Combined Antibacterial Effect of Sodium Hypochlorite and Root Canal Sealers against *Enterococcus faecalis* Biofilms in Dentin Canals

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Abstract

Introduction: The present study aimed to evaluate the antibacterial effect of the combined use of sodium hypochlorite (NaOCl) and root canal sealers on *Enterococcus faecalis* biofilms using a dentin infection model. **Methods:** Cells of *E. faecalis* were introduced into the dentinal tubules by centrifugation and incubated in brain-heart infusion for 3 weeks. The biofilms in dentin were first subjected to 5% NaOCl or sterile water for 10 minutes followed by an equal thickness of AH Plus (Dentsply International Inc, York, PA), Endosequence BC Sealer (Brasseler USA, Savannah, GA), or MTA Fillapex (Angelus Indústria de Produtos Odontológicos S/A, Londrina, Brazil) placed on the root canal wall of the dentin specimens for 7, 30, and 60 days. Gutta-percha and water were used in a similar manner as controls. The proportions of dead and live bacteria inside the dentinal tubules were assessed by confocal laser scanning microscopy and viability staining. **Results:** The combined use of NaOCl and sealers (30 and 60 days) killed significantly more bacteria than NaOCl or sealers alone ($P < .05$). NaOCl + MTA Fillapex was the most effective antibacterial combination by killing 83% bacteria in dentin tubules in 60 days. Thirty and 60 days of exposure to the sealers resulted in significantly more dead bacteria in dentin biofilms than 7-day exposures ($P < .05$). **Conclusions:** The placement of root canal sealer after NaOCl treatment enhanced antibacterial effects against *E. faecalis* in the dentinal tubules. Little additional effect was obtained after 30 days of exposure to sealers. *(J Endod 2015;41:1294–1298)*

Key Words

Confocal laser scanning microscopy, dentin, *Enterococcus faecalis*, sealer, sodium hypochlorite

A critical objective of endodontic treatment of a tooth with apical periodontitis is to eliminate the microorganisms within the root canal system. Bacteria and their by-products are important etiologic factors in pulpal necrosis and periapical pathosis (1, 2). Although sodium hypochlorite (NaOCl) is well-known as an effective disinfectant with a wide antimicrobial spectrum in root canal treatment (3), evidence suggests that persistent microorganisms after endodontic treatment can survive and interfere with healing (4). Different microorganisms in the root canal infection may invade dentinal tubules where the viable bacteria may be better protected from intracanal irrigants than in the main canal (5–7). The use of root canal sealers with antibacterial activity is assumed to further reduce the number of remaining microorganisms, aiming to maximize killing of bacteria in infected dentin.

Many different techniques have been used to measure the effectiveness of treatment procedures against bacteria in dentinal tubules (5, 8–10). One of the major challenges with the traditional dentin block model has been the difficulty to obtain a strong, deep, and comparable infection in dentin. The lack of a predictable, heavy presence of bacteria in dentin canals makes it difficult to measure the comparative effectiveness of various disinfecting agents (9, 11). Therefore, a new model of dentin infection was developed to secure a predictable, dense, and deep penetration of bacteria in dentin canals using centrifugation (11). Results obtained using this model and viability staining with confocal laser scanning microscopy (CLSM) have shown reproducible data on dentin disinfection in different conditions, including different biofilm age (12), length of disinfectant exposure (13), and combinations of disinfecting agents (14).

The antimicrobial effect of NaOCl (13, 15) and root canal sealers (16) in dentin infection has recently been reported using the dentin infection model, showing up to 78% and 46% of bacterial killing, respectively, depending on the time of exposure and NaOCl concentration. Therefore, it is hypothesized that the use of both of these strategies may have an additive or synergistic effect on the killing of bacteria cells. So far, such evidence is lacking.

By using this dentin infection model, the present study aimed to evaluate the long-term antibacterial effectiveness of NaOCl followed by different root canal sealers against *E. faecalis* biofilms in dentin using viability staining and CLSM.

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Materials and Methods

Dentin Specimen Preparation

Thirty-two single-rooted caries-free 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), 64 semicylindrical dentin halves were fractured and shaped to $4 \times 4 \times 2$ mm in size. The cementum layer on the external dentin surface was removed. The smear layer on both sides of the specimen was removed by immersion in 5% NaOCl (EMD Chemicals Inc, Darmstadt, Germany) and 6% citric acid (Sigma-Aldrich, St Louis, MO) (pH = 4.0) each for 4 minutes in an ultrasonic bath (Sankei Giken Industry Co Ltd, Tokyo, Japan).

