Antimicrobial Effects of Novel Triple Antibiotic Paste–Mimic Scaffolds on *Actinomyces naeslundii* Biofilm

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Abstract

Introduction: *Actinomyces naeslundii* has been recovered from traumatized permanent teeth diagnosed with necrotic pulps. In this work, a triple antibiotic paste (TAP)–mimic scaffold is proposed as a drug-delivery strategy to eliminate *A. naeslundii* dentin biofilm. **Methods:** Metronidazole, ciprofloxacin, and minocycline were added to a polydioxanone (PDS) polymer solution and spun into fibrous scaffolds. Fiber morphology, mechanical properties, and drug release were investigated by using scanning electron microscopy, microtensile testing, and high-performance liquid chromatography, respectively. Human dentin specimens ($4 \times 4 \times 1$ mm^3, n = 4/group) were inoculated with *A. naeslundii* (ATCC 43146) for 7 days for biofilm formation. The infected dentin specimens were exposed to TAP-mimic scaffolds, TAP solution (positive control), and pure PDS (drug-free scaffold). Dentin infected (7-day biofilm) specimens were used for comparison (negative control). Confocal laser scanning microscopy was done to determine bacterial viability. **Results:** Scaffolds displayed a submicron mean fiber diameter (PDS = 689 ± 312 nm and TAP-mimic = 718 ± 125 nm). Overall, TAP-mimic scaffolds showed significantly ($P \leq .040$) lower mechanical properties than PDS. Within the first 24 hours, a burst release for all drugs was seen. A sustained maintenance of metronidazole and ciprofloxacin was observed over 4 weeks, but not for minocycline. Confocal laser scanning microscopy demonstrated complete elimination of all viable bacteria exposed to the TAP solution. Meanwhile, TAP-mimic scaffolds led to a significant ($P < .05$) reduction in the percentage of viable bacteria compared with the negative control and PDS. **Conclusions:** Our findings suggest that TAP-mimic scaffolds hold significant potential in the eradication/elimination of bacterial biofilm, a critical step in regenerative endodontics. *(J Endod 2015;41:1337–1343)*

Key Words

Antibiotic, bacteria, disinfection, electrospinning, nanofibers, pulp, regeneration, root canal, scaffold, stem cells

Regeneration of the pulp-dentin complex is highly dependent on the effective elimination of bacterial overgrowth within the root canal system (1, 2). Several microbial species have been identified inside root canals, including but not limited to *Actinomyces naeslundii*. A gram-positive filamentous, rod-shaped facultative anaerobe commonly found in the gastrointestinal tract (3), *A. naeslundii* has been increasingly correlated to the ability of biofilm formation and to failed endodontic therapy (4–6). Recently, *A. naeslundii* was detected in initial samples recovered from 10 of 15 traumatized permanent immature teeth diagnosed with necrotic pulps (7), suggesting the species may be relevant to regenerative endodontics.

The traditionally advocated substances used in endodontics to combat root canal infection, including sodium hypochlorite and calcium hydroxide, have shown ineffectiveness, mainly in cases of biofilm-related persistent infections (8, 9). In recent years, antibiotic mixtures such as triple antibiotic paste (TAP), a mixture of metronidazole (MET), ciprofloxacin (CIP), and minocycline (MINO) (10, 11), has gained increased evidence because of its clinically proven disinfection role within the revascularization strategy (12, 13). Regardless of the promising clinical evidence of pulp-like tissue regeneration, TAP uses a considerably high concentration (1 g/mL) of these antibiotics, which has demonstrated harmful effects on cell viability/proliferation (14, 15).

In light of this, the use of antibiotic-containing scaffolds as drug-delivery systems has demonstrated significant clinical potential (2, 16) when compared with TAP from a cytocompatibility standpoint. To our knowledge, no attempt has been made to develop and establish an *A. naeslundii* biofilm on dentin and understand its susceptibility to antimicrobial substances used in regenerative endodontics. We report for the first time the antimicrobial effects of a novel TAP-mimic scaffold on *A. naeslundii*–infected human dentin biofilm by using confocal laser scanning microscopy (CLSM).

