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Araştırma/ Research Article

IN-VITRO ANTIBACTERIAL EFFICIENCY OF IRRIGATION REGIMENS AGAINST BIOFILM OF ENTEROCOCCUS FAECALIS

İRRİGASYON REJİMLERİNİN ENTEROCOCCUS FAECALIS BİOFİLMİ ÜZERİNE İN-VİTRO ANTİBAKTERİYEL ETKİNLİĞİ

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ABSTRACT

Objectives: The aim of this study was to compare the antibacterial efficiency of 2.25% sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX), and gaseous ozone to biofilm of *Enterococcus faecalis* (*E. faecalis*) generated on cellulose nitrate membrane filters.

Materials and methods: After an overnight culture of *E. faecalis* grown in tryptic soy (TYC) broth, an aliquot of 100µl of microorganisms was seeded onto 13.0-mm diameter cellulose nitrate membrane filters (Sartorius®, UK) and incubated for 48 to 72 h at 37ºC in an aerobic atmosphere. Twenty membranes were used for each test agent. Then, biofilm formation test agents, gaseous ozone, and 0.9% NaCl (positive control) were applied at time intervals. Application periods for NaOCl and CHX were 5 to 10 minutes, whereas ozone application with Ozonytron^{x} (Bizonix, GmbH, Germany) was 80 to 120 seconds. Next, from each of the samples, 100µl aliquots were put onto the TYC agar plates. Incubation colonies were calculated as colony forming units (CFU/mL) per membrane and observed by scanning electron microscopy (SEM). Data were analyzed using the Wilcoxon test, Kruskal-Wallis test, and Mann-Whitney U-test at $p<0.05$.

Results: All of the irrigation regimens disgregated and removed the biofilm compared to the positive control ($p<0.05$).However, both 2.25% NaOCl and 2% CHX caused a significantly higher degree of biofilm disgregation than ozone application ($p<0.05$).

Conclusions: The irrigation regimen of 2.25% NaOCl and 2% CHX was more effective in eliminating biofilm formation of *E. faecalis.* However, the long-term irrigation regimen of ozone was found to be a successful alternative for endodontic treatment.

Key-words: Biofilm, Ozone, Enterococcus faecalis

ÖZET

Amaç: Çalışmanın amacı % 2,25 sodyum hipoklorid (NaOCl), %2 klorheksidin (CHX) ve ozon gazının selüloz nitrat membran filtreler üzerinde oluşturulan *Enterococcus faecalis* (*E. faecalis*) biyofilmi üzerindeki antibakteriyal etkinliğini karşılaştırmaktır.

Materyal method: Triptik soy (TYC) besiyerinde bir gece *E. faecalis* kültüre edilerek, 100 µl mikroorganizma sıvısı 13,0-mm çapında selüloz nitrat membran filtreler (Sartorius®, İngiltere) üzerine ekildi ve 48-72 saat 37ºC'de aerobic bir atmosferde inkübe edildi. Her test ajanı için 20 membran kullanıldı. Test ajanları, ozon gazı ve %0,9 NaCl (pozitif kontrol) zaman aralıklarında biyofilm formasyonuna uygulandı. NaOCl ve CHX için uygulama periyotları 5 ve 10 dak. iken; Ozonytron^{x} (Bizonix, GmbH, Almanya) cihazı ile ozon gazı 80 ve 120 saniye uygulandı. Her örnekten 100µl sıvı TYC agarlara konuldu. İnkübasyon kolonileri membran yüzeyinde koloni oluşturan birimler (CFU/mL) olarak hesaplandı ve SEM ile gözlemlendi. Veriler Wilcoxon, Kruskal-Wallis ve Mann-Whitney U-testleri kullanılarak $p<0,05$ güven aralığında değerlendirildi.

Bulgular: Pozitif kontrol grubu ile kıyaslandığında tüm irrigasyon rejimleri biyofilmi bozuntuya uğrattı ($p<0,05$). Öte yandan hem %2,25 NaOCl hem de %2 CHX ozon grubundan istatistiksel olarak anlamlı farklılık yaratacak düzeyde yüksek biyofilm bozunumu oluşturdu ($p<0,05$).

Sonuç: %2,25 NaOCl ve %2 CHX rejimleri *E. faecalis* biyofilm formasyonunun eliminasyonunda daha etkilidir. Öte yandan daha uzun süreli ozon gazı uygulamaları endodontik tedavi için alternatif bir irrigasyon rejimi olabilir.

