Comparative Evaluation of Antimicrobial Efficacy of Sodium Hypochlorite, MTAD, and Tetraclean Against *Enterococcus faecalis* Biofilm

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Abstract

The aim of this study was to compare the antimicrobial efficacy of 5.25% NaOCl, BioPure MTAD (Dentsply Tulsa Dental, Johnson City, TN), and Tetraclean (Ogna Laboratori Farmaceutici, Milano, Italy) against *Enterococcus faecalis* biofilm generated on cellulose nitrate membrane filters. After incubation, the membrane filters were transferred into tubes containing 5 mL of the selected antimicrobial solution test agent or NaCl 0.9% (positive control) and incubated for 5, 30, and 60 minutes at 20°C. After each period of time, the test agents were vortexed for 60 seconds to resuspend the microorganisms. Ten-fold serial dilutions were generated in reduced transport fluid. Each dilution was plated onto a brain heart infusion plates. The plates were then incubated for 48 hours in an aerobic atmosphere at 37°C and colony-forming units per membrane was calculated. Statistical analysis showed that only 5.25% NaOCl can disgregate and remove the biofilm at every time; however, treatment with Tetraclean caused a high degree of biofilm disgregation in every considered time intervals as compared with MTAD (T5 $p < 0.05$, T30 $p < 0.01$, and T60 $p < 0.001$). *(J Endod 2007;xx:xxx)*

Key Words

Biofilm, *Enterococcus faecalis*, irrigants

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Microorganisms are essential in the development of periradicular diseases and are the major causative factors associated with endodontic treatment failures (1). In infected and necrotic root canal systems, bacteria grow mostly in sessile biofilms, aggregates, and coaggregates in which they are embedded in an extracellular matrix material (2, 3). Biofilms are disrupted, and the microbial load is reduced by mechanical instrumentation, irrigation with tissue-lytic and microbicidal solutions, and antimicrobial medicaments in the root canal. Irrigants are used during the endodontic treatment to flush out loose debris, to lubricate the dentinal walls, to dissolve organic matter in the canal, and to have antimicrobial effects (4). Different concentrations of sodium hypochlorite (NaOCl) were used as root canal irrigants in the past 7 decades because of its well-known antimicrobial action and its ability to dissolve tissue (5).

Previous studies have shown that instrumentation and antibacterial irrigation with sodium hypochlorite would eliminate bacteria in 50% to 75% of infected root canals at the end of the first treatment session, whereas the remaining root canals contain recoverable bacteria (6, 7). In their study Nair et al. (8) showed that 88% of root canal–treated mandibular molars revealed residual infection of mesial roots after instrumentation, irrigation with NaOCl, and obturation in a one-visit treatment. Because of these limitations, a better root canal irrigant is still being searched for.

BioPure MTAD (Dentsply Tulsa Dental, Johnson City, TN) has been described as a universal irrigating solution (9). Torabinejad et al. (10) have shown that MTAD is able to safely remove the smear layer and that it is effective against *Enterococcus faecalis*, and it can also eliminate bacteria in human root canals that had been infected by whole saliva (11). A new irrigant, Tetraclean (Ogna Laboratori Farmaceutici, Milano, Italy), has been developed. It is a mixture of doxycycline hiclate at a lower concentration than MTAD, an acid, and detergents. It is able to eliminate microorganisms and smear layer in dentinal tubules of infected root canals with a final 4-minute rinse. *E faecalis* has been often isolated from teeth with endodontic failed treatments (12). Consequently, recent laboratory studies have focused on evaluating the effectiveness of root canal irrigants and medicaments against *E faecalis*. Many of these studies have grown the bacterial strains as planktonic cultures (bacteria in suspension) (10). However, planktonic bacteria do not usually comply with the in vivo growth condition found in an infected tooth in which bacteria grow as a biofilm on the dentinal wall (2). Bacterial biofilm is made by bacteria in sessile form embedded in a polysaccharide matrix. This structure makes it more difficult for drugs to reach bacteria. Therefore, all studies about the "clinical" action of endodontic irrigants should be conducted with bacteria in "biofilm form." Up to now, however, very few studies about the action of antimicrobic irrigants against biofilm have been published. As a result, recent laboratory studies have attempted to evaluate the efficacy of antimicrobial agents used in root canal treatment against *E faecalis* grown as a biofilm (13). The aim of this study was to assess the antimicrobial efficiency of endodontic irrigants against *E faecalis* biofilms.