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 with *E. faecalis*

E. faecalis VP3-181 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 harvested and suspended in BHI broth. Cell density was spectrophotometrically standardized to 3×10^6 colony-forming units/mL. Following a protocol described in detail by Ma et al (11), 500 μL *E. faecalis* suspension in BHI broth was added to each filter tube with the dentin specimen inside. The tubes were centrifuged at 1400*g*, 2000*g*, 3600*g*, and 5600*g* in a sequence twice each for 5 minutes. A fresh solution of bacteria was added between every centrifugation. All tubes were then incubated at 37°C in BHI broth in air for 24 hours after centrifugation to facilitate bacterial recovery. Filter tubes with infected dentin specimens were incubated for 3 weeks under the same conditions to allow biofilm growth and maturation in the dentinal tubules. Fresh BHI broth was changed once a week.

Disinfection of Dentin with NaOCl

The dentin specimens were taken out of each tube, and the surrounding composite was removed, rinsed in sterile water for 1 minute, and air dried. The outer surfaces (cemental sides) of the specimens were sealed with nail varnish. The 64 infected dentin halves with 3-week-old *E. faecalis* biofilms were randomly allocated to 2 major groups, 5% NaOCl and sterile water, with 32 specimens in each group. A droplet of 50 μL NaOCl or water was placed on the root canal wall of the specimens for 10 minutes; a new droplet was added after 5 minutes of exposure. The specimens were then rinsed with sterile water for 1 minute.

Sealer Placement

Three different endodontic sealers, AH Plus (Dentsply International Inc, York, PA), Endosequence BC Sealer (Brasseler USA, Savannah, GA), and MTA Fillapex (Angelus Indústria de Produtos Odontológicos S/A, Londrina, Brazil), were used after exposure to NaOCl or sterile water. Thirty-six dentin specimens were randomly divided into 6 groups with 6 specimens in each sealer-containing 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. Sterile water and gutta-percha (Brasseler USA) without sealer (used after NaOCl or sterile treatment) were used as the control groups with 16 and 12 specimens, respectively. 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 sterile water control group, 50 μL sterile water was placed on the dentin surface. Four specimens in the sterile water group were used for the evaluation of the instant killing effect by 5% NaOCl on day 0 (Table 1). All other dentin samples were placed at 37°C in 100% relative humidity for 7, 30, and 60 days.

Confocal Laser Scanning Microscopic Examination

Two semicylindrical dentin halves (4 samples after fracturing the halves) of each group were examined at each time point by viability staining and CLSM. The sealers were scraped off the root canal wall dentin. The semicylindrical 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 confocal laser scanning microscopic examination as previously described (11).

LIVE/DEAD BacLight Bacterial Viability stain (Molecular Probes, Eugene, OR) containing SYTO 9 and propidium iodide was used for staining of a total of 128 fractured dentin specimens according to 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 bacterial cells using a 20× lens with an additional zoom of 2×. Four areas on the border of the root canal were randomly chosen on each of the 4 specimens in each group per time point for confocal laser scanning microscopic scanning. Images were acquired by EZ-C1 v.3.40 build 691 software (Nikon Canada) at a 512×512 pixel scan area. A stack of 20 slices (0.5-mm step size) was scanned for each area (0.30×0.30 mm) on each sample.

The confocal laser scanning microscopic data were processed by Imaris 7.2 software (Bitplane Inc, St Paul, MN). The thresholds of the red and green fluorescences were manually set according to their respective fluorescence 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 treatments were subjected to univariate analysis of variance 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

Confocal laser scanning microscopic results showed a homogenous penetration of *E. faecalis* deep into the dentinal tubules of the dentin specimens (Fig. 1). Five percent NaOCl and root canal sealers killed 66%–68% and 27%–55% bacteria in dentin tubules, respectively, when used alone (Table 1). The proportion of killed bacteria increased significantly during the first 30 days of exposure to the 3 sealers ($P < .05$) but remained stable after that ($P > .05$).

Significantly more bacteria were dead when NaOCl and sealers (exposure for 30 and 60 days) were used in combination than when used alone ($P < .05$) (Table 1). No significant difference was found between NaOCl alone and NaOCl + sealer treatment at 7 days of sealer exposure ($P > .05$) (Table 1). Thirty- (Fig. 1*A4–E4*) and 60-day (Fig. 1*A6–E6*) exposure to the sealers resulted in significantly more dead bacteria in dentin than the 7-day (Fig. 1*A2*–E2) exposure ($P < .05$) (Table 1), whereas no statistically significant increase of the proportion of dead bacteria was detected between 30- (Fig. 1*A3–E4*) and 60-day samples (Fig. 1*A5–E6*) ($P > .05$). The combination of NaOCl and MTA Fillapex (Fig. 1*C1–C6*) exhibited the highest effectiveness by killing 83% of the bacteria in dentin ($P < .05$)