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Biofilm Formation within the Interface of Bovine Root Dentin Treated with Conjugated Chitosan and Sealer Containing Chitosan Nanoparticles

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Abstract

Objective—To assess biofilm formation within sealer-dentin interfaces of root segments filled with gutta-percha and sealer incorporated with chitosan (CS) nanoparticles, without and with canal surface treatment with different formulations of CS.

Methods—Standardized canals of 4 mm bovine root segments (n=35) were filled with gutta-percha and Pulp Canal Sealer incorporated with CS nanoparticles without surface treatment (group CS), or after surface treatment with phosphorylated CS (group PHCS), CS-conjugated Rose Bengal and photodynamic irradiation (group CSRB) and a combination of both PHCS and CSRB (group RBPH). The control group was filled with gutta-percha and unmodified sealer. After 7 d of setting, specimens were aged in buffered solution at 37° C for 1 or 4 wks. Monospecies biofilms of *Enterococcus faecalis* were grown on specimens for 7 d in a chemostat-based biofilm fermentor. Biofilm formation within the sealer-dentin interface was assessed with confocal laser scanning microscopy.

Results—In the 4-wk aged specimens only, the mean biofilm areas were significantly smaller than in the control for CS (p=0.008), PHCS (p=0.012) and RBPH (p=0.034). Percentage of biofilm-covered interface also was significantly lower than in the control for CS (p=0.024) and PHCS (p=0.003). CS, PHCS and RBPH did not differ significantly.

Conclusions—Incorporating CS nanoparticles into the zinc-oxide eugenol sealer inhibited biofilm formation within the sealer-dentin interface. This effect was maintained when canals were treated with phosphorylated CS, and it was moderated by canal treatment with chitosan-conjugated Rose Bengal and irradiation.

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The authors deny any conflict of interest related to this study.

Keywords

biofilm; chitosan; confocal laser scanning microscopy; endodontic sealer *Enterococcus faecalis*; nanoparticles; sealer-dentin interface

Introduction

Endodontic treatment is focused on reduction of the microbial load within the root canal system to enable healing (1), and on prevention of future microbial ingress by completely filling the canal with stable materials (2, 3). The use to these ends of antimicrobial irrigants and medicaments may alter the surface characteristics of root dentin and compromise its mechanical properties (4). Further, the specific use of chelating solutions demineralizes the root dentin (5), exposing collagen to possible degradation by bacterial and host mediated factors (6). Degradation of root dentin collagen over time may contribute to breakdown of the interface between the canal wall dentin and root filling, and allow bacterial recolonization of the dentin-filling interface in the long-term after treatment (6).

Root canal sealers used for root filling are expected to exert an antimicrobial effect to aid in controlling infection within the root canal system (7). The antimicrobial constituents contained by most sealers, must be released from the sealer matrix (8) to be able to diffuse into the dentinal tubules (9, 10). Once set, the release of antibacterial constituents is associated with disintegration of the sealer (11), which may compromise the sealer-core and sealer-dentin interfaces, making them vulnerable to bacterial recolonization.

Chitosan (CS) is a nontoxic cationic biopolymer that possesses lasting antibacterial properties (12). When incorporated into a zinc-oxide eugenol (ZOE) sealer, CS nanoparticles improved the sealer's antibacterial properties in membrane-restricted assays (8), suggesting that CS nanoparticles can potentially diffuse from the sealer and penetrate into dentinal tubules and anatomical complexities. In addition to the ability of CS to be incorporated into sealers, the chemical structure of CS also permits the preparation of numerous derivatives (13) that may be used for canal surface treatment.

Presence of phosphorylated chitosan (PHCS) at the dentin interface may enable biomineralization of collagen matrices upon contact with saliva (14). The biomineralized collagen forms a barrier against bacterial recolonization (14), that can inhibit bacterial adherence to tooth surfaces (15). Moreover, the immobilization of Rose Bengal, a photo sensitizer used in photodynamic therapy (PDT), onto CS leads to a covalent bond resulting in CS-conjugated Rose Bengal (CSRB) (16). When applied in conjunction with PDT, CSRB exhibited antibacterial properties and crosslinked dentin collagen (17, 18), enhancing the mechanical properties of the collagen matrix and improving its resistance to bacterial-mediated enzymatic degradation (19). Crosslinking of the collagen could also potentially stabilize the sealer-dentin interface and make it more resistant to breakdown and consequent bacterial recolonization in the long-term after treatment.

This study was designed to explore potential benefits of CS applications in endodontics, including incorporation into a ZOE-based sealer and canal surface treatment. The purpose was to assess biofilm formation at the sealer-dentin interface, when the dentin surface is treated with PHCS or CSRB and CS nanoparticles are incorporated within the sealer, using a bovine tooth model.