Materials and Methods

The methodology used was adapted from Spratt et al. (14) with some modifications. Biofilms of *E faecalis* strain ATCC 29212 were generated on cellulose nitrate membrane filters. An overnight culture of *E faecalis* grown in brain heart infusion (BHI) broth (Difco Co; Becton Dickinson, Sparks, MD), adjusted to 0.5 Mc Farland

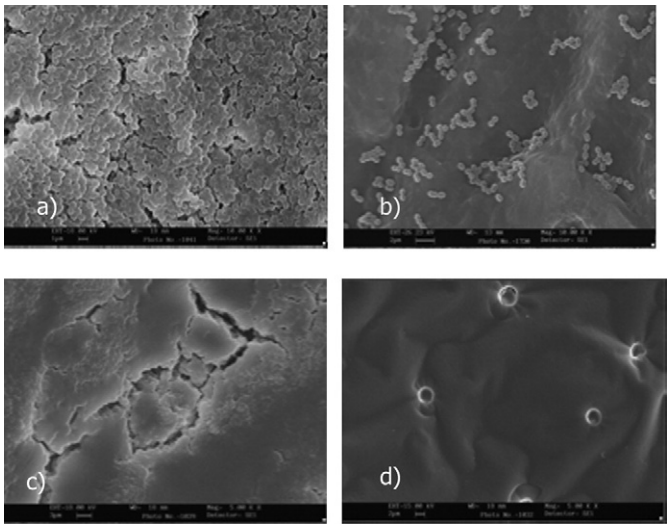

Figure 1. (a) *E faecalis* biofilm on cellulose nitrate membrane filter after 48 hours of incubation (original magnification ×10,000). (b) *E faecalis* biofilm after 30 minutes of contact with Tetraclean (original magnification ×5,000). (c) Undisgregated biofilm after 30 minutes of contact with MTAD (original magnification ×5,000). (d) Biofim completely removed after 30 minutes of contact with 5.25% NaOCl (original magnification ×5,000).

scale (1×10^8 CFU/mL), was used. An aliquot of 20 μL of *E faecalis* was seeded onto 13.0-mm diameter cellulose nitrate membrane filters (0.22-μm pore diameter; Millipore Corporation, Bedford, MA), which were placed on the surfaces of BHI agar plates. Nine membranes were used for each plate. Plates containing membranes were then incubated for 48 hours at 37°C in an aerobic atmosphere. The efficiency of the method for biofilm generation was observed in a pilot study visually and by scanning electron microscopy (SEM) (Fig. 1). After incubation, membrane filters were removed aseptically from the agar plate and transferred carefully to avoid any disruption of the biofilm into tubes containing 5 mL of the selected antimicrobial solution test agent, NaCl 0.9% (positive control), and 5.25% NaOCl (negative control) and incubated for 5, 30, and 60 minutes at 20°C. After each period of time, the membrane filters were then carefully transferred aseptically into tubes containing 5 mL of neutralizing broth (D/E Neutralizing Broth, Difco Co) for 5 minutes to stop the antimicrobial action of the test agents and vortexed for 60 seconds to resuspend the microorganisms. Ten-fold serial dilutions were generated in reduced transport fluid. Each dilution was plated onto BHI plates. The plates were then incubated for 48 hours in an aerobic atmosphere at 37°C and colony-forming units (CFU) per membrane were calculated. Controls were exposed to sterile saline for the same periods. Three replicates were performed for each antimicrobial agent and control.

Results

The results of this study are shown in Figure 2; 5.25% NaOCl was the only irrigant capable of removing biofilm after only 5 minutes, whereas the same effect was reached by Tetraclean after 60 minutes. Biopure MTAD was unable to reach this goal at every considered time. Furthermore, the use of Tetraclean was able to reduce 90% bacteric load after 5 minutes and >99.9% after 30 minutes of application. The bacterial load reduction using Biopure MTAD has not been significant after 5 minutes, whereas it has been much lower after 30 minutes than using Tetraclean or 5.25% NaOCl.

