The Synergistic Antimicrobial Effect by Mechanical Agitation and Two Chlorhexidine Preparations on Biofilm Bacteria

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Abstract

Introduction: Irrigation of the root canal with antibacterial solutions is considered an essential part of root canal treatment in endodontics. The purpose of this study was to investigate whether mechanical agitation (ultrasonic or sonic) improves the effectiveness of chlorhexidine against biofilm bacteria in vitro. Methods: Collagen-coated hydroxyapatite (CHA) disks were exposed to dispersed subgingival plaque for 3 weeks at 37°C. The multispecies biofilms established were subjected for 1 and 3 minutes to CHX-Plus (Vista Dental Products, Racine, WI) and 2% chlorhexidine (CHX), with or without mechanical agitation. After treatment, the amount of dead bacteria in biofilms was analyzed by viability staining and confocal laser scanning microscopy (CLSM). The morphology of biofilms, with or without mechanical agitation, was also examined by CLSM. Results: The structure of the biofilm did not show any obvious change when the solutions surrounding the biofilm were exposed to continuous ultrasonic or sonic agitation. The combined use of mechanical agitation and chlorhexidine had a more pronounced antimicrobial effect against the biofilms than either one alone. Sonic activation (EndoActivator; Advanced Endodontics, Santa Barbara, CA) showed the highest levels of bactericidal activity with CHX-Plus after both exposure times. The proportion of killed bacteria also depended on the type of irrigant (p < 0.001) and the time of exposure (p < 0.001). Conclusions: The low-intensity ultrasonic or sonic agitation that does not disrupt biofilm or disperse the biofilm bacteria improves the action of disinfectants against biofilm bacteria. (J Endod 2010;36:100–104)

Key Words
Antimicrobial, biofilm, chlorhexidine, CHX-Plus, confocal laser scanning microscopy, sonic, ultrasonic
reduction after root canal irrigation (16, 17) encompassed the use of colony-forming unit (CFU) counts of planktonic bacteria as the gold standard method for evaluating disinfection efficacy. However, numerous in vitro studies have shown the ability of multiple bacteria to form a biofilm architecture on root canal walls (18–20). Therefore, the aim of this study was to evaluate if such a synergistic antimicrobial effect exists between ultrasonic or sonic irrigation and irrigating solutions currently used in root canal treatment. A three-dimensional analysis through confocal laser scanning microscopy (CLSM) was used to visualize antibacterial efficacy in a setup that closely mimics the in vitro multispecies biofilm. The null hypothesis was that different irrigating solutions have a similar antimicrobial effect against biofilms.

Materials and Methods

Sterile hydroxyapatite (HA) disks (0.38-inch diameter by 0.06-inch thickness; Clarkson Chromatography Products, Williamsport, PA) were used as the biofilm substrate. The HA disks were coated with bovine dermal type I collagen (10 μg/mL collagen in 0.012 N HCl in water) (Cohesion, Palo Alto, CA). Collagen coating of the HA disks (CHAs) was performed by overnight incubation at 4°C in the wells of a 24-well tissue culture plate containing 2 mL of the collagen solution as previously described (21). Subgingival plaque on the first or second upper molars of each of three healthy volunteers was collected and mixed in Brain Heart Infusion broth (BHI). The CHA disks were placed in the wells of a 24-well tissue culture plate containing 1.80 mL of BHI. Each well was inoculated with 0.2 mL of dispersed subgingival plaque, containing a minimum of 3.2 × 10^7 CFU/mL. The disks were incubated under anaerobic conditions (AnaeroGen; OXOID, Hampshire, UK) at 37°C for 21 days; fresh medium was changed once a week.

After 21 days of anaerobic incubation in BHI broth, specimens for the CLSM were rinsed in 0.85% physiologic saline for 2 minutes to remove the culture broth. The disks were then immersed in either 2 mL of 2% chlorhexidine digluconate (CHX) freshly prepared from a 20% stock solution (Sigma Chemical Co, St Louis, MO) or CHX-Plus (Vista Dental Products, Racine, WI), which is composed of 2% chlorhexidine gluconate with wetting agents and surface modifiers for 1 or 3 minutes.