Materials and Method

Synthesis of TAP-mimic Electrospun Polymer Scaffolds

Polydioxanone (PDS II; Ethicon, Somerville, NJ) filaments were subjected to an undying process to remove the violet color. In brief, PDS filaments were immersed in dichloromethane (Sigma-Aldrich, St Louis, MO) for 2 days (16–19). A 10 wt% PDS solution was prepared in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich).

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MET, CIP, and MINO (Sigma-Aldrich) were added to the polymer solution at 25 wt% concentration (relative to the total PDS [600 mg] weight, ie, 150 mg of each drug) and mixed together under stirring (16–19). Pure PDS (control) and the TAP-mimic polymer solutions were spun into scaffolds (ie, flow rate 2 mL/h, 18-cm distance, and electrical voltage between 15 and 19 kV). The polymer solutions were individually placed into plastic syringes (Becton, Dickinson and Company, Franklin Lakes, NJ) fitted with a metallic 27-gauge blunt needle, and the fibers were collected on an aluminum foil covered rotating mandrel at room temperature (RT) (16–19). To ensure complete elimination of any residual solvent, the scaffolds were dried for 48 hours under vacuum at RT and then stored at 4°C (16–19).

Fiber Morphology, Mechanical Characterization, and Drug Release

Fiber morphology of the scaffolds was investigated under a scanning electron microscope (FE-SEM, Model JSM-6701F; JEOL, Tokyo, Japan). The fiber diameter was measured (Image-J; National Institutes of Health, Bethesda, MD). The scaffolds' mechanical properties were determined by tensile testing (expert 5601; ADMET, Norwood, MA) of specimens (15×3 mm^2, $n = 10$/group/condition) under dry and wet conditions (24-hour incubation in phosphate-buffered saline [PBS]) (16, 17).

Evaluation of the drug release from TAP-mimic scaffolds (15×15 mm^2, $n = 4$) was done via high-performance liquid chromatography (HPLC). The scaffolds were weighed and then immersed in PBS (10 mL) for 28 days. Aliquots (1 mL) were removed from each solution at selected time points (1, 3, 7, 14, and 28 days) and replaced with an equal amount of fresh PBS at each collection (16, 18). Aliquots (10 μL) were analyzed by using HPLC-UV equipment (Agilent 1100 System, Palo Alto, CA; Zorbax SB-phenyl chromatography column), which consisted of a binary mobile phase of solvent systems A (0.1% formic acid in ddH_2O, v/v) and B (0.1% formic acid in acetonitrile, v/v) in a gradient elution. Test parameters were described in detail elsewhere (16, 18). The percentage of the released drugs was then calculated on the basis of the initial weight of the scaffolds (16, 18).

