Antibacterial Efficacy of Octenisept, Alexidine, Chlorhexidine, and Sodium Hypochlorite against *Enterococcus faecalis* Biofilms

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Abstract

Introduction: The purpose of this study was to evaluate the antibacterial effectiveness of Octenisept (OCT; Schülke & Mayr GmbH, Norderstedt, Germany), 1% alexidine (ALX) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and 2% chlorhexidine (CHX) against *Enterococcus faecalis* biofilm using confocal laser scanning microscopy. **Methods:** Root dentin discs were prepared from extracted human teeth, sterilized, and inoculated with *E. faecalis* strain (ATCC 29212) to establish 3-week-old biofilm model. Infected dentin discs were exposed to OCT (n = 20), 1% ALX (n = 20), and 2% CHX (n = 20) for 10 minutes. Dentin discs (n = 15) exposed to 5.25% sodium hypochlorite (NaOCl) were used as a positive control, whereas specimens exposed to saline (n = 15) were used as a negative control. After exposure, the dentin discs were stained with fluorescent LIVE/DEAD *Bac*Light dye (Invitrogen Molecular Probes, Eugene, OR) and analyzed with confocal laser scanning microscopy to determine the proportion of dead cells in the biofilm. Statistical analysis was performed using the Kruskal-Wallis and Mann-Whitney U tests ($P < .05$). **Results:** The highest proportion of dead cells was found in the 5.25% NaOCl group (94.14%; range, 92.30%–98.20%) compared with the experimental groups ($P < .05$). A significantly greater proportion of dead cells was found in the OCT group (74.14%; range, 70.03%–78.96%) compared with the 1% ALX and 2% CHX groups ($P < .05$). The proportion of dead cells was 43.89% (range, 24.86%–55.63%) and 42.78% (range, 25.45%–55.06%) in the 1% ALX and 2% CHX groups, respectively, with no statistical significant difference between the 2 groups ($P > .05$). **Conclusions:** NaOCl had significantly greater antimicrobial activity against *E. faecalis* biofilms compared with OCT, CHX, and ALX. OCT was more effective than CHX and ALX. *(J Endod 2017;43:643–647)*

Key Words

Alexidine, biofilm, chlorhexidine, *Enterococcus faecalis*, Octenisept

Significance

NaOCl was the most effective antimicrobial solution against *E. faecalis* biofilms grown on dentin discs. Octenisept was more effective than CHX and alexidine and, in view of its low toxicity and substantivity, could be considered as an alternative to these solutions.

Evidence has shown that microbial infection of the root canal system is the primary etiologic factor in pulpal and periapical disease (1). Free-floating microorganisms in the root canal system can attach to one another and form mature biofilms (2). Bacteria in mature biofilms have inherent resistance to antimicrobial agents, which makes it difficult to eradicate the biofilms from the root canal system (3).

The goal of root canal treatment is to prevent and treat apical periodontitis by eliminating microbial biofilms from the root canal system (4). The current cleaning and shaping techniques alone are unable to provide a bacteria-free root canal, and, therefore, a chemical irrigant is required to assist in reducing bacterial numbers and their toxic by-products (5). The most commonly used irrigating solutions are sodium hypochlorite (NaOCl) and chlorhexidine (CHX). NaOCl has potent antibiofilm activity and the ability to dissolve organic tissues (6). However, it has high toxicity and can cause allergic reactions (7). By contrast, CHX has antibacterial action and lower tissue toxicity than NaOCl (8), but it is unable to eradicate biofilm or dissolve organic tissue (9, 10).

Several antimicrobial disinfectants used in the medical field have been evaluated as irrigants in endodontics, such as Octenisept (OCT; Schülke & Mayr GmbH, Norderstedt, Germany) and alexidine (ALX). OCT was introduced in 1990 as a mucous membrane antiseptic. It contains 0.1% octenidine dihydrochloride/2% phenoxyethanol. Phenoxyethanol, an ethanol derivate, serves as a preservative and synergistically improves the antibacterial activity of octenidine. OCT has a broad antimicrobial spectrum, is effective against biofilm-forming organisms (11), and has remarkable and substantive antimicrobial effects because it readily binds to negatively charged surfaces. Its efficacy remains unchanged in the presence of blood, mucin, and albumin (12). Moreover, it has been proven to be nontoxic after topical application in mice, and it is not absorbed via the skin, mucous membranes, or wounds (13). Several *in vitro* studies have shown that OCT has high antimicrobial effectiveness against bacteria when used as a root canal irrigant (14–16).

