Clindamycin-modified Triple Antibiotic Nanofibers: A Stain-free Antimicrobial Intracanal Drug Delivery System

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Abstract

Introduction: A biocompatible strategy to promote bacterial eradication within the root canal system after pulpal necrosis of immature permanent teeth is critical to the success of regenerative endodontic procedures. This study sought to synthesize clindamycin-modified triple antibiotic (metronidazole, ciprofloxacin, and clindamycin [CLIN]) polymer (polydioxanone [PDS]) nanofibers and determine *in vitro* their antimicrobial properties, cell compatibility, and dentin discoloration. **Methods:** CLIN-only and triple antibiotic CLIN-modified (CLIN-m, minocycline-free) nanofibers were processed via electrospinning. Scanning electron microscopy, Fourier-transform infrared spectroscopy (FTIR), and tensile testing were performed to investigate fiber morphology, antibiotic incorporation, and mechanical strength, respectively. Antimicrobial properties of CLIN-only and CLIN-m nanofibers were assessed against several bacterial species by direct nanofiber/bacteria contact and over time based on aliquot collection up to 21 days. Cytocompatibility was measured against human dental pulp stem cells. Dentin discoloration upon nanofiber exposure was qualitatively recorded over time. The data were statistically analyzed ($P < .05$). **Results:** The mean fiber diameter of CLIN-containing nanofibers ranged between 352 $\pm$ 128 nm and 349 $\pm$ 128 nm and was significantly smaller than PDS fibers. FTIR analysis confirmed the presence of antibiotics in the nanofibers. Hydrated CLIN-m nanofibers showed similar tensile strength to antibiotic-free (PDS) nanofibers. All CLIN-containing nanofibers and aliquots demonstrated pronounced antimicrobial activity against all bacteria. Antibiotic-containing aliquots led to a slight reduction in dental pulp stem cell viability but were not considered toxic. No visible dentin discoloration upon CLIN-containing nanofiber exposure was observed. **Conclusions:** Collectively, based on the remarkable antimicrobial effects, cell-friendly, and stain-free properties, our data suggest that CLIN-m triple antibiotic nanofibers might be a viable alternative to minocycline-based antibiotic pastes. *(J Endod 2017;■:1–8)*

Key Words

Antibiotic, clindamycin, disinfection, electrospinning, nanofibers, regeneration, stem cells

Significance

Collectively, based on the remarkable antimicrobial effects, cell-friendly, and stain-free properties, our data suggest that CLIN-m triple antibiotic nanofibers might be a viable alternative to minocycline-based antibiotic pastes.

Tooth loss in young children as a result of deep caries or trauma-induced pulpal necrosis can lead to complications in craniomaxillofacial growth and development, thus impacting their psychosocial well-being (1, 2). From a clinical standpoint, the management of pulpal necrosis in immature permanent teeth is challenging because of the abrupt interruption of root development resulting in thin dentinal walls, wide open apices, and an increased risk of cervical fracture (3–6). Calcium hydroxide and mineral trioxide aggregate apexification have been widely used to treat immature permanent teeth with necrotic pulps in an effort to obtain an aseptic environment and a calcified apical barrier (7). However, neither apexification therapy has induced complete root development (length and thickness) (5, 6), which compromises the long-term mechanical integrity of the tooth (3, 5–7).

A fairly novel alternative approach to apexification is regenerative endodontics, which aims to promote periapical healing, restitution of pulpal function, and root maturation through a combinatorial disinfection and intracanal stem cell recruitment approach with the use of antibiotic pastes (eg, triple antibiotic paste [TAP]) and evoked bleeding from the periapical tissues, respectively (7, 8). A seminal study by Sato et al (9) showed significant bacterial elimination in deep root canal dentin when using a mixture of metronidazole (MET), ciprofloxacin (CIP), and minocycline (MINO) in a pastelike consistency. Specifically, MET is a bactericidal imidazole that is highly effective against obligate anaerobic bacteria (10), CIP is a bactericidal broad-spectrum synthetic quinolone (11), and MINO is a bacteriostatic broad-spectrum tetracycline (9, 12). Despite the documented clinical efficacy associated with the use of TAP (1 g/mL), recent

