Dentin Extends the Antibacterial Effect of Endodontic Sealers against *Enterococcus faecalis* Biofilms

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Abstract

Introduction: The purpose of this study was to evaluate the antimicrobial effects of root canal sealers on *Enterococcus faecalis* biofilms in dentinal tubules by using a novel dentin infection model. **Methods:** Cells of *E. faecalis* were introduced into the dentinal tubules by centrifugation and incubated in brain-heart infusion broth for 3 weeks. An equal thickness of AH Plus, Endosequence BC sealer (BC sealer), and pulp canal sealer EWT (PCEWT) was placed on the root canal wall of the dentin specimens for 1, 7, and 30 days in humid conditions at 37°C. Gutta-percha and water were used in a similar manner as the tested sealers. The proportions of dead and live bacteria inside the dentinal tubules after exposure to root canal sealers were assessed by confocal laser scanning microscopy. **Results:** Significantly more bacteria were killed in the 3 sealer groups than in the 2 control groups ($P < .05$). BC sealer and AH Plus resulted in significantly more dead cells than PCEWT did. There was no statistically significant difference between BC sealer and AH Plus at any time point ($P > .05$). Thirty days of exposure to BC sealer and AH Plus resulted in significantly more dead bacteria in dentin than 7-day and 1-day exposures in the biofilms, whereas no statistically significant increase of the proportion of dead bacteria was detected between 7-day and 30-day PCEWT ($P > .05$). **Conclusions:** The 3 endodontic root canal sealers had antibacterial effects against *E. faecalis* in the dentinal tubules. BC sealer and AH Plus had superior antibacterial effects compared with PCEWT. The antibacterial effects of sealers in dentinal tubules continued after setting. *(J Endod 2014;40:505–508)*

Key Words

Confocal laser scanning microscopy, dentin, disinfection, *Enterococcus faecalis*, sealer

One of the main goals of endodontic treatment is to eliminate microorganisms from the infected root canal system. This is achieved through mechanical cleaning, irrigation with antibacterial solutions, as well as temporary sealing between appointments and adequate filling of the root canal (1). However, root canal treatment reduces but does not necessarily eliminate all microbes, and viable bacteria often remain in the dentinal tubules and lateral canals. Therefore, the use of root filling with sealers having antibacterial activity is considered beneficial in the effort to further reduce the number of remaining microorganisms or even eradicate the infection completely (2).

In earlier studies, the agar diffusion test was commonly used to assess the antimicrobial activity of endodontic sealers (3, 4). However, the test is no longer recommended for this purpose because of its lack of reliability (5). The agar diffusion test has since been replaced by the direct contact test (DCT), which better reflects the true antimicrobial potential of the various sealers in standardized settings (6). However, DCT also has limitations in predicting clinical performance because several important factors such as microanatomy and chemistry of the tooth and biofilm formation are not part of the experimental setting (7, 8). Although many DCT studies have found several sealers to be effective against *Enterococcus faecalis* (4, 6, 9), there is little evidence so far of their effectiveness against the microorganisms in infected dentin. By using bacterial culturing of powdered dentin, Saleh et al (10) reported that root filling with AH Plus or Grossman's sealer killed all *E. faecalis* cells in dentin at least to a depth of 300 μm.

Recently, a new dentin infection model was introduced to establish a standardized and deep penetration of bacteria into dentinal tubules by centrifugation (11). This model is used to measure the effectiveness of disinfecting solutions against *E. faecalis* biofilms in dentin and has produced reproducible results in standardized settings by using viability staining and confocal laser scanning microscopy (CLSM) (12, 13). Hence, the application of this new model might be suitable to provide a predictable platform for the measurement of antibacterial activity of endodontic sealers in infected dentin.

In the present study, the antibacterial effects of BC sealer, AH Plus, and pulp canal sealer EWT were tested against *E. faecalis* in dentinal tubules by using the new dentin infection model and CLSM.

Materials and Methods

Dentin Preparation

Fifteen single-rooted teeth extracted for orthodontic reasons were collected under a protocol approved by the ethics committee of the university. According to a previously described protocol (11), 30 semicylindrical dentin halves $4 \times 4 \times 2$ mm in size were fractured and shaped, followed by immersing in 5.25% NaOCl (EMD Chemicals Inc,

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Darmstadt, Germany) and 6% citric acid (Sigma-Aldrich, St Louis, MO) (pH 4.0) each for 4 minutes. The prepared dentin specimens, with their canal sides up, were placed on the bottom of the upper chamber of a Nanosep microfiltration tube (Pall Corp, Ann Arbor, MI). The gaps between the inner tube and the dentin specimen were sealed with a Revolution Formula 2 composite filling material (Kerr Co, Orange, CA) and light-cured for 20 seconds.

Dentin Infection

Enterococcus faecalis VP3-181 (14) was used as the test organism and grown in air at 37°C overnight on brain-heart infusion (BHI) agar (Becton-Dickinson, Sparks, MD) plates. The bacteria were suspended in BHI broth (Becton-Dickinson) and standardized spectrophotometrically to 3×10^6 colony-forming units/mL. *E. faecalis* suspension was sequentially centrifuged into the dentinal tubules in accordance with a previously described detailed protocol (11). All microfiltration tubes with infected dentin specimens inside were incubated at 37°C in BHI broth, which was changed weekly. After 3 weeks, the dentin specimens were removed from the tubes and rinsed in sterile water for 1 minute and air-dried. The outer surfaces (cemental sides) of the specimens were closed by nail varnish.