Antimicrobial Evaluation of *A. naeslundii* Biofilm-infected Dentin Specimens

Caries-free human mandibular canines were collected under an approved (protocol #1407656657) local Institutional Review Board protocol (Indiana University). Teeth were washed, and the soft tissue remains were removed before storage in thymol 0.1%. After crown sectioning by using a diamond disk, the teeth were sectioned along the buccolingual plane, obtaining 2 halves for dentin specimen ($4 \times 4 \times 1$ mm^3) preparation. The cementum was removed, and the specimens were wet-finished with SiC papers (600–1200 grit). All the specimens were immersed in 2.5% NaOCl and 17% EDTA (Inter-Med, Inc, Racine, WI) for 3 minutes each in an ultrasonic bath (L&R 2014 Ultrasonic Cleaning System; L&R Manufacturing Company, Keamy, NJ) to remove the smear layer (19). Last, the specimens were rinsed in saline solution for 10 minutes, sterilized in an autoclave (121°C for 20 minutes), and then randomly placed with the dentin side positioned upward in 24-well plates (Costar; Corning Life Sciences, Tewksbury, MA) before *A. naeslundii* (ATCC 43146) inoculation. In brief, 200 μL of 16- to 18-hour culture of the bacterial suspension (ca. 10^6 bacteria) was pipetted into each well containing 1.8 mL brain-heart infusion medium (Difco Laboratories Inc, Detroit, MI). The plates were kept (37°C) aerobically in an incubator for 7 days to allow biofilm formation. The broth was changed every other day. All the specimens were rinsed for 1 minute (2×) with PBS (Sigma-Aldrich) to remove nonadherent bacterial cells before treatment. Infected dentin specimens ($n = 6$/group) were randomly allocated to 3 experimental groups: pure PDS scaffolds (drug-free), TAP-mimic scaffolds (13.15 ± 0.3 mg, ca. 3.3 mg of each antibiotic), TAP solution (50 mg/mL of each of the drugs), and the negative control group (7-day biofilm untreated). The scaffolds (15×15 mm^2) were sterilized by ultraviolet irradiation (16–19) adapted to plastic inserts (CellCrown; Scaffdex Ltd, Tampere, Finland) and then placed into the wells containing the infected dentin specimens and 1 mL PBS (19). The plates were incubated under aerobic conditions at 37°C for 3 days, and the specimens were prepared for CLSM. Finally, the dentin specimens were rinsed in PBS (2×) to remove unbound bacteria and medium. Two specimens per group were prepared for scanning electron microscopy (SEM) imaging. The remaining specimens in each group were stained with the fluorescent LIVE/DEAD BacLight Bacterial Viability Kit L-7012 (Molecular Probes, Eugene, OR) containing SYTO 9 and propidium iodide (PI). The dyes were mixed in a 1:1 solution and applied to the specimens for 30 minutes at RT before CLSM analysis. Live bacteria presenting intact cell membranes were dyed green (SYTO 9), whereas dead bacteria with damaged membranes were stained red (PI) (20–23). A total of 16 dentin specimens ($n = 4$/group) and 5 randomly selected microscopic fields were scanned, starting from the edges of the specimen to obtain 20 measurements per group. Images were acquired with a confocal/2-photon Leica TCS SP8 system (Leica Microsystems Inc, Buffalo Grove, IL) by using Leica HC PL APO 40 × /1.3 oil immersion objective. Series of sections through the depth of tissue (Z-stacks) were collected by using optimal step size settings (0.35 μm); images were composed of 512 × 512 pixels (221×221 μm^2). Data quantification and 3-dimensional volume reconstruction were performed by using dedicated software (Imaris 7.7; Bitplane USA, South Windsor, CT) (23, 24). Volume images were processed to extract a statistical parameter of live and dead bacteria volume. Data were presented as a ratio of LIVE/DEAD bacteria.

Statistical Analysis

The mechanical data were analyzed (SigmaPlot version 12; Systat Software Inc, San Jose, CA) by using two-way analysis of variance and Tukey test for multiple comparison ($\alpha = 0.05$). For CLSM analysis, groups were compared for differences in the percentage of dead bacterial cells by using the mixed-model analysis of variance, with a fixed effect for group and a random effect for sample, to account for measurements at multiple areas on each specimen. A variance stabilizing transformation ($\sin^{-1}$[(percent dead)½) was used to satisfy the assumptions.

Results

Fiber Morphology, Mechanical Characterization, and Drug Release

A submicron mean fiber diameter was seen for both pure PDS (689 ± 312 nm) and TAP-mimic (718 ± 125 nm) scaffolds (Fig. 1*A* and *B*). Whereas PDS scaffolds exhibited a wider fiber diameter distribution (Fig. 1*C*), the TAP-mimic scaffold presented a gaussian distribution (Fig. 1*D*), with most of the fibers ranging from 600 to 800 nm. Mechanical performances of the bioactive scaffolds are displayed in Figure 1*E–G*. Data analysis revealed that the scaffold ($P = .020$) and storage condition factors ($P \leq .001$) were both significant, although their interaction was not ($P = .541$). Figure 2 reveals a burst release of the drugs within the first 24 hours, which was sustained for MET and CIP but not for MINO, which displayed a progressive concentration reduction within 7 days of incubation.