ALX, a bisbiguanide similar to CHX, has broad antimicrobial activity and helps to inhibit the immune response of major virulence factors, such as bacterial lipopolysaccharide and lipoteichoic acid, more effectively than CHX (17). Moreover, the cytotoxic activities of ALX have been shown to be comparable with those of CHX (18). Kim et al (19) showed that 1% ALX and 2% CHX had similar antibacterial effects against *Enterococcus faecalis*–infected dentin blocks. In addition, when ALX is associated with NaOCl,

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0099-2399/$ - see front matter

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http://dx.doi.org/10.1016/j.joen.2016.09.013

it does not form a reaction precipitate that would block the dentinal tubules, suggesting that ALX could be used as an irrigant with NaOCl (20).

Direct observation techniques using confocal microscopy can provide data on bacterial quantification; they evaluate bacterial invasion of the dentinal tubules, the microbial viability, and the architecture and spatial distribution of microbial biofilms (21). To date, no studies have used confocal laser scanning microscopy (CLSM) to evaluate the antibiofilm activity of OCT and ALX as endodontic irrigants. Therefore, the aim of this *in vitro* study was to evaluate the antibacterial effectiveness of OCT, 1% ALX, and 2% CHX against *E. faecalis* biofilm using CLSM.

Materials and Methods

Specimen Preparation

This study was approved by the Ethics Committee of the College of Dentistry Research Center, King Saud University, Riyadh, Saudi Arabia (PR 0020). Extracted human single-rooted teeth were collected. Roots with fracture lines, anatomic irregularities, previous endodontic treatment, or curvatures were excluded. The preparation of the specimens was adapted from Wu et al (22) with some modifications. The crown and the apical portion of the tooth were sectioned off with a diamond disc, and the middle third was sectioned with a low-speed sectioning saw (IsoMet 2000 Precision Saw; IsoMet, Buehler, IL) at 1000 rpm in a buccolingual direction under water cooling. One hundred $6 \times 8 \times 0.5$ mm (width × length × thickness) dentin discs were prepared. The dentin discs were treated with 17% EDTA in an ultrasonic bath for 1 minute to eliminate the smear layer and then rinsed in sterile saline for 1 minute. All dentin discs were sterilized with gamma radiation at a dose of 25 kGy. Two dentin discs randomly selected from each group were incubated in brain-heart infusion (BHI) broth for 24 hours at 37°C to ensure that there was no bacterial contamination.

Bacterial Strain and Biofilm Generation

E. faecalis (ATCC 29212) was plated in the BHI broth and incubated anaerobically at 37°C for 24 hours. The colonies were diluted in fresh BHI broth and incubated to match the turbidity equivalent to a 0.5 McFarland standard, corresponding to an optical density of 0.08 to 0.1 absorbance at 600 nm in a spectrophotometer. Sterilized dentin discs were placed in sterilized 12-well tissue culture plates (Nunc; Thermo Scientific, Darmstadt, Denmark) and inoculated with 3.0 mL *E. faecalis* suspension (1×10^8 colony-forming unit/mL) under anaerobic conditions for 21 days at 37°C. The inoculation was repeated every 72 hours using a 24-hour prepared culture to remove dead cells and ensure bacterial viability. After incubation, the specimens were aseptically removed from the wells and gently rinsed with sterile phosphate-buffered saline (PBS) for 1 minute to remove loosely attached planktonic bacteria. Two additional dentin discs were randomly selected from each group and examined using a confocal laser scanning microscope (ZEISS Cell Observer SD Spinning Disk Confocal Microscope; Carl Zeiss Microscopy, Jena, Germany) to verify the viability of *E. faecalis* biofilms.

