Antibiofilm Effects of Endodontic Sealers Containing Quaternary Ammonium Polyethylenimine Nanoparticles

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Abstract

Introduction: This study evaluated the antibiofilm effects of 2 endodontic sealers incorporated with quaternary ammonium polyethylenimine (QPEI) nanoparticles at a 2% concentration (w/w). **Methods:** The materials tested were AH Plus and Pulp Canal Sealer EWT (PCS) in the commercial unmodified form or containing 2% QPEI. Antibiofilm assays were conducted by using direct-contact and membrane-restricted tests for evaluation of bacterial viability in biofilms grown onto membranes or paper disks and the crystal violet microtiter-plate assay to evaluate the effects of sealer extracts on the biofilm biomass. Two *Enterococcus faecalis* strains (ATCC and an endodontic isolate) were used. **Results:** Direct contact and membrane-restricted antibiofilm tests revealed that PCS 2% was the only material to promote total killing of *E. faecalis* ATCC biofilms. All the materials significantly reduced bacterial counts in *E. faecalis* ATCC biofilms when compared with the positive control in both tests ($P < .05$). In the direct test against *E. faecalis* RW35, PCS 2% was significantly more effective than the other materials and was the only one that showed significantly lower counts than the positive control ($P < .05$). In the crystal violet assay, only AH Plus 2% presented optical density readings significantly lower than the positive control of the ATCC strain ($P < .05$). No other significant effects on the biofilm biomass of the 2 *E. faecalis* strains were observed for any of the sealers tested ($P > .05$). **Conclusions:** Addition of QPEI nanoparticles improved the killing ability of PCS against biofilms of both *E. faecalis* strains and the effects of AH Plus on the biomass of biofilms from the ATCC strain. *(J Endod 2014;40:1167–1171)*

Key Words

Biofilms, endodontic sealers, *Enterococcus faecalis*, nanoparticles, quaternary ammonium

Apical periodontitis has been recognized as a biofilm-induced disease (1). Although the currently available methods for chemomechanical debridement have been shown to promote significant microbial reduction in infected canals, complete eradication is still unattainable in most cases (2, 3). Studies have demonstrated that biofilms present in areas such as isthmus, lateral canals, and apical ramifications may remain untouched after root canal instrumentation and irrigation procedures (4, 5). Not surprisingly, it has been shown that treated teeth with persistent disease are commonly associated with biofilms present in these areas (6). Therefore, reduction of the critical bacterial concentration and disruption of biofilms can predictably improve the chances for a positive periradicular response to endodontic treatment.

Endodontic filling materials with antimicrobial properties have the potential to eliminate or entomb microorganisms that persisted after chemomechanical procedures. Preventing bacterial recontamination is also a goal to be reached with filling materials. Ideally, root canal sealers should exhibit antibacterial and antibiofilm effects. They should be used in a way that permits the material to reach residual bacterial biofilms in remote areas of the root canal system. Although several studies have reported the antimicrobial activity of some endodontic sealers (7–10), most investigations used either the direct contact test (DCT) or the agar diffusion test. Both methods have significant limitations. Whereas DCT evaluates the effects against planktonic bacteria, agar diffusion test requires diffusion of the test material through the culture medium and does not differentiate bactericidal from bacteriostatic effects. In the clinical condition, some factors such as tissue remnants, smear layer, and previous dressings or filling materials may prevent close contact between the sealer and residual bacteria. To simulate these conditions, some studies have proposed changes in the methods to test the antibacterial activity of the sealers, including a membrane-restricted assay in which a membrane is placed between the sealer and the biofilm (11). Although there have been many investigations on the antibiofilm effects of irrigants and medicaments, root canal sealers have not been consistently studied.

Incorporation of nanoparticles of different antimicrobial compounds has emerged as an approach to increase the antimicrobial activity of endodontic sealers (8, 10, 12, 13). It has been previously reported that incorporation of quaternary ammonium polyethylenimine (QPEI) nanoparticles into various composite resins, provisional cements, and root canal sealers promoted strong and long-lasting antibacterial properties. Besides, this increase in the antibacterial activity did not introduce significant changes on other biological, physicochemical, and mechanical properties of the materials (8, 14–16).

The purpose of the present study was to evaluate the effects of 2 endodontic sealers, unmodified or loaded with QPEI nanoparticles at 2% w/w concentration, on

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0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2013.12.021

biofilms formed by 2 *Enterococcus faecalis* strains. The methods used were the direct contact and membrane-restricted antibiofilm assays and the crystal violet microtiter-plate test.

Materials and Methods

Materials Tested

Two endodontic sealers were tested, AH Plus (Dentsply, DeTrey, Konstanz, Germany) and Pulp Canal Sealer EWT (PCS) (Kerr, Italia Srl, Salerno, Italy). QPEI nanoparticles were synthesized via reductive amination and incorporated into the sealers at 2% w/w by using the methodology reported before (8).

Antibiofilm Activity: Direct Contact and Membrane-restricted Experiments

E. faecalis strains ATCC 29212 and RW35 (isolated from a root canal with post-treatment apical periodontitis) were grown overnight at 37°C in trypticase soy broth (Difco, Detroit, MI). Bacterial inoculum was standardized to an optical density (OD) of 0.1 (600 nm). Biofilms were grown on filter paper disks (4 mm in diameter; Whatman GmbH, Dassel, Germany) or cellulose nitrate membrane filters (0.22-μm pore size, 13 mm in diameter; Whatman GmbH). Disks and membranes were placed on the surface of *Mitis Salivarius* agar plates. Then, aliquots of 5 or 10 μL of the bacterial suspension were applied to the surface of each disk or membrane, respectively, and plates were stored for 48 hours at 37°C.