Anahtar kelimeler: Biofilm, Ozon, Enterococcus faecalis

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INTRODUCTION

The successful treatment of an infected root canal involves a combination of mechanical and chemical means.[1] Using mechanical instrumentation alone might reduce the number of bacteria in the root canal system by 50%. Antibacterial irrigants have to have the capability to penetrate non-instrumented surfaces.[2]

Enterococcus faecalis (*E. faecalis*) is commonly isolated from the root canals of teeth with persistent periapical periodontitis.[3] At times, it is the only bacterium that is isolated from these root canals. Further, its growth in root canal walls as biofilm, antibacterial resistance, and ability to adapt to harsh environmental changes make it responsible for numerous endodontic failures.[4] Several studies are focused on evaluating the efficacy of root canal irrigants, especially against *E. faecalis* .[3] In many of these studies, the researchers grew the bacterial strains as planktonic cultures (bacteria in suspension). However, planktonic cultures do not comply with the in-vivo growth condition found in an infected root canal in which bacteria grow as a biofilm on the dentinal walls.[5] In addition, bacteria members of a biofilm community can be up to 1,000 times more resistant to antimicrobial compounds than their planktonic counterparts.[6] Therefore, the authors revealed that all studies about the "clinical" action of root canal irrigants should be conducted with bacteria in biofilm form.[5,6]

Several irrigating solutions are used during endodontic treatment. In general, endodontic irrigants must not only have effective antimicrobial activity, but also exhibit relatively no cytotoxicity toward periapical and oral mucosal tissue.[7] Sodium hypochloride (NaOCl) is the most common irrigant of choice due to its antibacterial and tissue-dissolving effects.[1] On the other hand, it is toxic at high concentrations and weakens dentine by reducing its flexural strength and resilience. Therefore, root-treated teeth are becoming more susceptible to deformation and fractures.[8,9]. It has been reported that as much as 3% NaOCl has limited efficacy against high-pathogenic endodontic microorganisms.[7] However, side effects, such as hemorrhage, edema, and skin ulceration have been reported when high concentrations of NaOCl come into contact with oral tissues.[10] To a lesser degree than NaOCl, clorhexidine digluconate (CHX) also has substantive properties. As a result, it has also been recommended for root canal irrigation in combination with mechanical debridement .[11] However, CHX loses its antibacterial effect rapidly when combined with organic or inorganic molecules because of its strong positive charge.[12]. Further, it has been reported that CHX (2%) may cause mucosal desquamation, impaired wound healing, and tooth staining. In addition, a high cytotoxic potential has been demonstrated on epithelial cells.[7] Therefore, researchers are looking for an alternative endodontic antiseptic with high antimicrobial potential and fewer side effects.

Ozone (O_3) is a naturally occurring compound consisting of three oxygen atoms. The gaseous or aqueous phases of ozone have been shown to be a powerful and reliable antimicrobial agent against bacteria, fungi, protozoa, and viruses.[13] In this context, ozone is a possible alternative antiseptic agent in dentistry because of its reported high antimicrobial power and low likelihood of drug resistance. During the last few years, HealOzone (KaVo, Biberach, Germany) has been used in dentistry as an ozone generator. Moreover, gaseous ozone in a concentration of 4 g m^{-3} is already being used clinically for endodontic treatment. In addition, the concentration (4 g m^{-3}) of gaseous ozone used in endodontics has been shown to be less cytotoxic than NaOCl (2.5%).[7] Currently, there is growing interest in the use of ozone in oral health care. "Ozonytron^{X}" (Biozonix®, Mymed, Germany) is one of the newest devices for generating ozone. The objective and purpose of this study was to compare the antibacterial efficiency of 2.25% NaOCl, 2% CHX, and gaseous ozone generated by Ozonytron^{X} to biofilm of *E. faecalis* generated on cellulose nitrate membrane filters.