Treatment of Infected Specimens

The dentin discs were randomly divided into experimental (n = 60) and control (n = 30) groups. The experimental group was subdivided into 3 groups (n = 20/group) according to the irrigant solution as follows: the OCT group, OCT; the ALX group, 1% ALX dihydrochloride (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) prepared by dissolving 10 mg ALX powder per 1 mL methanol (Sigma-Aldrich, St Louis, MO); and the CHX group: 2% CHX (Vista Dental Products, Racine, WI). The positive control group (n = 15) was treated with 5.25% NaOCl, whereas the negative control group (n = 15) was treated with sterile saline. Each specimen was immersed in 2 mL of the tested solution for 10 minutes. The samples were gently washed for 1 minute with 5 mL PBS to remove the remnants of the irrigant solution and neutralized with their corresponding agent for 5 minutes to inhibit the continued antibacterial activity of the solution (Table 1).

TABLE 1. The Irrigant Solutions and Corresponding Neutralizing Agents

Irrigant solution	Neutralizing agent
Octenisept	Mixture of 3% Tween 80 (Sigma-Aldrich); 0.3% Lecithin (Sigma-Aldrich); and 0.1% Cystein (Sigma-Aldrich)
1% alexidine	0.3% Lecithin
2% chlorhexidine	Mixture of 3% Tween 80 and 0.3% Lecithin
5.25% sodium hypochlorite	5% sodium thiosulfate solution (Sigma-Aldrich)

Confocal Laser Scanning Microscopic Analysis

Each dentin disc was rinsed with 2 mL sterile PBS and stained with LIVE/DEAD *Bac*Light fluorescence dye (Invitrogen Molecular Probes, Eugene, OR) for 30 minutes. The dye contains 2 nucleic acid–binding dyes (SYTO 9 and propidium iodide) that are used to differentiate live and dead cells. Bacteria with intact cell membranes stain green, whereas those with damaged membranes stain red. A fresh mix at a 1:1 ratio was prepared for each dentin disc immediately before microscopic evaluation. Each disc was then rinsed with 2 mL PBS to remove excess dye. All samples were mounted on the confocal laser scanning microscope and observed using the 25× oil lens. The 4 corners of each dentin disc were scanned with a 2-μm step size at a resolution of 512×512 pixels. Simultaneous dual-channel imaging was used to display the green fluorescence (live cells) and red fluorescence (dead cells). The excitation/emission wavelengths were 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. Confocal laser scanning microscopic images of the biofilms were analyzed and quantitated with AxioVision Rel. 4.8.Ink (Carl Zeiss Microscopy). The volume ratio of red fluorescence to green and red fluorescence indicated the proportion of dead cells in each sample, from which the percentage of dead cells could be obtained. Statistical analysis was performed using the Kruskal-Wallis test to measure the mean value of the proportion of dead cells after exposure to different solutions. The Mann-Whitney U test was used for comparisons between irrigant solutions ($P < .05$).

Results

A total of 400 confocal laser scanning microscopic operative field "stacks" were evaluated for all samples. The confocal laser scanning microscopic images of the dentin discs that verified the formation of *E. faecalis* biofilm showed a homogenous and dense biofilm on the dentin surfaces (Fig. 1*A1–4*).

A significantly greater proportion of dead cells resulted in the samples treated with OCT (74.14%) followed by 1% ALX (43.89%) and 2% CHX (42.79%). The positive control group (5.25% NaOCl) significantly scored the highest proportion of dead cells (94.14%) compared with the experimental groups ($P < .05$), and the lowest proportion was observed with the saline group (13.10%). The Mann-Whitney U test showed a statistically significant difference among all of the groups ($P < .05$), except for the CHX and ALX ($P > .05$) groups (Table 2).

Confocal laser scanning microscopic images of 3-week-old *E. faecalis* biofilms exposed to OCT showed that the majority of bacterial biofilms were removed from the dentin surfaces, with some remaining dead cells attached to the dentin surfaces (Fig. 1*B1–4*). The confocal

