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Clin Oral Investig. 2016 March ; 20(2): 387–393. doi:10.1007/s00784-015-1577-2.**Antibacterial TAP-mimic Electrospun Polymer Scaffold – Effects on *P. gingivalis*-Infected Dentin Biofilm****Maria Tereza P. Albuquerque^{1,4}, Joshua D. Evans¹, Richard L. Gregory¹, Marcia C. Valera⁴, and Marco C. Bottino^{1,2,3,*}**¹ Department of Biomedical & Applied Sciences, Indiana University School of Dentistry (IUSD), Indianapolis, IN 46202, USA² Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA³ Department of Biomedical Engineering, Purdue School of Engineering and Technology, Indiana University-Purdue University Indianapolis, Indianapolis, IN. 46202, USA⁴ Graduate Program in Restorative Dentistry (Endodontics), Universidade Estadual Paulista, São José dos Campos Dental School, São José dos Campos, São Paulo, 12245-000, Brazil**Abstract**

Objectives—This study sought to investigate, *in-vitro*, the effects of a recently developed triple antibiotic paste (TAP)-mimic polymer nanofibrous scaffold against *Porphyromonas gingivalis* (*Pg*)-infected dentin biofilm.

Materials and Methods—Dentin specimens (4×4×1mm³) were prepared from human canines. The specimens were sterilized, inoculated with *Pg* (ATCC 33277), and incubated for one week to allow for biofilm formation. Infected dentin specimens were exposed for 3 days to the following treatments: antibiotic-free polydioxanone scaffold (PDS, control), PDS+25wt.% TAP (25 mg of each antibiotic [metronidazole, ciprofloxacin, and minocycline] per mL of the PDS polymer solution), or a saturated TAP-based solution (50 mg of each antibiotic per mL of saline solution). In order to serve as the negative control, infected dentin specimens were left untreated (bacteria only). To determine the antimicrobial efficacy of the TAP-mimic scaffold, a colony-forming unit (CFU/mL) (n=10/group) measurement was performed. Furthermore, additional specimens (n=2/group) were prepared to qualitatively study biofilm inhibition via scanning electron microscopy (SEM). Statistics were performed and significance was set at the 5% level.

Results—Both the TAP-mimic scaffold and the positive control (TAP solution) led to complete bacterial elimination, differing statistically (p<0.05) from the negative control group (bacteria only). No statistical differences were observed for CFU/mL data between antibiotic-free scaffolds (2.7 log₁₀ CFU/mL) and the negative control (5.9 log₁₀ CFU/mL).

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This article does not contain any studies with human participants performed by any of the authors.

Conclusions—The obtained data revealed significant antimicrobial properties of the novel PDS-based TAP-mimic scaffold against an established *Pg*-infected dentin biofilm.

Clinical relevance—Collectively, the data suggest that the proposed nanofibrous scaffold might be used as an alternative to the advocated clinical gold standard (*i.e.*, TAP) for intracanal disinfection prior to regenerative endodontics.

Keywords

Electrospinning; Scaffold; Antibiotic; Disinfection; Endodontics; Nanofibers

Introduction

Porphyromonas gingivalis is a Gram-negative, strictly anaerobic bacterium. It is commonly found in association with primary endodontic infections and/or periapical pathology [1-4]. Traditional endodontic treatment using antimicrobial substances, mechanical instrumentation, and obturation has demonstrated to be effective against most *P. gingivalis*-associated infections [5]. Even so, depending on the time and persistence of bacterial colonization, they can organize themselves into complex biofilms, mainly in the apical area. Indeed, recent findings have shown that *P. gingivalis* may also be related to the colonization of traumatized immature permanent teeth with pulpal necrosis [6].

Over the past decade, the clinical management of immature permanent necrotic teeth has changed considerably when compared to the conventional apexification treatment. In these cases, infection has been increasingly treated with pulp revascularization protocols using antimicrobial agents, including but not limited to irrigant solutions and calcium hydroxide [7-9]. Calcium hydroxide, though traditionally accepted as an intracanal medication, may present antimicrobial limitations against some resistant species or microbial biofilms [10]. In this way, for regenerative/revascularization purposes, the “gold-standard” triple antibiotic paste (TAP) (*i.e.*, ciprofloxacin/CIP, metronidazole/MET, and minocycline/MINO) has been used [11-13]. Regrettably, this antibiotic paste makes use of substantial amounts (1 g/mL) of the aforementioned antibiotics, which have been linked with toxic effects to stem cells key to the regenerative process [14]. In recent years, an innovative strategy has been considered to develop a low-concentration, yet antimicrobially-effective and biocompatible polymer-based nanofibrous electrospun scaffolds as a drug delivery system to promote intracanal biofilm eradication without jeopardizing stem cell viability and function [15,16]. Electrospinning has been considered as a highly effective process to obtain extracellular matrix (ECM)-mimicking structures with adequate chemistry and three-dimensional porous architectures [11, 15]. Moreover, it has enabled the synthesis of bioactive (*i.e.*, therapeutic drugs and growth factors) polymer-based nanofibrous scaffolds [11, 15-19]. Thus, the purpose of this *in-vitro* study was to investigate the effects of a recently developed antibiotic-containing TAP-mimic scaffold against an established *P. gingivalis*-infected dentin biofilm.