Material and Methods

Root segment specimens

Intact, freshly extracted bovine incisors ($N = 35$) were decoronated and 4 mm root segments produced from the cervical third of the roots using a slow-speed, water-cooled rotary diamond disc (Brasseler, Savannah, GA). The apical and coronal surfaces of the segments were polished with silicon carbide grinding papers (CarbimetVR, P1200, Buehler, Lake Bluff, IL). Canals were bored with a water-cooled straight fissure bur (FG57L, Brasseler USA, Savannah, GA). Root segments were autoclaved in distilled water at 121°C for 30 min, and their exterior surfaces sealed with 2 coats of nail varnish. Canals were irrigated with 2 mL of each 5.25% NaOCl, 17% EDTA and sterile distilled water as a final rinse.

Root canal surface treatment

CS nanoparticles were synthesized as previously described (8, 14, 17). The root specimens were randomly divided into 5 groups (Table 1; $n = 6\text{--}8/\text{group}$) differing in root canal surface treatment and incorporation of CS nanoparticles in a ZOE-based sealer (Pulp Canal Sealer EWT, SybronEndo, Orange, CA). Root canal surfaces were treated as follows:

- In Groups ZOE and CS, canals were immersed in deionized water for 30 min.
- In Group CSRB, canals were filled with 500 μL of 1 mg/mL CSRB for 15 min, excess solution was blotted from the canal lumen with sterile paper points without touching the walls, the lumen filled with 1 mL of perfluoro-decahydronaphthalene (PFC), and root specimens irradiated with noncoherent light (540 nm, LumaCare Inc., Newport Beach, CA) for 2 min each on the coronal and apical aspect (total energy of $40\text{ J}/\text{cm}^2$). PFC serves as an optical conduit and oxygen carrier for photodynamic therapy.
- In Group PHCS, canals were filled with 200 μL of PHCS (10 mg/mL) and incubated for 15 min at 37°C . The canal lumen was blotted dry with sterile paper points and filled for 15 min with 500 μL of saturated calcium hydroxide solution (40.5 mM) at 37°C . Canals were then rinsed with 2 mL of deionized water.
- In Group RBPH, canals were first treated as in Group CSRB and subsequently treated as in Group PHCS.

Root canal filling and aging

Gutta-percha (Sybron Endo, Orange, CA) was decontaminated with 5.25% NaOCl for 10 min and rinsed with sterile deionized water. Canals were blotted dry with sterile paper points. Sealer was mixed as per the manufacturer recommendation. In Group ZOE (control), canals were filled with unaltered sealer. In the remaining 4 groups, CS nanoparticles were added to the sealer powder component at a ratio of 15:100 (nanoparticles:sealer powder) (8), and manually prepared by mixing the powder and liquid components in a ratio of 3 g/mL. Canals were filled with gutta-percha and sealer via lateral condensation using hand spreaders (Patterson Dental, Montreal, QC, Canada). Excess gutta-percha was removed with a heated medium plugger set at 200°C (System B, Sybron Endo).

Filled root specimens were stored for 7 d at 37°C and 100% humidity (Hera Cell 150, Heraeus, Newton, CT), and any excess sealer removed with a size 15 scalpel blade under sterile conditions. Specimens were subjected to aging at 37°C in a buffered solution containing 20 mM HEPES buffer solution (pH 7.0), 1.5 mM calcium as CaCl_2 and 0.9 mM phosphate as KH_2PO_4 (20). They were aged for 1 or 4 wks ($n = 3\text{--}4/\text{group}/\text{time}$) while replacing the buffered solution every 2 d.

Biofilm cultivation

Aged specimens were suspended in a chemostat-based biofilm fermentor to cultivate monospecies biofilms of *Enterococcus faecalis* (ATCC 29212) at 37° C (21, 22). Continuous flow of fresh Brain Heart Infused broth (BD Bioscience, Sparks, MD) was adjusted to 0.72L/d and dilution rate $D = 0.075/\text{hr}$ to mimic the resting salivary flow rate (23). Specimens were aseptically removed after 7 d and gently rinsed with sterile phosphate buffered solution (PBS).