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evidence shows not only toxic effects on various cell types (13–15) but also significant dentin discoloration (9, 16, 17) and potential antiangiogenic activity because of the presence of MINO (18–20). Meanwhile, clindamycin (CLIN), a bacteriostatic lincosamide (21, 22) known for its efficacy against a broad spectrum of endodontic bacteria (ie, gram-positive aerobes and most anaerobic bacteria), seems to be a clinically viable alternative to MINO. Thus, this study sought to synthesize clindamycin-modified (CLIN-m, minocycline-free) triple antibiotic polymer nanofibers as a biocompatible, stain-free, and potentially proangiogenic intracanal drug delivery system for regenerative endodontics.

Materials and Methods

Synthesis and Characterization of CLIN-containing Antibiotic Nanofibers

CLIN-only and CLIN-m triple antibiotic (CLIN, CIP, and MET) nanofibers were processed via electrospinning. Polydioxanone (PDS) suture filaments (PDS II; Ethicon, Somerville, NJ) were cut into pieces and soaked in dichloromethane (Sigma-Aldrich, St Louis, MO) at room temperature for 48 hours to remove the sutures' purple color (23–25). Next, undyed PDS suture filaments were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (Sigma-Aldrich) at 10 wt% under stirring conditions. CLIN- and CLIN-m–containing polymer (PDS) solutions were separately synthesized by dissolving 210 mg (ie, 35 wt% relative to the total PDS weight, 600 mg) of each antibiotic followed by 48 hours of vigorous stirring. Electrospinning under optimized parameters (1.5–2.0 mL/h, 18-cm distance, and 18 kV) was performed using a laboratory-designed apparatus (26). Antibiotic-free PDS fibers (control) were synthesized as previously reported (23–26). After electrospinning, the fibers were vacuum dried (48 hours) followed by storage at 4°C until use (27).

Fiber morphology was evaluated using a field emission scanning electron microscope (Model JSM-6701F; JEOL, Tokyo, Japan). The samples were mounted on Al stubs and sputter coated using Au-Pd before imaging. The mean fiber diameter was calculated from 25 single fibers per image (4 images/group) using ImageJ software (National Institutes of Health, Bethesda, MD) (24). Fourier-transform infrared spectroscopy (FTIR) (ATR/FTIR-4100; JASCO, Easton, MD) was performed for each antibiotic powder and the processed fibers to confirm incorporation of the chosen antibiotics (24). The mechanical strength of the CLIN-containing fibers (15×3 mm^2, n = 10/group) was gauged under dry and wet conditions (24 hours of incubation in phosphate-buffered saline, PBS) and determined by tensile testing (26).

Antimicrobial Properties

The antimicrobial efficacy of electrospun nanofibers and antibiotics containing aliquots generated through the incubation of nanofiber samples (over time assessment) were evaluated against *Actinomyces naeslundii* (*An* [ATCC 43143; American Type Culture Collection, Manassas, VA]), *Enterococcus faecalis* (*Ef*, ATCC 29212 [American Type Culture Collection]), *Aggregatibacter actinomycetemcomitans* (*Aa*, ATCC 33384 [American Type Culture Collection]), and *Fusobacterium nucleatum* (*Fn*, ATCC 25586 [American Type Culture Collection]) through agar diffusion–based assays (28).

Disc-shaped (ϕ = 5 mm) samples were weighed and disinfected by ultraviolet (UV) light (30 minutes each side). *Fn* and *Aa* were anaerobically cultured for 24 hours in 5 mL brain-heart infusion supplemented with 5 g/L yeast and 5% volume vitamin K + hemin. Meanwhile, *Ef* and *An* were aerobically cultured for 24 hours in 5 mL tryptic soy broth. One hundred microliters of each broth was swabbed onto blood agar plates to form a bacterial lawn that was then divided into 3 zones: 10 μL 0.12% chlorhexidine (positive control), 10 μL distilled water (negative control), and the fiber disc-shaped samples (23, 29). After 2 days of incubation, the zones of growth inhibition were measured (in mm).