Materials and methods

Processing of a TAP-mimic Polymer-based Scaffold

Polydioxanone (PDS II[®], Ethicon, Somerville, NJ, USA) suture was selected as the scaffold polymer. In brief, violet-colored PDS sutures were cut (ca. 2-3 cm) and incubated in dichloromethane (Sigma-Aldrich, St. Louis, MO, USA) at room temperature (RT) for 2 days to remove the dye [15-19]. Next, clear PDS pieces were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma-Aldrich). Metronidazole, ciprofloxacin, and minocycline (Sigma-Aldrich) were added to the polymer solution at a concentration of 25 mg/mL relative to the total PDS [600 mg] weight (*i.e.*, 150 mg of each antibiotic) and mixed together under stirring to obtain the TAP-mimic scaffold (*i.e.*, PDS+25wt.%TAP). Pure PDS (control) and TAP-mimic polymer solutions were spun into scaffolds using a custom-made electrospinning system. The polymer solutions were then loaded into distinct 5-mL plastic syringes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) fitted with a metallic 27-gauge blunt-tip needle and placed in a syringe pump (Legato 200, KD Scientific Inc., Holliston, MA, USA) to be dispensed at a rate of 2 ml/h. The fibers were collected at RT on an aluminum foil-covered rotating (120 revolutions per minute, rpm), grounded stainless steel drum ($\phi = 4$ cm) placed 18 cm away from the needle tip. During electrospinning, electrical voltage between 15-19 kV was applied to the needle using a high voltage power supply (ES50P-10W/DAM, Gamma High-Voltage Research, Inc., Ormond Beach, FL, USA). After electrospinning, the scaffolds were removed from the mandrel and placed under vacuum at RT for 48 h to ensure complete elimination of any residual solvent prior to storage at 4°C [15-19].

Antimicrobial Evaluation of *P. gingivalis* Biofilm-infected Dentin Specimens

This study was approved (protocol #1407656657) by the local Institutional Review Board (Indiana University). Twenty-four recently extracted, caries-free, non-restored human canine teeth were cleansed and stored in 0.1% thymol until used [18,19]. The crowns were sectioned using a diamond disc. Next, the teeth were cut along the bucco-lingual plane to obtain two halves for dentin specimen ($4 \times 4 \times 1$ mm³) preparation [19]. The cementum was removed and the specimens were wet-finished with SiC papers (600-1200-grit). The specimens were soaked in 2.5% NaOCl and 17% ethylenediamine tetraacetic acid (EDTA, Inter-Med, Inc., Racine, WI, USA) solutions for 3 min each in an ultrasonic bath (L&R 2014 Ultrasonic Cleaning System, L&R Manufacturing Company, Keamy, NJ, USA) to remove the smear layer, rinsed in saline solution for 10 min, and then autoclaved (121°C for 20 min) [18,19]. Next, the specimens were aseptically placed in sterile 24-well plates (Costar, Corning Life Sciences, Tewksbury, MA, USA) with the dentin side positioned upwards. A 200- μ L *Pg* (ATCC 33277) bacterial suspension (ca. 10^6 bacteria) containing 1.8 mL of Brain Heart Infusion media supplemented with 5% (v/v) Vitamin K and Hemin (BHI, Difco Laboratories Inc., Detroit, MI, USA) was added to each well. The plates were incubated in anaerobic jars (37°C) for 7 days to allow for biofilm formation. The broth was changed every two days. All the specimens were rinsed for 1 min (2 \times) in PBS (Sigma-Aldrich) to remove non-adherent bacterial cells prior to treatment. Infected dentin specimens (n=12/group) were randomly divided into four groups: pure PDS (antibiotic-free scaffold), TAP-mimic scaffolds (weight=12.32 \pm 0.92 mg, which accounts for ca. 3.1 mg of the antibiotics),