For mechanical agitation, an ultrasonic tip (E7 of Varios 350 LUX) (Nakanishi Inc, Kanuma, Japan) or an EndoActivator (sonic) tip (size 35/04) was placed 5 mm above the top of the biofilm, which was immersed in irrigant. Ultrasonic or sonic energy was set at medium power while the tip was moved back and forth at the same level above the biofilms. Samples treated with saline for corresponding time periods were used as controls. After the exposure to the disinfecting agents or saline, the specimens were gently rinsed in saline. Two biofilm disks were used for each agent at different exposure times. The LIVE/DEAD BacLight Bacterial Viability kit L-7012 for microscopy and quantitative assays (Molecular Probes, Eugene, OR) containing separate vials of the two component dyes (SYTO 9 and propidium iodide in 1:1 mixture) in solution was used for staining the biofilm bacteria following the manufacturer’s instructions. Fluorescence from the stained cell was viewed using a CLSM (Nikon Eclipse C1; Nikon Canada, Mississauga, ON). A 488-nm argon ion laser and a 543-nm HeNe laser were used for the confocal scanning; simultaneous dual-channel imaging was used to display green and red fluorescence.

CLSM images of the biofilms were acquired by the software EZ-C1 v. 3.40 build 691 (Nikon) at a resolution of 512 × 512 pixels. The mounted specimens were observed by using a 0.5–μm step size. Confocal LIVE/DEAD images were analyzed and quantified by using the MeVisLab package (available from www.mevislab.de/). The volume ratio of red fluorescence (dead cells) to green and red fluorescence (dead and live cells) indicated the portion of killed cells for each medicament.

Figure 1. Three-dimensional constructions of CLSM scans of 3-week-old multispecies biofilms after treatment with mechanical agitation and/or disinfecting agents for 1 minute. Biofilm treated with the (A) combination of EndoActivator and physiologic saline, (B) CHX-Plus treatment, (C) the combination of EndoActivator and 2% CHX treatment, and the (D) combination of EndoActivator and CHX-Plus treatment. Green, viable cells; Red, dead cells.
After segmentation and reconstruction of the data, the distribution of microbial populations in the three-dimensional biofilm model was analyzed. The method of evaluation of antimicrobial effect against biofilm was described in a previous study (21). The results were subjected to univariate analysis or a t test, when necessary, at a significance level of p < 0.05.

Results

The CLSM observations revealed that the biofilm adhered firmly to the surface of collagen-coated HA. A confluent mixture of some single cells and an abundance of clusters of cells were seen, with occasional small lacuna. Ultrasonic or sonic agitation did not cause any obvious changes in the structure of biofilm (Fig. 1).

The use of sonic or ultrasonic vibration alone had no effect on bacterial viability in 3-week-old biofilm (Fig. 1 and Table 1). The combined use of ultrasonic or sonic vibration and chlorhexidine showed a better antimicrobial effect against biofilms than chlorhexidine alone. The volume of killed cells was significantly correlated with the time of exposure, the type of medicament, and treatment groups (sonic, ultrasonic, and no mechanical agitation) (p < 0.001). The volume of killed cells by CHX-Plus was significantly higher than by 2% CHX at both time periods in both the ultrasonic and the sonic group (p < 0.001). The proportion of killed cells increased significantly with the increasing time of medicament exposure (1 and 3 minutes). For 2% CHX, sonic agitation showed a higher level of bactericidal activity than ultrasonic agitation at 1 minute (p < 0.05) and 3 minutes of exposure time (p < 0.05). In the CHX-Plus group, sonic agitation and ultrasonic agitation were equally effective at 1 minute (p > 0.05), and both were significantly more effective than the control group and all CHX groups (p < 0.001).

Discussion

Although in vivo effectiveness is the ultimate test for the performance of antibacterial solutions, in vitro testing can also provide useful information about the potency and spectrum of the activity of the various substances. In vitro tests generally allow better control of the experimental conditions than in vivo testing. The multispecies biofilm model used in the present study closely mimics the in vivo biofilm (21) and allowed standardized comparison of the efficacy of the antimicrobial agents. This in vitro biofilm model is not a perfect replication of the root canal biofilm; however, it does capture some key characteristics of the in vivo situation, including the thickness of the biofilm and the presence of spirochetes (21). The bacteria were attached to each other and to the collagen coated hydroxyapatite as in endodontic biofilms.

During this study and pilot experiments, the minimum distance between the ultrasonic (or sonic) tip and the biofilm surface, at which mechanical agitation did not disrupt or disperse the bacteria, was defined as 5 mm. Scanning electron microscopy indicated that the biofilm with no treatment and after exposure to ultrasonic and sonic agitation was mostly intact (data not shown). If the ultrasonic vibration is brought to direct contact with the biofilm or close enough to cause detachment or disruption of the biofilm, it is no longer possible to quantitatively measure the effectiveness of the disinfecting solutions by CLSM. Although the enhancement of antibacterial activity by ultrasonic or sonic agitation defined as the “bioacoustic effect” in the present and previous studies is beyond dispute (22, 23), the precise mechanisms of the enhanced killing have not been identified and may be different in each situation. The enhancement is often speculatively attributed to ultrasonic and sonic vibrational interactions with bubbles (cavitation events) (24), to a reduction in boundary layer thickness...
because of turbulence or microconvection (25), to “oscillatory enhanced dispersion” caused by oscillatory flow in channels (26), or to increased microconvection from ultrasonic and sonic heating (25, 27). Irrespective of the mechanism of effect, even at a 5-mm distance as in the present model, both ultrasonic and sonic activation improved the effectiveness of both chlorhexidine solutions in biofilm killing. This may be promising with regard to the effect of the two types of agitation in the root canal system in areas far beyond the reach of the tip of the sonic/ultrasonic instruments.