Sealer Placement

Three different types of endodontic sealers, Endosequence BC sealer (Brasseler USA, Savannah, GA) used as the bioceramic sealer, AH Plus (Dentsply International Inc, York, PA) used as the epoxy resin sealer, and pulp canal sealer EWT (PCEWT) (SybronEndo, Glendora, CA) used as the zinc oxide–eugenol sealer, were studied. Sterile water and gutta-percha (Brasseler USA) without sealer were used as control groups. Thirty dentin specimens were randomly divided into 5 groups with 6 specimens in each group. All sealers were prepared in strict compliance with the manufacturers' instructions. Each freshly prepared sealer was placed on the dentin surface of the root canal wall to achieve an approximate thickness of 0.5 mm. For the gutta-percha control group, gutta-percha points were cut to match the width of the semicylindrical dentin halves and pressed against the root canal wall of the specimens. In the water control group, 50 μL sterile water was placed on the dentin surface. All dentin samples were placed at 37°C in 100% relative humidity for 1, 7, and 30 days. Two semicylindrical dentin halves (4 samples after fracturing the halves) of each group were examined at each time point by CLSM and viability staining to determine the proportions of live and dead bacteria.

CLSM Examination

For the CLSM, the sealer was scraped off the root canal wall dentin. The specimens were rinsed in sterile water for 1 minute and vertically fractured through the root canal into 2 halves to expose a fresh surface of longitudinally fractured dentinal tubules for CLSM examination as previously described (11).

The fluorescent LIVE/DEAD BacLight Bacterial Viability stain (Molecular Probes, Eugene, OR) containing SYTO 9 and propidium iodide was used for staining of a total of 60 fractured dentin specimens following the manufacturer's instruction. A confocal laser scanning microscope (Nikon Eclipse C1; Nikon Canada, Mississauga, ON, Canada) was used to view the fluorescence from the stained cells. The mounted specimens were observed by using a 20× lens with an additional zoom of 2×. Four randomly selected areas on the border of the root canal were chosen on each of the 4 specimens in each group per time point for CLSM scanning. Images were acquired by the software EZ-C1 v.3.40 build 691 (Nikon) at a 512 × 512 pixel scan area. A stack of 20 slices (0.5-μm step size) was scanned at the 4 areas (0.30 × 0.30 mm for each area) on each sample. The raw data of CLSM were processed by Imaris 7.2 software (Bitplane Inc, St Paul, MN). The threshold of the red and green fluorescences was manually set according to their raw intensity and kept consistent for each sample. Live/dead ratios of the infected dentinal tubules were then automatically calculated by the software. The volume ratio of red fluorescence to green and red fluorescence indicated the proportion of killed cells. The proportions of dead cell volume after exposure to different sealers at different setting times were subjected to univariate analysis of variance by using SPSS 16.0 (SPSS Inc, Chicago, IL). Post hoc multiple comparisons were used to isolate and compare the results at a significance level of $P < .05$.

Results

A homogenous and dense penetration of *E. faecalis* deep into the dentinal tubules of the dentin specimens from the root canal was confirmed by CLSM (Fig. 1). From 5% to 7% of the bacteria inside the dentin were dead in the control groups exposed to sterile water and gutta-percha at each time point examined (Table 1) (Fig. 1*A1–B3*). The 3 sealers killed significantly more bacteria than control groups at 1, 7, and 30 days ($P < .05$). The proportion of killed bacteria increased during the 30 days of exposure to the 3 sealers. BC sealer and AH Plus sealer showed statistically significant increase of bacterial killing at all 3 time points ($P < .05$) (Table 1) (Fig. 1*C1–D3*); however, for the PCEWT sealer the difference between the 7-day and 30-day time points was not significant ($P > .05$) (Fig. 1*E2* and *E3*). At 30 days, almost half of the bacteria were killed by BC sealer (45%) and AH Plus (46%). Dead bacteria could be seen throughout the 200-μm-deep scanned dentin area (Fig. 1*C3* and *D3*). No statistically significant difference in bacterial killing was found between BC sealer and AH Plus ($P > .05$) (Table 1). In the PCEWT group, fewer bacteria were killed than in the BC sealer and AH Plus groups at each time interval (Table 1) (Fig. 1*C1–E3*).

Discussion

Antimicrobial properties of sealers may help to eliminate residual microorganisms after chemomechanical preparation of the root canal system. One of the challenges in endodontic research has been the lack of standardized protocols for the testing of the antimicrobial effect of sealers. As a quantitative and reproducible approach, DCT is able to simulate the contact of the test microorganism with endodontic sealers and measure bacterial growth in the presence and absence of the tested materials (9). Thus, the effect of sealers on microbial viability at various stages of the setting reaction can be examined by DCT, providing information on both bactericidal and bacteriostatic effects (5, 6). However, limitations also exist with the DCT method because it does not provide opportunity for the consideration of factors such as microanatomy and chemistry of the tooth and biofilm formation.

The survival of bacteria may be partly attributed to their invasion into dentinal tubules where biofilms can be formed; thus, bacteria in dentin may be protected from disinfecting agents in the root canal and also from the direct antibacterial effect of sealers. Heling and Chandler (15) evaluated the antibacterial activity of 4 root canal sealers by using a dentin block model to measure the optical density of the infected dentin powder-containing broth (16). Although this study provides information on the antimicrobial effect inside dentinal tubules, the original dentin structure was destroyed in the process of bacterial collection. In addition, culturing is not an optimal method to verify non-logarithmic differences between samples. By also using bacterial culturing from dentin chips obtained by bur, Saleh et al (10) reported complete killing of *E. faecalis* from dentin up to a depth of 300 μm.

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