Effect of Long-term Exposure to Endodontic Disinfecting Solutions on Young and Old *Enterococcus faecalis* Biofilms in Dentin Canals

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Abstract

Introduction: The purpose of this study was to evaluate the antimicrobial activity on *Enterococcus faecalis* biofilms in dentin canals of short-term and long-term exposure to different endodontic disinfecting solutions by using a dentin infection model and confocal laser scanning microscopy. **Methods:** Dentinal tubules in semi-cylindrical dentin blocks were filled with *E. faecalis* by centrifugation and incubated to form 1-day-old and 3-week-old biofilms. The young and mature biofilms in dentin were subjected to sterile water, 2% chlorhexidine, 2% sodium hypochlorite (NaOCl), and 6% NaOCl for 3, 10, and 30 minutes. After treatments, the proportion of bacteria killed by the disinfectants was analyzed by confocal laser scanning microscopy by using LIVE/DEAD bacterial viability stain. **Results:** The proportion of killed bacteria was lower after 3 minutes than after 10 and 30 minutes of exposure to the disinfecting agents ($P < .05$). The killing of bacteria in the *E. faecalis* biofilms was fastest during the first 3 minutes and slowed down greatly after 10 minutes. Six percent NaOCl was the most effective antibacterial solution against both the 1-day-old and 3-week-old biofilms ($P < .05$). No significant difference in bacterial killing was detected between 2% chlorhexidine and 2% NaOCl ($P > .05$). Significantly more cells were killed in young biofilms than in old biofilms in all groups ($P < .05$). **Conclusions:** The killing of bacteria in infected dentin by disinfecting solutions is time-dependent. However, little additional killing is obtained after the first 10 minutes of exposure. *(J Endod 2014;40:509–514)*

Key Words

Biofilm, chlorhexidine, dentinal tubules, *Enterococcus faecalis*, sodium hypochlorite

The etiologic role of intracanal microorganisms and their by-products is well-established in the development and progression of pulpal and periapical diseases (1–4). There is also evidence suggesting that periapical healing occurs at a higher rate in teeth with bacteria-free root canals after endodontic treatment (5). Therefore, one of the most important goals of root canal treatment is to eliminate bacteria from the infected root canal systems through instrumentation and use of disinfecting agents (6). However, in many if not most cases, some bacteria may still persist in the root canal system.

Dentin is considered to be a mineralized connective tissue that is porous because of the presence of dentinal tubules (7). Several microorganisms can invade radicular dentinal tubules, where they are better protected from intracanal irrigants and medicaments (7–12). *Enterococcus faecalis* is a gram-positive, facultatively anaerobic coccus and a frequently isolated species in root-filled teeth with persistent periapical lesions. Its ability to attach itself to dentin, invade dentinal tubules, and form biofilm communities contributes to its resistance to irrigating solutions and intracanal medicaments (13). For these reasons, *E. faecalis* is often chosen to induce *in vitro* bacterial biofilms in the dentinal tubules for disinfection studies.

In recent years, several advances have been made in the study of bacteria and bacterial biofilms in dentin by using confocal laser scanning microscopy (CLSM). Zapata et al (14) showed that the discrimination between live and dead bacteria in infected dentinal tubules could be done by CLSM after staining with the cell viability dyes fluorescein diacetate and propidium iodide (PI). Parmar et al (15) demonstrated that green and red fluorescent bacteria were visible within the dentinal tubules of infected root sections when examined by CLSM. A novel noninvasive model of infected dentinal tubules was established by Ma et al (16) to test the effectiveness of dentin disinfection by using CLSM. The portion of dead *E. faecalis* cells after 1-minute and 3-minute exposure to disinfecting agents was presented as the volume ratio of red fluorescence to green-and-red fluorescence in 3-dimensional (3D) reconstructions. Only short-term exposure times to the disinfecting agents were used in this and subsequent studies that used the new model (16–18). The proportion of cells killed by 6% sodium hypochlorite (NaOCl) in 1 or 3 minutes was 35% and 61%, respectively, which means that many bacteria were still alive (18). It is therefore hypothesized that long-term exposure to disinfection solutions may kill more bacteria cells, even to complete elimination of viable bacteria. However, there are no data available about the antibacterial effect of long-term exposure by the disinfecting agents against bacterial biofilms in infected dentinal tubules.

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Therefore, the aim of this present study was to evaluate the antibacterial effectiveness against *E. faecalis* biofilms in dentin canals of short-term and long-term exposure to 2% chlorhexidine (CHX) and 2% and 6% NaOCl (3, 10, and 30 minutes) as revealed by CLSM and viability staining.

Materials and Methods

Preparation of Dentin Specimens

Approval to collect caries-free single-rooted human teeth extracted for orthodontic reasons was obtained from the Science and Ethics Commission of Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China. Twenty-four teeth were kept in 0.01% NaOCl solution at 4°C until processed further (19). By using a hand curette, soft tissue and bone fragments were removed from the root surfaces. According to a previously described protocol (16), a cylindrical root dentin block was horizontally sectioned from each single-rooted tooth at 1 mm below the cementoenamel junction by using a 0.6-mm-thick precision diamond saw (Isomet 5000; Buehler Ltd, Lake Bluff, IL) at 1000 rpm with water cooling to a uniform length of 4 mm. The root canals inside the blocks were enlarged to 1.5 mm with a size #6 Gates Glidden drill (Tulsa Dentsply, Tulsa, OK) at 300 rpm under water cooling. A thin groove was made in the middle of the cylindrical specimen by a low-speed handpiece with a small round bur (Tulsa Dentsply), and each dentin block was fractured with a blade and a hammer into 2 semi-cylindrical halves. The outer surface of the 48 halves was ground by 600-grit silicon carbide paper (Carbine; Buehler Ltd) to remove the root cement and to obtain a standard thickness of 2 mm. To make the dentin half fit to the inner wall of a filter tube with a 0.45-μm pore size (Pall Corporation, Ann Arbor, MI), 48 dentin specimens were shaped by using a low-speed handpiece with a fine carbide bur (Tulsa Dentsply) at 300 rpm under water cooling. Finally, the refined sample was approximately 4 × 4 × 2 mm.