Outcome assessment

Specimens were stained with dihydroethidium (AnaSpec, Fremont, CA) and fluorescence viewed in a confocal laser scanning microscope (CLSM) (Zeiss LSM700; Carl Zeiss Canada Ltd, Toronto, ON, Canada). The excitation/emission wavelengths were 535/610 nm for dihydroethidium. Two additional filled, non-inoculated specimens were similarly stained (negative control). Root specimens were placed in a slide chamber with sterile PBS and their cross sections observed by using a 63-fold objective lens. CLSM images were acquired using ZEN 2009 Software® (Zeiss). The border of the root canal was identified, and the sealer-dentin interface at 4 cardinal points scanned with the CLSM up to a depth of 150 μm (1 μm step size). Images and Z-stacks were analyzed for maximum biofilm area. In addition, the 4-wk aged specimens were examined for percentage of biofilm-covered interface. Representative specimens were longitudinally split, dehydrated, mounted on stubs, platinum sputtered and evaluated in a scanning electron microscope (SEM) (Hitachi S-2500; Sapporo, Japan) operated at 10 kV to morphologically verify the presence of *E. faecalis*.

Analysis

The maximum biofilm area within the sealer-dentin interface, and the percentage of biofilm-covered interface, were analyzed across all groups with Kruskal-Wallis and post-hoc analysis with Dunn's multiple comparisons tests. Significance was set at 5% level.

Results

Qualitative analysis of CLSM micrographs revealed stained bacteria in all inoculated root specimens, with densely packed bacteria evident on the coronal and apical surfaces of the specimens, as well as at the sealer-dentin interfaces (Fig. 1). The largest biofilm area at the sealer-dentin interface was observed in the ZOE control group. The SEM analysis confirmed the morphology of *E. faecalis* in all inoculated specimens.

Quantitative CLSM analysis of the 1-wk aged root specimens revealed no significant differences ($p > 0.05$) among the groups (Fig. 2). In the 4-wk aged specimens, a significant difference was observed among the groups in interfacial biofilm area ($p = 0.034$) and percentage of biofilm-covered interface ($p = 0.035$). Compared to the ZOE control, the mean biofilm areas (Fig. 3) were significantly smaller for all of CS ($p = 0.008$), PHCS ($p = 0.012$) and RBPH ($p = 0.034$), with no significant differences among the three groups. The percentage of biofilm-covered interface (Fig. 4) was significantly smaller than in the ZOE control group, for both CS ($p = 0.024$) and PHCS ($p = 0.003$), with no significant difference between the two groups. CSRB did not significantly differ from the control in regards to both outcome measures.

Discussion

Novel biomaterials require evaluation with physiologically relevant models. Use of the chemostat-based biofilm fermentor in this study was based on our group's previously established non-destructive experimental model (21), which was subsequently adapted for

assessment of the interface of root canal sealers with root dentin in simulation of intraoral pathogenic conditions (22). Unlike in the previous studies (24, 25), bovine roots were used in the current study, because their large canals facilitated standardized application of surface treatments with CSRB and PHCS. Also, the large canals could be bored to produce parallel walls, in order to maximize the focal depth of CLSM while accounting for the transparency of materials (26). Studies (24, 25) have indicated that bovine teeth are morphologically and histochemically similar to human teeth and may be considered as a suitable surrogate for human teeth.

The aging of specimens in the buffered aqueous environment containing physiologically available inorganic ions simulated the exposure of filled root canals to the oral environment. The duration of aging and composition of the buffered solution was previously established in biomineralization studies (14, 20). Care was taken to carry out canal surface treatments with precision, to maximize the exposure of the root dentin to the treatment agents. Surface treatments with PHCS also included the subsequent application of calcium hydroxide to initiate the biomineralization process using clinically available materials (27).

After aging, the interfaces of sealer with root dentin were challenged with *E. faecalis*, an endogenous oral bacterial species frequently isolated in secondary root canal infections (28). This bacterial species forms monospecies biofilms over root dentin, survives disinfection regimens and nutritional deprivation, and invades filled canals and dentinal tubules (28).

Interfacial bacterial ingress occurred throughout all specimens; however, significant differences in biofilm formation were noted only among the 4-wk aged groups. The longer aging period may have exploited the potential benefits of CS-mediated treatments and solubility factors related to the sealer (29). The specimens filled with CS-incorporated sealer without surface treatment presented less biofilm formation at the sealer-dentin interface, suggesting an inhibitory effect on biofilm formation. When incorporated within the sealer, the CS nanoparticles may leach out of the sealer mass providing diffusible antibacterial properties, while at the same time inhibiting the surface adherence of *E. faecalis* (8). The surface charge of nanoparticulates can influence its interaction with dentin/bacteria. Positively charged nanoparticulates in an aqueous suspension can adhere to negatively charged dentin surface. Even though the force of adhesion between nanoparticulates and dentin may be weak, when bacteria recolonize the root canal, the nanoparticulates will electrostatically interact with the negatively charged bacterial cell to destroy them readily. Nevertheless, further experiments are required to understand whether the inhibition of bacterial adherence by these nanoparticulates is by the killing of bacteria in the vicinity or by the direct effect of nanoparticulates on the bacteria-substrate interaction.