Biofilms are a preferred way of microbial existence because they provide protection against existing physical forces and chemical attack. The biofilm structure is especially important for the calculation of diffusion rates of antimicrobials or nutrients through a biofilm (13) and for the evaluation of the mechanical properties of a biofilm (28). Microscopy, scanning electron microscopy, and colony-forming unit counts have been used for the evaluation of the antimicrobial effect against the biofilm by various substances. The analysis of microbial biofilms by CLSM yields stacks of digital images that can be combined to give a three-dimensional view of the biofilm. In the present study, mechanical agitation was effective in enhancing the efficacy of the chlorhexidine products, whereas ultrasonics and sonics alone, under these conditions, did not influence bacterial viability in the biofilms. A previously proposed hypothesis as to why the ultrasonic produced more cell killing was that the ultrasonic was breaking up the biofilm and allowing antibiotic access to the interior parts of the biofilm. However, the confocal microscopy observations in the present study showed that the application of ultrasonic and sonic vibration did not change the structure of the biofilm or the spatial arrangement of the cells. The bioacoustic effect in this study may be related to the enhancement of disinfectant transport to deeper layers of the biofilm or through the cell membrane. Carmen et al (29) found that ultrasonic significantly increased transport of gentamicin across Escherichia coli and Pseudomonas aeruginosa biofilms that normally blocked or slowed gentamicin transport when not exposed to ultrasound. This enhanced transport may be partially responsible for the increased killing of biofilm bacteria exposed to combinations of antibiotic and mechanical agitation.

Sonic irrigation is different from ultrasonic irrigation because it operates at a lower frequency (1–6 kHz) and produces a smaller shear stress. Some studies have reported that passive ultrasonic irrigation removed more dentin debris from the root canal than did sonic irrigation because there was a positive relationship between streaming velocity and frequency (5, 8, 30). In fact, the antimicrobial effect cannot be described by a simple relationship between velocity, gas fraction, and bubble size but involves the interactions between these variables. Visual observation of more bubbles exiting along the EndoActivator file during irrigation indicates that bubbles do not exit in a perfect linear stream, but their flow is turbulent and chaotic, thus creating a column of bubbles instead of a line of bubbles is a better result. The formation of microbubbles gradually increases in diameter until they collapse provoking very effective small implosions, which produce an irregular agitation of the irrigant. On the other hand, the end of the tip of the EndoActivator has a bigger amplitude than the upper part. Moreover, the oscillating patterns of the sonic devices are different compared with ultrasonically driven instruments. When the EndoActivator tip was placed 5 mm over the top of the biofilm in this study, the acoustic stream was connected to the rapid movement of the irrigating solution in a vortex around biofilm.

In addition to mechanical agitation, the effectiveness of killing biofilm bacteria is dependent on the chemistry of the antibacterial agent and the contact time. The differences in the physical properties of CHX-Plus and CHX may also have an effect on the transmission of ultrasonic and sonic energy by the irrigant. Besides chlorhexidine gluconate, CHX-Plus contains modifiers to reduce the surface tension. Recent studies (21, 31) showed that CHX-Plus killed bacteria much faster in monospecies or multispecies biofilm than 2% CHX. Furthermore, in the present study, much more bubbles were formed in CHX-Plus, particularly smaller bubbles, and they are less prone to coalesce than bubbles in CHX. These factors may partly explain why CHX-Plus appears to be more efficient than CHX during mechanical agitation. It is not surprising that the amount of chlorhexidine crossing the biofilm increased with time because more time was allowed for chlorhexidine to accumulate in the biofilm. More work remains to be done to identify and take advantage of ultrasonic or sonic agitation in enhancing the action of disinfectants against biofilms.

Whatever the molecular mechanism may be, these results have shown that a synergistic relationship exists between ultrasonic or sonic agitation and chlorhexidine enhancing the antimicrobial effect against biofilm bacteria. The enhanced antimicrobial activity did not negate the normal absorption or the binding interactions between the disinfectant and components of the biofilm; these probably occur as usual. Several additional local factors in the root canal environment may affect the function of the various disinfecting solutions. Therefore, conclusions from the present study must be drawn with caution.

Acknowledgments

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References