Statistical analysis was performed by using two-way analysis of variance followed by Bonferroni post hoc test. A test of homogeneity of variances revealed that data were normally distributed (Cochran, Bartlett test; T5 $p = 0.4$, T30 $p = 0.67$, and T60 $p = 0.35$). Statistical significance was set at $p < 0.05$. The overall analysis of variance revealed a highly significant either for treatment effect ($F[3,8,rsqb] = 177.87$, $p < 0.00$) or for interaction effects (treatment × time) ($F[6,16] = 5.86$, $p < 0.002$).

Bonferroni post hoc analysis showed that treatment with Tetraclean induced an increase in biofilm disgregation in every considered time intervals compared with the positive control NaCl 0.9% (T5 $p < 0.01$, T30 $p < 0.001$, and T60 $p < 0.001$), MTAD (T5 $p < 0.05$, T30 $p < 0.01$, and T60 $p < 0.001$), and NAClO 5% (T5 $p < 0.0001$, T30 $p < 0.00001$, and T60 $p < 0.00001$).

Discussion

In any natural environment, bacteria show the tendency to aggregate in adherent microbic communities. The biofilm formation is present on any surface that comes in contact with natural liquids. The formation of biofilms follows the same stages (15) starting with the formation of a conditioning film; the adhesion of planktonic bacteria takes place on this surface, and this attachment may be strengthened through polymer production and the unfolding of cell-surface structures. In the third stage, the biofilm is already formed, and, in its growth, a continuous detachment of bacteria to the planktonic phase is present. These detached cells serve as a steady source for chronic infection (16). This phenomenon starts after initial adhesion, and the number of bacteria released is related to the number of bacteria forming the biofilm. Moreover, the stage of biofilm is a defense of the microbic community against host defenses and against antimicrobic agents (17). Central to the theme of biofilm control is the use of surfactants, antimicrobial agents, and preservatives. The microbic communities grown in biofilm are remarkably difficult to eradicate with antimicrobial agents; bacteria in mature biofilm can resist the action of antibacterial irrigants, but the reasons for this resistance are not completely understood. Bacteria in biofilm are 2- to 1,000-fold more resistant than the corresponding bacteria in planktonic form (18, 19). Biofilm bacteria may also show a different phenotype that could increase an enhanced resistance, and this could be because of different metabolic pathways. Biofilm bacteria might not express the drug target; they have been found to be more resistant to amoxicillin, doxycycline, and metronidazole.

Furthermore, the "biofilm model"seems to be more realistic than the "direct contact method" to test antimicrobic agents. Spratt et al. (14) used a simple model to evaluate the activity of several irrigants against 5 different root canal bacterial isolates. This study reported that sodium hypochlorite is the most effective agent tested.

In a study published by Sena et al. (20), the capability of different irrigants with andwithout agitation against 5 strains of bacteria was

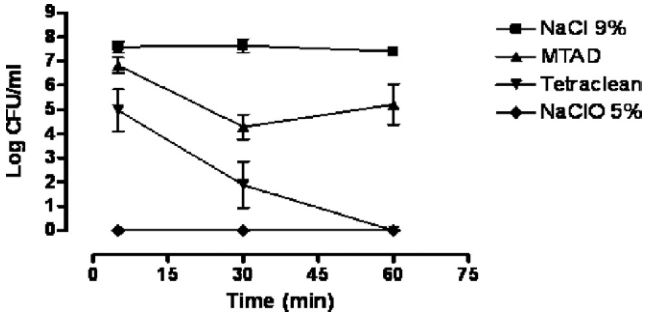

Figure 2. Bonferroni post hoc analysis showed that treatment with Tetraclean induced an increase in biofilm disgregation in every considered time intervals compared with the negative control NaCl 0.9% (T5 $p < 0.01$, T30 $p < 0.001$, and T60 $p < 0.001$) and MTAD (T5 $p < 0.05$, T30 $p < 0.01$, and T60 $p < 0.001$).

