Antimicrobial Efficacy of Triple Antibiotic–eluting Polymer Nanofibers against Multispecies Biofilm

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Abstract

Introduction: The elimination of microbial flora in cases of immature permanent teeth with necrotic pulp is both key and a challenging goal for the long-term success of regenerative therapy. Recent research has focused on the development of cell-friendly intracanal drug delivery systems. This *in vitro* study aimed to investigate the antimicrobial action of 3-dimensional (3D) tubular-shaped triple antibiotic–eluting nanofibrous constructs against a multispecies biofilm on human dentin. **Methods:** Polydioxanone polymer solutions, antibiotic-free or incorporated with metronidazole, ciprofloxacin, and minocycline, were electrospun into 3D tubular-shaped constructs. A multispecies biofilm consisting of *Actinomyces naeslundii*, *Streptococcus sanguinis*, and *Enterococcus faecalis* was forced inside the dentinal tubules via centrifugation in a dentin slice *in vitro* model. The infected specimens were exposed to 2 experimental groups (ie, 3D tubular-shaped triple antibiotic–eluting constructs and triple antibiotic paste [TAP]) and 2 control groups (7-day biofilm untreated and antibiotic-free 3D tubular-shaped constructs). Biofilm elimination was quantitatively analyzed with confocal laser scanning microscopy. **Results:** Confocal laser scanning microscopic (CLSM) analysis showed a dense population of viable (green) bacteria adhered to dentin and penetrated into the dentinal tubules. Upon 3D tubular-shaped triple antibiotic–eluting nanofibrous construct exposure, nearly complete elimination of viable bacteria on the dentin surface and inside the dentinal tubules was shown in the CLSM images, which was similar ($P < .05$) to the bacterial death promoted by the TAP group but significantly greater when compared with both the antibiotic-free 3D tubular-shaped constructs and the control (saline). **Conclusions:** The proposed 3D tubular-shaped antibiotic-eluting construct showed pronounced antimicrobial effects against the multispecies biofilm tested and therefore holds significant clinical potential as a disinfection strategy before regenerative endodontics. *(J Endod 2017;■:1–6)*

Key Words

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

Significance

Nanofiber-based drug delivery systems are a more cell-friendly disinfection strategy than the currently used triple antibiotic paste, which, in turn, may contribute to better and more predictable regenerative outcomes.

In general, endodontic infection consists of between 1 and 12 bacterial species living in symbiosis in the root canal system (1–3). This colonization encompasses mostly facultative and strict anaerobic bacteria with the ability to proliferate via interactions among bacterial cell proteins that establish a complex spatial structure known as biofilm (1–3). Several bacterial species have been identified as settling into the root canal of fully developed (mature) permanent teeth, including *Enterococcus faecalis* and *Streptococcus sanguinis* (4, 5). Meanwhile, a recent study found *Actinomyces naeslundii* to be most prevalent in traumatized immature permanent teeth with necrotic pulps (6).

Over the years, numerous clinical strategies have been suggested for root canal disinfection. Mechanical and chemical approaches, including but not limited to endodontic files, irrigant solutions, and intracanal medicaments associated or not with emerging technologies (eg, passive ultrasonic irrigation and photoactivated-induced passive irrigation) (7–12), have been used in both mature and immature permanent teeth. More specifically, among the presently available options, triple antibiotic paste (TAP, an equal parts mixture [1 g/mL] involving metronidazole [MET], ciprofloxacin [CIP], and minocycline [MINO]) has been recommended before evoking bleeding in regenerative-based procedures (13–16). Of note, although the intracanal application of TAP may offer some advantages (ie, effective disinfection and a decrease in conceivable systemic complications compared with antibiotic administration [eg, antibiotic-resistant strains, cytotoxicity, and allergic reactions]), TAP has been shown to promote notable tooth discoloration (13, 14, 16, 17) and significant dental stem cell (pulp and apical papilla) death (18, 19) when used at considerably high ($\geq$1 mg/mL) concentrations.

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Because of the aforementioned concerns, recent research has focused on the development of cell-friendly intracanal drug delivery systems (20–31). Electrospinning is known to be a straightforward nanotechnology-based technique capable of fabricating not only tissue scaffolds for regenerative medicine but also antibiotic-eluting polymer nanofibers for use in drug delivery (10, 21–25, 27–31). An accumulating body of evidence suggests that antibiotic nanofibers (single, dual, or triple antibiotics) may provide clinical benefit because substantial antimicrobial properties have been consistently seen when evaluated against dentin biofilm models (21–23, 31). Indeed, the unique release mechanism of antibiotics from polydioxanone (PDS) nanofibers (ie, initial burst release and sustained action for up to 14 days) (22, 25, 29) has shown the ability to promote bacterial cell death inside the dentinal tubules (22, 31).

In order to mimic clinical use, a recent study (31) investigated the ability of dental pulp stem cells to attach and proliferate on dentin surfaces that were previously exposed to the triple antibiotic–eluting nanofibers. No deleterious effects on stem cell attachment and viability were seen upon dentin exposure to the antibiotic-eluting nanofibers (31). By focusing on the clinical impact and translation of electrospun antibiotic-eluting polymer nanofibers for root canal disinfection before regenerative endodontics, this *in vitro* study aimed to investigate the antimicrobial action of 3-dimensional (3D) tubular-shaped triple antibiotic–eluting nanofibrous constructs against a multispecies biofilm (*A. naeslundii*, *S. sanguinis*, and *E. faecalis*) on human dentin.