For the DCT, approximately 40 μL of each fresh sealer was placed onto the biofilms formed on paper disks. For the membrane-restricted test, a sterile filter membrane was placed over the biofilm-containing membrane before placement of the sealer. Four disks or membranes were used for each sealer. The contact time in both tests was 30 minutes at 37°C. After exposure, the bulk of sealer was wiped off from the surface of the paper disk in the DCT. In the membrane-restricted test, the filter membrane with the sealer was discarded. As positive controls, biofilm-containing disks and membranes without any contact with sealer were used; negative controls consisted of sterile disks and membranes exposed to the sealers.

Disks and membranes were transferred to vials containing 2 mL sterile phosphate-buffered saline (PBS) and slightly agitated to remove unattached cells. Then they were transferred to another flask containing 2 mL PBS and vortexed for 30 seconds, followed by ultrasonication for 5 minutes. After serial 10-fold dilutions, bacterial survival was determined by culturing 10-μL aliquots of each dilution onto *Mitis Salivarius* agar plates. Colony-forming units were counted after incubation for 24 hours at 37°C.

Antibiofilm Activity: Crystal Violet Assay

Test samples were prepared according to International Standard Organization 10993-5:2009 (17). Briefly, 0.1 g of each sealer was immersed in 1 mL saline solution and incubated for 24 and 48 hours at 37°C in a humidified chamber. The final extracts were collected. A third experimental group consisted of a heavy suspension of each sealer prepared as follows. The sealers were mixed according to the manufacturer's instruction (2.5 g each) and left to set for 48 hours at 37°C in a humidified chamber. Afterwards, the sealer specimens were ground in a 6750 freezer mill (Spex, Metuchen, NJ) operated at the liquid nitrogen temperature, and 2 g of powder from each material powder was mixed with 20 mL saline solution.

Biofilm biomass was visualized and quantified with a modified crystal violet binding assay. The bacterial strains used were the same as those used in the antibiofilm experiment described above. Inoculum was prepared in trypticase soy broth (Difco) supplemented with 1% glucose (Merck, Whitehouse Station, NJ) according to the 0.5 McFarland standard from an overnight culture of each bacterial strain. After agitation by vortex, 200-μL aliquots of the bacterial suspension were distributed in wells of a 96-well microtiter plate (tissue culture–treated polystyrene, flat bottoms, model 92096 TPP; Techno Plastic Products, Trasadingen, Switzerland) and incubated for 24 hours at 37°C. The content of each well was aspirated, and the wells were rinsed 3 times with 200 μL PBS (pH 7.2) to remove loosely attached cells. Sealer extracts or heavy suspensions were applied at 200 μL per well for 120 minutes at 37°C. After removal of the sealer samples, each well was washed 3 times with PBS, and then adhering bacteria were stained for 20 minutes with 200 μL 0.1% crystal violet solution at room temperature. Excess stain was rinsed off by copious washing with distilled water. Plates were overturned and air-dried, and the dye bound to the adhering cells was solubilized with 150 μL 95% ethanol for 5 minutes. To quantify the biofilm biomass remaining after treatment, absorbance (590 nm) of the crystal violet solution was measured by using an enzyme-linked immunosorbent assay reader (Model 680; Bio-Rad Laboratories, Hercules, CA). For the positive control, saline was used instead of the test substance. For the negative control, sterile culture broth was used. All assays were performed with 4 repetitions on 2 separate occasions. The cutoff value for OD measurements was defined as 3 standard deviations above the mean OD of the negative control (18). Therefore, final OD values were expressed as average OD value reduced by the cutoff value.

Statistical Analysis

Data were analyzed by using the Statistical Package for the Social Sciences software, version 20.0 (SPSS, Chicago, IL). The antibiofilm effects of the test materials in all assays and against the 2 *E. faecalis* strains were compared by 1-way analysis of variance and Tukey post hoc test. The statistical significance level of 5% ($P < .05$) was established for all analyses.

Results

The direct contact and membrane-restricted antibiofilm assays provide information about the effects of the materials tested on the viability of cells forming the biofilm. Biofilm formation on the filter disks and membranes was confirmed by scanning electron microscopy (data not shown). Results showed that PCS 2% was the only material to promote total killing of *E. faecalis* ATCC biofilms in both direct and membrane-restricted tests (Fig. 1). This material was significantly better than all the other materials tested and the positive control ($P < .01$). In addition, all the materials significantly reduced bacterial counts in *E. faecalis* ATCC biofilms when compared with the positive control in both tests ($P < .05$). In the DCT against *E. faecalis* RW35, PCS 2% was significantly more effective than the other materials and the only one to promote significantly lower bacterial counts than the positive control ($P < .05$). No material showed significant killing effects in the membrane-restricted assay against strain RW35 (Fig. 1).

The microtiter plate assay using crystal violet provides information on the effects of the materials on the biofilm biomass. In this experiment, AH Plus with or without QPEI showed overall the lowest OD values, but statistical analysis revealed that only AH Plus 2% showed OD readings significantly lower than the positive control of the ATCC strain ($P < .05$) (Fig. 2). No other significant effects on the biofilm biomass of the 2 *E. faecalis* strains were observed for any of the sealers tested ($P > .05$).

Discussion

The biofilm mode of growth is a microbial strategy to survive in most environments and endure adverse conditions (19). In an