investigated. In this study, all strains were eliminated by 5.25% sodium hypochlorite after 30 seconds, both with or without agitation, whereas 2.5% sodium hypochlorite showed the same action against *E faecalis* after 5 minutes only for *E faecalis* the "agitation group." *E faecalis*, a saprophytic component of the enteric flora, is the bacteria most commonly isolated in endodontic retreatment of apical periodontitis (21) where it is often isolated either as a monoinfection or mixed with one or more species. According to Molander et al. (22), *E faecalis* can survive in a quiescent phase with low metabolic activity for a period of time. Distel et al. (23) have shown that in both short-term and long-term incubation periods, *E faecalis* colonized medicated root canals with possible biofilm formation in the long-term experiments. This is the reason why *E faecalis* is often used in studies regarding the efficiency of the endodontic irrigants in cleaning the root canal system. Two new irrigants, MTAD BioPure and Tetraclean, have recently been proposed as the final rinse in endodontic treatment. Both these irrigants are a mixture of doxycycline, citric acid, and a surfactant. Using the "direct method," a study made by measuring zones of inhibition on agar plates has shown that MTAD was as effective as 5.25% NaOCl in eradicating (10). A final rinse of MTAD, used in combination with 1.3% NaOCl as the root canal irrigant, seems to be significantly more effective than 5.25% NaOCl in disinfecting root canal systems contaminated with whole saliva (11) or with *E faecalis* (24). Furthermore, when the "biofilm method" is used, BioPure MTAD efficacy in root canal disinfection seems to be much lower. Dunavant et al. (25) have shown that only NaOCl is able to kill the whole bacteria population organized in biofilm and that its activity is strictly correlated to its concentration. The percentage of bacteria killed by BioPure MTAD was only 16.08%.

These findings could be correlated with the bacteriostatic activity of doxycycline; therefore, no destructive activity of this mixture may be present. Kho and Baumgartner (26) compared the action of 1.3% NaOCl/BioPure MTAD versus 5.25% NaOCl/17% EDTA in the apical 5 mm of teeth infected with *E faecalis*. After irrigation, the root canal apexes were resected and pulverized to expose *E faecalis* in dentinal tubules. In this study, there were no differences in antimicrobial efficacy for irrigation with 5.25% NaOCl/17% EDTA versus 1.3% NaOCl/BioPure MTAD in 5-mm apical dentinal tubules infected with *E faecalis*. In a recent study, Clegg et al. (27) reported that only 6% and 3% NaOCl were capable of disrupting and removing the biofilm, but only the 6% concentration was capable of both rendering bacteria nonviable and physically removing the biofilm; 1.6% NaOCl/BioPure MTAD was capable of disrupting but not eliminating bacteria. Tetraclean has shown a good antimicrobial activity when used against bacteria in the planktonic phase (28), but it seems to also have an excellent antimicrobial activity against *E faecalis* in an "ex vivo" model (29). Tetraclean has shown the lowest value of surface tension, and this could increase the adaptation of the mixture to dentinal walls and to biofilm (30). As seen in the results of other studies, even in the present study, 5.25% NaOCl was the only irrigant capable of eliminating biofilm at every tested time, whereas MTAD BioPure seems to be unable to remove biofilm at any tested time.

Tetraclean was able to eliminate biofilm after 60 minutes of contact, but its action of disrupting the biofilm was statistically better in every considered time interval compared with the NaCl 0.9% (positive control) and the MTAD one. Moreover, it must be emphasized that Tetraclean removes 90% of the bacteria present in biofilm after 5 minutes of contact and >99% after 30 minutes. According to Torabinejad et al. (10), the action against bacteria of these irrigants should be caused by the doxycycline present in the mixture, but this is a bacteriostatic antibiotic and it cannot kill bacteria. Moreover, BioPure MTAD contains a quantity of doxycyclin three times greater than Tetraclean, but it shows a lower effect in removing biofilm; the better action of the latter in disrupting biofilm should be caused by the synergic effect of different components of the mixture rather than the concentration of antibiotics. In any case, only 5.25% NaOCl seems to be able to remove completely the biofilm organized on the surface of the membrane, whereas "new" irrigants fail in this action; Tetraclean, compared with MTAD BioPure, shows a better action, but the goal of the total disappearance of the biofilm is reached only after 30 to 60 minutes of irrigation. This is too long for a clinical use of this irrigant; however, Tetraclean seems to cause a valid reduction in bacteria after only 5 minutes of use (Fig. 1c).

According to this work, further studies should be performed to understand the correct action and the correct sequence of different irrigants against bacteria both in the plaktonic phase and organized in biofilm on the surface of the root canal wall or inside the dentinal tubules.

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