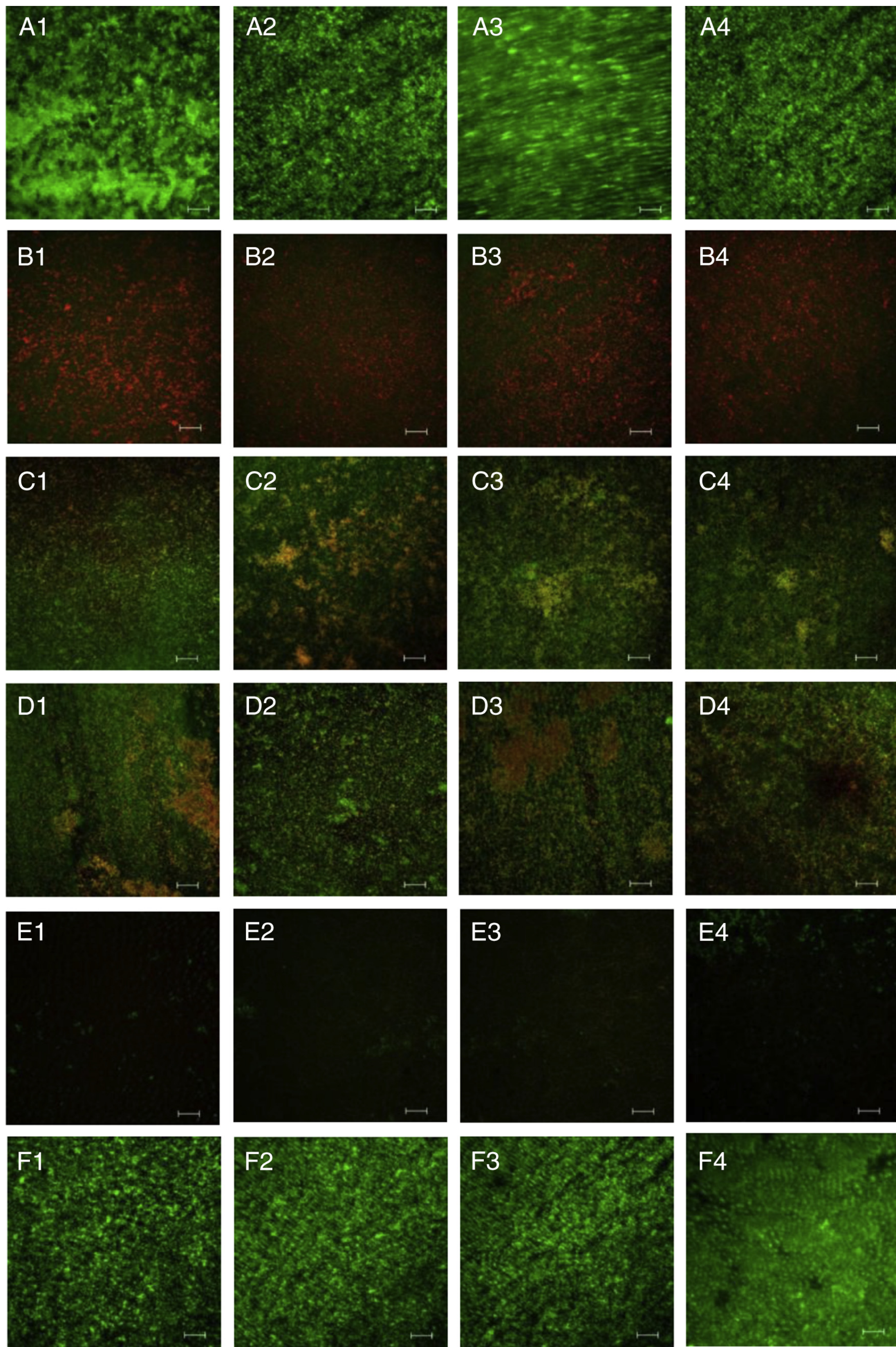

Figure 1. (*A1–4*) Confocal laser scanning microscopic images of 3-week-old *E. faecalis*–infected dentin discs after 10 minutes of exposure to (*B1–4*) OCT, (*C*1–*4*) 1% ALX, (*D1–4*) 2% CHX, (*E1–4*) 5.25% NaOCl, and (*F1–4*) saline. All images are shown at 25× magnification. The green cells represent cells with intact membranes, whereas the red cells are damaged or dead. All bars represent 50 μm.