MATERIAL AND METHODS

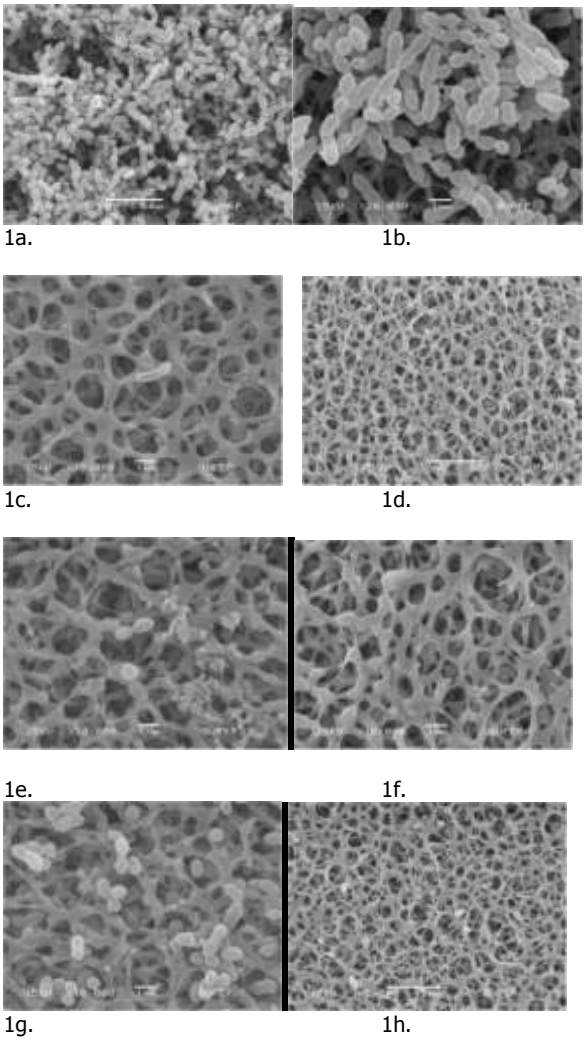
Biofilms of *E. faecalis* strain *ATCC 29212* were generated on cellulose nitrate membrane filters (Sartorius®, UK). After an overnight culture of *E. faecalis* grew in tryptic soy (TYC) broth, an aliquot of 100µl of microorganisms was seeded onto 13.0-mm diameter cellulose nitrate membrane filters and incubated for 48 to 72 h at 37ºC in an aerobic atmosphere. Twenty membranes were used for each test agent. Next, biofilm formation test agents,

gaseous ozone, and 0.9% NaCl (positive control) were applied at time intervals. Application periods for 2.25% NaOCl and 2% CHX were 5 and 10 minutes, whereas ozone application with Ozonytron^{X} (Biozonix®, Mymed, Germany) was performed 80 and120 seconds, according to the manufacturer's directions. After each period of time to stop the action of the antimicrobial test agents, the membrane filters were carefully transferred aseptically into tubes containing 5 ml of neutralizing broth (D/E, Neutralizing Broth, Difco) for 5 minutes and vortexed for 60 seconds. In addition, 100µl aliquots were put onto the TYC agar plates. After 48-hour incubation in an aerobic atmosphere at 37°C, colonies were calculated as colony forming units (CFU/mL) per membrane. Three samples from each group and the initial biofilm formation (negative control) generated on membranes were placed in a fixing solution (14) and observed by scanning electron microscopy (SEM).The data were analyzed with the Wilcoxon test, Kruskal-Wallis test, and Mann-Whitney U-test at $p<0.05$.

RESULTS

The three irrigants tested were 2.25% NaOCl, 2% CHX, and gaseous ozone. Specifically, 0.9% NaCl was used as a positive control. The mean log counts of the number of bacteria are shown in Table 1. All irrigation protocols induced a significant increase in biofilm disgregation at every time interval compared to the positive control, 0.9% NaCl ($p<0.05$). Further, 2% CHX was the only irrigant that was capable of killing 100% of the bacteria after only 5 minutes ($p<0.05$), whereas the same effect was achieved by 2.25% NaOCl after 10 minutes. Although ozone application did produce an increase in biofilm disgregation, neither 80 seconds nor 120 seconds of gaseous ozone application accomplished this goal. Also, the 120-second application of ozone killed significantly more bacteria than the 80-second application ($p<0.05$).

The SEM images showed undisgregated biofilm of *E. faecalis* on positive and negative control samples (Figs 1a-b). No biofilms were observed on any of the membrane surfaces treated with 2% CHX (Figs 1c-d). Small numbers of deformed *E. faecalis* were observed on the membrane surfaces treated with 2.25% NaOCl for 5 and 10 minutes (Figs 1e-f). Although small colonies were observed on the surfaces treated with ozone for 80 seconds, the decrease in the number of bacteria and physiological changes in bacteria were observed on the membrane surfaces treated with ozone for 120 seconds (Figs 1g-h).



1a. 1b. 1c. 1d. 1e. 1f. 1g. 1h.