Materials and Methods

Fabrication of Triple Antibiotic–Eluting Polymer Nanofibers

A PDS (Ethicon, Somerville, NJ) monofilament absorbable suture processed from the polyester poly(p-dioxanone) was dissolved in hexafluoro-2-propanol (Sigma-Aldrich, St Louis, MO) at a 10 wt% concentration (21–31). Next, MET, CIP, and MINO (Sigma-Aldrich) were added to the polymer solution at 35 wt% relative to the total PDS weight (210 mg of each antibiotic) (22, 31). Antibiotic-free (control) PDS solution was also prepared. After overnight stirring, each solution was loaded into 5-mL plastic syringes (Becton; Dickinson and Company, Franklin Lakes, NJ) fitted with a metallic 27-G blunt tip needle. An electrospinning system consisting of a high-voltage source (ES50P-10W/DAM; Gamma High-Voltage Research Inc, Ormond Beach, FL), a syringe pump (Legato 200; KD Scientific Inc, Holliston, MA), and a Teflon-coated collecting steel mandrel (1.5 ± 0.02 mm) connected to a high-speed mechanical stirrer (BDC6015; Caframo, Wiarton, ON, Canada) was used to spin 3D tubular-shaped constructs under optimized parameters (26, 30). All constructs were dried under a vacuum for 48 hours to entirely remove any remaining solvent (21–31).

Multispecies Biofilm Model and Confocal Laser Scanning Microscopic Evaluation of Bacterial Viability

This study was approved (protocol #1407656657) by the local institutional review board protocol (Indiana University). Sixteen caries-free human canines were collected from deidentified patients and used to prepare the dentin slices. In brief, after thorough cleaning and crown sectioning using a low-speed water-cooled wafering diamond blade (Isomet, Buehler, Lake Bluff, IL), the roots were horizontally sectioned at 3 mm apical to the cementoenamel junction to obtain 1.5 ± 0.1-mm thick dentin slices. Subsequently, the dentin slices were wet finished with SiC papers (up to 1200-grit) to obtain a 1-mm uniform thickness. The root canals were enlarged using a round bur (2.5 mm in diameter) at low speed (300 rpm) under water cooling. In order to remove the smear layer, all the dentin slices were incubated in an ultrasonic bath containing 2.5% sodium hypochlorite followed by 17% EDTA (Inter-Med, Inc, Racine, WI) solutions for 3 minutes each. All the dentin slices were rinsed in saline solution for 10 minutes and autoclaved at 121°C (22, 31). The sterile slices were then randomly placed inside microcentrifuge tubes containing 300 μL suspension (approximately 10^6 bacteria) of each of the following bacteria: *A. naeslundii* (ATCC 43146), *S. sanguinis* (ATCC 10556), and *E. faecalis* (ATCC 29212), totaling 900 μL bacteria suspension. Following a previously established protocol, an optimized sequence of centrifugal cycles (2× each) at 1400 g, 2000 g, 3600 g, and 5600 g for 5 minutes was performed to allow for bacterial penetration (32). The bacterial suspension was renewed between every centrifugal cycle. The infected slices were then allocated to 24-well plates containing 1 mL fresh and sterile brain-heart infusion + 1% sucrose. The plates were incubated under aerobic conditions (37°C and 5% CO_2) for 7 days for biofilm formation. The broth was replaced every other day to ensure bacterial viability. Scanning electron microscopy (JSM-5310LV; JEOL, Tokyo, Japan) was performed following a routine sample preparation protocol to qualitatively evaluate biofilm formation and overall morphology over the dentin substrate (22, 23). After 7 days, the dentin slices were gently rinsed with sterile phosphate-buffered saline (Sigma-Aldrich) to remove loosely bound bacteria.

The infected dentin slices ($n = 4$/group) were randomly allocated into 2 experimental groups (ie, 3D tubular-shaped triple antibiotic–eluting nanofibrous constructs and TAP) and 2 control groups (7-day biofilm untreated and antibiotic-free 3D tubular-shaped antibiotic-free PDS nanofibrous constructs). Tubular-shaped constructs (1 ± 0.1 mm in height and 2.5 mm in diameter, average weight 2.5 mg [ie, $\sim$ 900 μg antibiotics in total]) were ultraviolet sterilized (30 min/side) and fitted inside the infected root canal space. TAP was spatulated into a creamy consistency by mixing 50 mg each of MET, CIP, and MINO with 1 mL sterile distilled water and applied into the root canal space of the infected dentin slices. The medicaments remained for 7 days. To maintain a humid environment and prevent both the 3D constructs and TAP from drying out, a damp cotton ball saturated with 50 μL distilled water was placed on top of each specimen. To assess the antimicrobial activity, all samples of each group ($n = 4$/group) were prepared for confocal laser scanning microscopic (CLSM) analysis. In brief, the dentin slices were stained with the fluorescent LIVE/DEAD BacLight viability kit L-7012 (Molecular Probes Inc, Eugene, OR) containing SYTO 9 and propidium iodide (32–34). Two random areas in each dentin slice were analyzed using a mosaic technique of 3D reconstruction, wherein 9 subareas of 300×300 μm were merged, totaling 18 areas per sample. The areas were selected starting from the root canal space toward the cementum side for imaging on a confocal laser scanning microscope (Leica SP2 CL5Mt; Leica Microsystems Inc, Heidelberg, Germany) using a 40× lens. The sequence of segments through the depth of tissue (Z stacks) was collected by using optimal step size settings (0.35 μm); the images were composed of 512×512 pixels. They were evaluated and quantified using dedicated software (Imaris 7.2 software; Bitplane Inc, St Paul, MN). The excitation emission maxima for the dyes were approximately 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide, respectively.

Statistical Analysis

The percentages of live/dead bacteria were compared for differences of dead cells using a mixed-model analysis of variance with a fixed effect for group and a random effect for sample to account for

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