Dentin surface treatments with different formulations of CS did not enhance the inhibitory effect of the CS-incorporated sealer. Surface treatment of dentin collagen with PHCS, in the presence of calcium and phosphate ions, was shown to induce biomimetic mineralization of the collagen matrix (14), forming a barrier against bacterial adherence (14). As no additional inhibitory effect on biofilm formation was observed after canal surface treatment with PHCS, it appeared that either no collagen mineralization occurred or that the mineralization achieved was insufficient to prevent bacterial adherence. Nevertheless, canal surface treatment with PHCS maintained the inhibitory effect of the CS-incorporated sealer.

Surface treatment with CS-conjugated Rose Bengal combined with PDT appeared to moderate the inhibitory effect of the CS-incorporated sealer. PDT has emerged as an effective method for killing bacteria (30) by using a specific-wavelength light to activate a nontoxic photosensitizer in the presence of oxygen, to produce a singlet oxygen species that kills microorganisms (17, 31). Importantly, the reactive oxygen species generated also can

crosslink collagen by reacting with particular amino acids (32), resulting in increased resistance and toughness of dentin (19). In the context of the current study model, the crosslinking of collagen was expected to stabilize the sealer-dentin interface against breakdown during the aging period, which in turn was expected to inhibit interfacial biofilm formation. The inefficacy of the PDT protocol in this study in inhibiting interfacial biofilm formation could be due to the irradiation energy level used, derived from a previous antibacterial study (17), which was double that used in our previous crosslinking investigation (17). This energy level could be inadequate for achieving effective crosslinking of dentin collagen within root canals. Alternatively, there could be a lack of interaction between the CS-conjugated photosensitizer (Rose Bengal) and the CS nanoparticles within the sealer. The negatively charged Rose Bengal molecule (anionic) of the CS-conjugated Rose Bengal could have interfered with the CS nanoparticles (cationic), altering their antibacterial effect at the interface (8). However, further investigation is required to explain this finding.

While dentin surface treatments with the two formulations of CS had no apparent additional effect on biofilm formation at the root dentin-sealer interface, these might still provide structural benefits related to root dentin. The PHCS-mediated collagen mineralization (14) might play a role in countering some of the detrimental surface changes produced by the use of conventional root canal irrigants (5, 33). The CSRB-mediated crosslinking of collagen (32) also strengthened the dentin (19). These potential benefits warrant investigation; however, assessment of the effects of the experimental treatments on the root dentin *per se* was not within the scope of this study.

Our present and previous studies (8) have highlighted the antibacterial potential of the incorporation of CS nanoparticles with ZOE-based sealers. Further studies are required to assess the *in vivo* efficacy of such sealer modification. The canal surface treatments tested herein require further optimization and long-term microbiological and structural analysis to evaluate their feasibility in root canal treatment.

Conclusions

Within the parameters of this study model, incorporating chitosan nanoparticles into zinc-oxide eugenol sealer inhibited biofilm formation within the sealer-dentin interface. This inhibitory effect was maintained when canals were surface-treated with phosphorylated chitosan, but it was moderated when canals were surface-treated with chitosan-conjugated Rose Bengal followed by PDT.

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Figure 1. Representative CLSM micrographs from Z-stack series of 4-wk aged bovine root specimens, showing *E. faecalis* biofilms within sealer-dentin interfaces. (A) ZOE control group, (B) CS group, (C) CSRB group, (D) PHCS group, (E) RBPH group.

Figure 2. Mean *E. faecalis* biofilm area at the sealer-dentin interfaces of 1-wk aged bovine root specimens. No significant differences were noted among all groups ($p=0.126$).

Figure 3. Mean *E. faecalis* biofilm area at the sealer-dentin interfaces of 4-wk aged bovine root specimens. Groups CS, PHCS and RBPH had significantly smaller mean biofilm areas than the control group ZOE. No significant differences were noted between CS, PHCS and RBPH groups.

Figure 4. Percentage of *E. faecalis* biofilm covered interface of 4-wk aged bovine root specimens. Groups CS and PHCS had significantly lower percentage of biofilm covered interface values than the control group ZOE. No significant differences were noted between CS and PHCS.

Table 1

Experimental groups

Groups	Sealer used	Surface treatments
ZOE (control)	ZOE-based sealer	Sterile deionized water
CS		Sterile deionized water
PHCS		Phosphorylated chitosan
CSRB	ZOE-based sealer with CS nanoparticles	Chitosan-conjugated Rose Bengal
RBPH		Chitosan-conjugated Rose Bengal followed by phosphorylated chitosan