Figure 1(a-h): SEM images of the inhibition of *E.faecalis* biofilm formation with irrigation regimens on membrane filters.

a. Untreated membrane surfaces after 48-72 hours incubation of *E.faecalis* biofilm (Negative control).
b. Undisgregated *E.faecalis* biofilm after 10 minutes of contact with 0.9% NaCl (Positive control).
c. *E.faecalis* biofilm completely removed after 5 minutes of contact with 2% CHX.
d. *E.faecalis* biofilm completely removed after 10 minutes of contact with 2% CHX
e. *E.faecalis* biofilm after 5 minutes of contact with 2.25% NaOCl
f. *E.faecalis* biofilm after 10 minutes of contact with 2.25% NaOCl
g. *E.faecalis* biofilm after 80 seconds of contact with gaseous ozone
h. *E.faecalis* biofilm after 120 seconds of contact with gaseous ozone

Table 1. Antimicrobial activity against *E.faecalis* biofilm of different irrigation regimens.

rrigants	N	Initial CFU/membrane	5 min/80 sec CFU/membrane	10 min/120 sec CFU/membrane
%0.9 NaCl	10	$1,5X10^{8a}$	$2,3x10^{8a,A}$	$1,4x10^{8a,F}$
%2 CHX	10	$1,5X10^{8a}$	$0^{b,B}$	$0^{b,G}$
%2.25 NaOCl	10	$1,5X10^{8a}$	$15,8^{c,D}$	$0^{d,G}$
O_3	10	$1,5X10^{8a}$	$1471,2^{e,E}$	$293,6^{f,H}$

The lower cases in the same line indicates significant differences (p<0,05).
The upper cases in the same column indicates significant differences (p<0,05).

DISCUSSION

The primary aim of endodontic treatment is to eradicate or substantially reduce the microbial load in the root canal system. Several irrigating solutions or chemicals like CHX, NaOCl, and latterly MTAD BioPure and Tetraclean, are used during endodontic treatment.[5] But still NaOCl and CHX are the most frequently studied agents for endodontic treatment.[11,15] Although studies with similar results have been reported when NaOCl and CHX were compared.[16,17] other investigations have shown either that NaOCl presents better activity than CHX or that CHX has better antimicrobial activity than NaOCl[11]. Dunavant et al.[15] found that only NaOCl is able to kill the entire bacteria population organized in *E. faecalis* biofilm. They also reported that its activity is strictly correlated to its concentration. These differences may have been caused by differences in the concentration and type of compared agents, period of time used, or experimental methodology.

New therapeutic strategies for treating apical periodontitis should consider not only antimicrobial effects, but also their influence on the host immune response.[18] NaOCl is recommended for endodontic treatment, particularly at higher concentrations, because it has greater antimicrobial activity than diluted solutions. However, higher concentrations are associated with increased toxicity as opposed to lower concentrations.[8,9] Authors suggest that NaOCl should be used with caution, adding that the use of 0.5% NaOCl for 30 minutes can prevent the inadvertent extrusion of concentrated NaOCl.[10] Similarly, it has been reported that CHX has side effects, such as mucosal desquamation and tooth staining, and high cytotoxic potential.[7] Given the demand for relative non-toxicity toward periapical and oral mucosal tissue for endodontic irrigants, gaseous ozone is currently used in endodontics as an alternative oral antiseptic.[1,7,13]

In the present study, 2.25% NaOCl was preferred to reduce the irritation potential of NaOCl. In the literature, 2.5% and 2.25% NaOCl are commonly recommended[7] Additionally, 2% CHX was utilized as another preferred agent in endodontic treatment in this study.[11] The agents were used with the contact times of 5 and 10 minutes.

In this study, *E. faecalis*, a gram-positive facultative anaerobe, was selected because it is commonly found in the root canals of failing endodontically treated cases. According to Molander et al.[19], *E. faecalis* can survive in a quiescent phase with low metabolic activity for a period of time. Although **Candida albicans** is frequently observed mainly in refrectory infections and has also an important virulence factor, **E. faecalis** has the important ability to penetrate the dentinal tubules, exhibits strong adhesion to collagen, and shows resistance to irrigation solutions usually used during the instrumentation of root canals. This is why *E. faecalis* has been used in numerous studies to test the efficacy of endodontic irrigants so do in the present study.[5,7,11] Depending upon the methodology-direct contact, agar diffusion, or contaminated dentin test, the irrigation agents can demonstrate efficacy or not.[11] In accordance with Giardino et al.[5], in the present study, a "biofilm model" was used because it seemed to be more realistic to test the efficacy of selected irrigation agents.