TABLE 2. Median and Range Values of Dead Cells (%) after 10 Minutes of Exposure to Irrigant Solutions

Irrigant solution	Median (range, %)*
Sodium hypochlorite	94.14 (92.30–98.20)[a]
Octenisept	74.14 (70.03–78.96)[b]
Alexidine	43.89 (24.86–55.63)[c]
Chlorhexidine	42.79 (25.45–55.06)[c]
Saline	13.10 (7.10–16.28)[d]

*Data labeled with different letters are significantly different from each other ($P < .05$).

laser scanning microscopic images of the 1% ALX group showed that the upper layers of the biofilms were more affected by the irrigant solution than the deeper layers with small patches of dead bacteria attached to the live bacterial biofilm (Fig. 1*C1–4*); similar findings were noted in the 2% CHX group (Fig. 1*D1–4*). The 5.25% NaOCl group showed almost complete biofilm removal from dentin surfaces. Although clean dentin surfaces were observed, residual bacterial biofilms appeared firmly attached to the dentin structure (Fig. 1*E1–4*). In contrast, the saline group showed intact biofilm layers and numerous live bacteria attached to dentin surfaces (Fig. 1*F1–4*).

Discussion

It has been well established that endodontic disease is a biofilm-mediated infection (23). Therefore, the elimination of bacterial biofilms is an essential element for the successful outcome of endodontic treatment. In the present study, a homogenous and dense *E. faecalis* biofilm was observed with CLSM in all samples evaluated to verify the biofilm formation on dentin surfaces. This observation confirmed the presence of the well-established 3-week-old biofilm.

The results of this study showed that the proportion of dead cells was significantly higher in the OCT group (74.14%). OCT killed the majority of *E. faecalis* in biofilm; this finding is in agreement with previous studies (14–16) although some have reported a higher antibacterial effect of OCT than was found in this study. This inconsistency might be caused by the different methods of antibacterial evaluation (culture-based vs CLSM) and the nature of the bacteria (planktonic vs biofilm). The antibiofilm effect of OCT could be a result of its ability to penetrate through the biofilm matrix (24).

This study revealed limited antibiofilm effects of 2% CHX and 1% ALX with no significant difference ($P > .05$) between them. This could be caused by the inactivation of cationic bisbiguanides by the organic matter of the biofilm (25) and the limited penetration of the extracellular matrix of the biofilm (26). The limited antibiofilm activity and the lack of organic tissue dissolution with CHX resulted in our study being in agreement with previous studies (24, 27) and inconsistent with others (28, 29). This inconsistency might be because of the longer exposure time (24 hours) and the method of evaluation (colony-forming unit technique). The limited antibiofilm of 1% ALX is in agreement with a previous study (19).

The results of this study showed that the proportions of dead cells were significantly higher in the 5.25% NaOCl group (94.14%) compared with the experimental groups ($P < .05$), demonstrating almost complete removal and dissolving of *E. faecalis* biofilms. Our findings concur with previous studies showing the potent antibiofilm effect of 5.25% NaOCl (10, 30). This is because of the ability of NaOCl to dissolve organic tissue and attack the biofilm's extracellular matrix (6). Although this solution has the ability to diffuse through the biofilm, its efficacy might decrease because of the buffering effects of the dentin and the organic matter of the biofilm; therefore, residual bacterial biofilms appeared firmly attached to the dentin structure (31).

E. faecalis was selected in this study because it is commonly detected in the root canals of teeth associated with persistent periradicular lesions (32). It has a high binding ability to the dentin surface and the ability to grow in a biofilm style (33). Therefore, dentin discs were used as a substrate for biofilm formation. Three-week-old *E. faecalis* biofilm was shown to be mature and more resistant to disinfecting solutions than young biofilm (30, 34). In young biofilm, the bacteria are in the active and exponential growth phase, and neither the structural development of the biofilm nor the production of the extracellular polymeric matrix has been completed. Therefore, a 3-week incubation period was used in this study to insure biofilm maturation.

An *in vitro* study showed that 10 minutes of exposure to an irrigant solution is the maximum effective time to kill 3-week-old *E. faecalis* biofilms in a dentin infection model (34). Therefore, in the present study, the infected dentin discs were exposed to the irrigant solutions for 10 minutes.

In conclusion, the present study showed that 5.25% NaOCl had significantly greater antimicrobial activity against *E. faecalis* biofilms compared with OCT, CHX, and ALX. OCT was more effective than CHX and ALX.

Acknowledgments

The authors thank the College of Dentistry Research Center (PR 0020), Deanship of Research in King Saud University, and the Cell Biology Department at King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia for their support of this research.

Supported by the King Abdulaziz City for Science and Technology (GSP-36-236).

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