For the aliquots, square-shaped (15×15 mm) samples (n = 3/group/bacteria, 4.0 ± 0.2 mg) from each nanofibrous mat were cut, disinfected, and rinsed (2×) with sterile PBS. Each sample was placed in an individual glass vial with sterile PBS (5 mL at 37°C); 500-μL aliquots were drawn on days 1, 7, 14, and 21 and replaced with an equivalent amount of fresh PBS. The aliquot samples were stored at −20°C until use. Bacterial plates were prepared and cultured as aforementioned, and after 2 days of either aerobic or anaerobic incubation, the diameters (in mm) of the clear zones of growth inhibition were measured (23, 28).

Colony-forming Units per Milliliter

An and *Ef* were specifically selected based on their association with immature trauma-induced pulpal necrosis (30). Square-shaped (15×15 mm) electrospun samples (n = 6/group/specie) were cut, disinfected, fixed to a plastic sample mount (CellCrown; Scaffdex Ltd, Tampere, Finland), and placed individually into 24-well plates. Both *An and Ef* were aerobically cultured overnight in 50 mL tryptic soy broth, and 2 mL inoculated broth was placed into each well to be aerobically incubated for 3 days (28). The samples were removed, rinsed with saline (2×), and placed in 3-mL vials with PBS (n = 4/group/species), which were sonicated and vortexed to remove biofilm bacteria for enumeration. A 1:100 saline dilution was prepared; 100 μL dislodged biofilm solution was spiral plated onto blood agar plates, which were aerobically incubated (37°C for 24 hours) and counted. Two samples per group were fixed in buffered 2.5% glutaraldehyde solution (Sigma-Aldrich) and dehydrated in ascending ethanol solutions before scanning electron microscopic (SEM) imaging.

Cytocompatibility

UV light-disinfected rectangular-shaped (4.0 ± 0.2 mg, n = 4/group) samples were individually placed into the wells of 24-well plates containing 5 mL sterile alpha-modified Eagle medium (Gibco Invitrogen Corporation, Grand Island, NY), supplemented with 10% FBS (Atlanta Biologicals Inc, Flowery Branch, GA), and incubated at 37°C. Aliquots (500 μL) were collected at 1, 7, 14, 21, and 28 days to evaluate cell toxicity over time (23). Human dental pulp stem cells (DPSCs) (Lonza, Walkersville, MD) obtained from permanent third molars were cultured in low-glucose Dulbecco modified Eagle medium containing 10% FBS and 1% penicillin-streptomycin (Sigma-Aldrich) in a humidified incubator at 37°C with 5% CO_2. The cells were seeded at a density of 3×10^3/well (100 μL cell suspension) on 96-well tissue culture plates. After 4 hours of incubation, the media was removed and replaced with the collected aliquots (100 μL) that were adjusted to 10% FBS and 1% penicillin-streptomycin. After incubation, 40 μL CellTiter 96 AQueous One Solution Reagent (Promega Corporation, Madison, WI) was allowed to react with the media for 2 hours before reading the absorbance at 490 nm in a microplate reader (BioTek Instruments Inc, Winooski, VT) against blank wells. The DPSCs cultured with the media were used as the positive control (23).

Dentin Discoloration

Antibiotic-free (PDS), CLIN-only, and CLIN-m electrospun fibers were processed as detailed previously. The electrospun samples (n = 3/group) were disinfected by UV light (30 minutes each side) and individually mounted in plastic inserts (CellCrown) (27). Fifteen human, caries-free, nonrestored canines were used in conformity with the rules and guidelines of the Indiana University Institutional

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