Authors revealed that some possible factors facilitating *E. faecalis*'s long-term survival are its ability to adhere to dentin, invade dentinal tubules, and form communities organized in biofilms.[11,20] In this context, the bacteria's location becomes one of the most important aspects to be considered in the treatment of infected root canals. Estrella et al.[11] revealed that when bacteria are lodged within the dentinal tubules or in deep layers, *E. faecalis* can be more resistant to the antibacterial action of NaOCl and CHX. In addition, Estrela et al.[23] reported that when a medicament does

not reach the target microorganism, its killing potential cannot be realized. For this reason, a better root canal irrigant that can be effective in the deeper layers of dentin and dentinal tubules is still needed.

As a gas, ozone has diffusion capacity in the deeper layers of dentin and dentinal tubules.[13] It has been proposed as an alternative oral antiseptic in dentistry. Further, results of studies have shown that ozone in the gaseous or aqueous phase has strong oxidizing power with reliable microbial effects.[1,7,13,20-22] It has been reported that oxidation mediated by ozone destroys the cell walls and cytoplasmic membranes of bacteria and fungi. After the membrane is damaged by oxidation, the permeability increases and ozone molecules can enter the cells and cause microorganisms to die.[7] Nagoyoshi et al.[21] showed that ozonated water had nearly the same antimicrobial activity against bacteria (*E. faecalis* and *S. mutans*), invaded dentinal tubules of root canals as 2.5% NaOCl, and showed a low level of toxicity against cultured cells. Hems et al.[1] found that NaOCl was superior to ozonated water in killing *E. faecalis*, whereas gaseous ozone had no effect. Moreover, Estrela et al.[23] reported that the irrigation of infected root canals with ozonated water, 2.5% NaOCl, 2% CHX, and gaseous ozone for 20 minutes was insufficient to inactivate *E. faecalis.*

Reports on the effect of ozone on bacteria in root canals are controversial. In addition, HealOzone was used in all previous studies. This is the first study to examine the efficacy of gaseous ozone generated by Ozonytron^{x} as an alternative antiseptic against *E. faecalis.* In addition, the effect of gaseous ozone against *E. faecalis* biofilm generated on nitrate membranes has not been evaluated. In the present study, the density of gaseous ozone generated by Ozonytron^{x} was 100%. Moreover, the generator was used for 80 and 120 seconds, according to the manufacturer's instructions for endodontic treatment.

Unlike those of many previous studies, the results of this study indicate that 5-minute contact with 2% CHX was more effective than 2.25% NaOCl in killing *E. faecalis* grown in biofilms. This finding is consistent with that reported by Oncag et al.[24], who found that 2% CHX with a contact time of 5 minutes was more effective than 5.25% NaOCl in extracted teeth infected with *E. faecalis.* These findings suggest that NaOCl should have contact with the bacteria for a substantial period so that it can act upon the bacterial cells[24,25] In this context, in the present study, 10-minute contact time of 2.25% NaOCl had similar effective antibacterial action with CHX. Although the use of ozone in the tested density (100%) and application periods (80 and 120 seconds) induced a significant increase in biofilm disgregation, the microorganisms were not completely eliminated in either time interval. On the other hand, a 120-second application time of gaseous ozone was significantly more effective than an 80-second application. The findings of this investigation are consistent with those of previous studies,[1,7,13,23] which indicated that highly concentrated gaseous ozone may be time-dependently more effective against the biofilm test model. The manufacturer's recommended application time was between 80 and 240 seconds. In this investigation, given that we aimed to test the effectiveness of gaseous ozone at a high density with average application times, the generator was used at the highest density (100%) and with contact times of 80 and 120 seconds.

The use of ozone is justified as a new irrigating agent with antimicrobial action. However, results of studies examining its efficacy against endodontic pathogens have been inconsistent. Further, there is little information regarding the most appropriate application time and concentration. This is the first study investigating the efficacy of gaseous ozone generated by a different device from the other generator used in many studies. In addition, studies should be undertaken with Ozonytron^{X}, which is one of the newest devices for generation of ozone. Studies should be conducted to investigate the applicability of ozone in different clinical and laboratory situations.

CONCLUSIONS

Based on the results of this study, CHX and NaOCl are highly effective in eliminating *E. faecalis* grown in biofilm. However, our results suggest that the application of gaseous ozone time-dependently might be useful for root canal irrigation.

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