequently, SPO demonstrated an appreciable antibacterial effect in terms of *S. aureus* elimination. Although there were significant differences compared with the aforementioned studies such as irrigation time (35-38, 40, 41) kinds of samples (35-41) and bacteria type (35-40), the results were found similar to each other.

Conclusion

Within the limitations of this *in vitro* study, it can be concluded that all irrigants used in study had a remarkably applicable antibacterial effect against *S. aureus* in root canals. Moreover, super-oxidized water indicated a remarkable and similar bactericidal effect to that of traditional NaOCl against *S. aureus* biofilms.

Source of funding
None declared

Conflict of interest
None declared

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Corresponding Author:
Recai ZAN
Department of Endodontics
Faculty of Dentistry Cumhuriyet University
58140 Sivas, Turkey
Phone: +90 346 2191010 / 2701
e-mail: drrecaizan@hotmail.com