The samples were rinsed with 5.25% NaOCl and 6% citric acid (pH 4.0) for 4 minutes in an ultrasonic bath (Sankei Giken Industry Co Ltd, MIE, Tokyo, Japan) to remove the smear layer and then immersed in sterile water for 1 minute. Each semi-cylindrical half with the canal side up was placed in a filter tube. Composite resin (Kerr Co, Orange, CA) was used to seal any gap between the specimen and the inner wall of the tube and light-cured for 20 seconds.

Infection of Dentin with *E. faecalis*

E. faecalis VP3-181 was grown on brain-heart infusion (BHI) agar (Becton-Dickinson, Sparks, MD) plates at 37°C in air overnight. The bacteria were harvested, checked for purity, and suspended in BHI broth. The cell density was adjusted in a spectrophotometer to 3×10^6 colony-forming units/mL (optical density at 405 nm = 0.05) in BHI broth.

Following a protocol described in detail by Ma et al (16), 500 μL of bacterial suspension was transferred into each filter tube including 1 semi-cylindrical dentin piece. The tubes were centrifuged at 5000, 6000, 8000, and 10000 rpm in a sequence twice each for 5 minutes. The bacterial suspension that penetrated the dentin piece into the lower compartment was discarded, and a new 500 μL of bacterial suspension was added to the upper compartment for the next centrifugation. Twenty-four randomly selected filter tubes with the dentin piece were incubated in sterile BHI broth in air at 37°C for 1 day, whereas another 24 tubes were incubated for 3 weeks under the same conditions to allow biofilm growth and maturation in the dentinal tubules. Fresh broth was changed once a week for the 3-week specimens.

Disinfection of Dentin with Irrigants

After incubation for 1 day or 3 weeks, the infected dentin pieces were taken out of the filter tubes, the surrounding composite was removed, and they were washed with sterile water for 1 minute and air-dried. The cemental side of each dentin specimen was sealed with nail varnish to close the open dentin canals. The 48 infected dentin halves with 1-day-old and 3-week-old *E. faecalis* biofilms were randomly allocated to the following 4 different disinfecting medications, with 6 specimens in each group: sterile water (control), 2% CHX (Sigma-Aldrich, St Louis, MO), 2% NaOCl (Canada Safeway Ltd, Calgary, Alberta, Canada), and 6% NaOCl (Canada Safeway Ltd). A droplet of 50 μL of each disinfecting solution was placed on the root canal wall of the specimens. Fifty microliters fresh CHX or NaOCl solution was added every 5 minutes in the 10-minute and 30-minute groups. The specimens were washed with sterile water for 1 minute and fractured vertically through the root canal into 2 halves to expose a fresh surface of longitudinally fractured dentinal tubules. The fracture surfaces were examined with CLSM and viability staining as previously described (16).

Examination with CLSM

The LIVE/DEAD BacLight Bacterial Viability kit L-7012 (Molecular Probes, Eugene, OR) containing SYTO 9 and PI was used to stain live and dead biofilm bacteria in the infected dentinal tubules following the manufacturer's instructions (20). Bacteria with intact cell membranes stain fluorescent green with SYTO 9, whereas bacteria with damaged membranes stain red with PI. The excitation/emission maxima for the dyes are approximately 480/500 nm for SYTO 9 and 490/635 nm for PI. A total of 96 dentin specimens were rinsed with phosphate-buffered saline for 1 minute and then were viewed by CLSM (Nikon Eclipse C1; Nikon Canada, Mississauga, ON, Canada) by using a 20× lens with an additional zoom of 2×.

Five random areas of 0.3 × 0.3 mm starting from the border of root canal were selected for CLSM examination and 3D reconstruction analysis in each specimen. A stack of 20 slices (0.5-μm step size) was obtained for each scan. Altogether, 480 scans were performed of the 1-day-old or 3-week-old infected dentin specimens. The CLSM images at a resolution of 512 × 512 pixels were captured by EZ-C1 v. 3.40 build 691 software (Nikon). The live cell volume (green fluorescence) and dead cell volume (red fluorescence) were obtained semiautomatically by using Imaris 7.2 software (Bitplane Inc, St Paul, MN). The thresholds of the red and green fluorescences were manually set according to the raw intensity of the confocal files, and the 3D reconstruction and volume calculation were performed automatically by the software. The proportion of dead cell volume was calculated with the volume ratio of red fluorescence to green-and-red fluorescence (16–18). Univariate analysis of variance was applied to analyze the differences between the proportions of dead cell volume by different disinfection by using SPSS 16.0 software (SPSS Inc, Chicago, IL). Post hoc multiple comparisons were used to isolate and compare the results at a significance level of $P < .05$.

Results

The presence of *E. faecalis* deep in the dentinal tubules of the dentin specimens incubated for 1 day or 3 weeks was verified by CLSM scans and 3D reconstructions (Figs. 1 and 2). The proportion of dead cells varied from 24% to 88% in the disinfectant groups, whereas only 2%–5% of the cells were dead in the control group (sterile water only) in 1-day-old and 3-week-old biofilms.

The proportion of dead *E. faecalis* cell volume depended on the exposure time, type and concentration of the disinfecting solution, and age of the bacterial biofilm (Table 1). Significantly more bacteria in

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