TAP solution (50 mg/mL of each of the antibiotics), and one negative control (7-days biofilm untreated). The scaffolds (15×15 mm²) were sterilized by UV-irradiation [15,17-19], adapted to plastic inserts (CellCrown,™ Scaffdex Ltd, Tampere, Finland), and then placed in wells containing the infected dentin specimens immersed in 1 mL of PBS [18,19]. For the TAP and negative control groups, the specimens were immersed in 1 mL of TAP and saline solution, respectively. The plates were incubated in anaerobic jars at 37°C for 3 days. The specimens were then prepared for biofilm assay (CFU/mL) by gently washing twice in PBS, followed by placement into vials containing 2 mL of PBS. The samples were sonicated for 10 s (Vibra Cell; Sonics & Materials, Inc., Newtown, CT, USA) and vortexed for 30 s [15, 18-19]. Then a 10-fold serial dilution was prepared (10⁻¹–10⁻⁴) in PBS in which 100 µL of dislodged *P. gingivalis* biofilm solution was spread onto blood agar plates (Fisher Scientific, Pittsburgh, PA, USA). The plates were incubated in anaerobic jars at 37°C for 5 days before the colonies were counted. Scanning electron microscopy (SEM, JSM-5310LV, JEOL, Tokyo, Japan) was carried out (n=2/group) to qualitatively assess biofilm inhibition. In detail, the dentin specimens were harvested, rinsed with PBS to remove unbound bacteria, and fixed in buffered 2.5% glutaraldehyde (Sigma-Aldrich). Prior to SEM imaging, the specimens were dehydrated in an ascending ethanol series, mounted on Al stubs, and sputter-coated with Au-Pd [18,19].

Statistical Analysis

CFU/mL results were analyzed using the Kruskal-Wallis test, followed by multiple pair-wise group comparisons using the Dunn test ($P < 0.05$).

Results

Figure 1 shows the bacterial counts (log₁₀ CFU/mL) after 3 days of dentin biofilm exposure to the distinct scaffolds. The highest bacterial counts (5.9 log₁₀ CFU/mL) were associated with dentin specimens exposed to the pure PDS scaffolds. Meanwhile, the 25 wt.% TAP-mimic polymer scaffold and the positive control (*i.e.*, TAP solution) led to complete bacterial elimination, differing statistically ($p < 0.05$) from the negative control (bacteria only). No statistical differences were observed for CFU/mL data between antibiotic-free PDS-based polymer scaffolds (2.7 log₁₀ CFU/mL) and the negative control (5.9 log₁₀ CFU/mL).

SEM images revealed a significant amount of bacteria-forming microbial biofilms after 7 days of *P. gingivalis* inoculation (Figure 2). Dentin specimens exposed to antibiotic-free PDS scaffolds demonstrated the presence of viable bacteria on the surface (Figure 3A), agreeing with the CFU/mL findings. SEM images indicated that both the TAP-solution treated and 25 wt.%TAP-mimic scaffold were able to eradicate most of the viable bacteria without removing them from the dentin surface (Figure 2). The bacteria were not considered viable due to the observation of irregularities and porosities in their membrane surfaces (Figures 3B and 3C). Furthermore, the SEM images also revealed the formation of small, insoluble agglomerates over the dentin surface (Figure 3C) after treatment with the TAP solution.

Discussion

This study demonstrated the antimicrobial clinical potential of a recently proposed TAP-mimic antibiotic delivery system [19] against strictly anaerobic bacteria (*P. gingivalis*). Predicting the long-term success of endodontic treatment is mainly dependent on the achievement of a bacteria-free environment [20]. However, root canal disinfection still represents a challenge due to the complex anatomy of the root canals and/or the non-optimal chemical substances usually used during treatment (*e.g.*, irrigant solutions and intracanal medicaments) [21,22]. Literature has proven that failure of canal disinfection frequently occurs when microbial biofilms are present inside or outside the root canal [23,24]. In contrast to the conventional treatment (*i.e.*, apexification), regenerative endodontics involves not only root canal disinfection, but also the association between bacterial elimination and development of a “pulp-like” tissue containing differentiated odontoblasts, in which the dentin walls increase in thickness and length, resulting in proper root maturation [11].

P. gingivalis is a virulent pathogen usually related to primary endodontic infection and is found in clinical cases of apical periodontitis [25]. In the present study, this bacterium was chosen, based on the occurrence of this pathogen in cases of traumatized necrotic immature permanent teeth [26]. This investigation succeeded in effectively developing and eliminating a 7-day-old *P. gingivalis* biofilm, despite the difficulties related to handling with strictly anaerobic species. The CFU/mL counting and SEM images in the control group demonstrated the viability of the bacteria and the presence of *P. gingivalis* biofilm on both the dentin surface and inside the dentinal tubules (Figure 2). Nonetheless, it is not possible to quantify bacteria viability and the effectiveness of medicaments inside dentinal tubules through a colony-counting assay. To evaluate the ingrowth of biofilm into dentinal tubules requires the use of confocal laser scanning microscopy (CLSM). Currently, *P. gingivalis* presents survival restrictions that make it difficult to be assessed using the aforementioned technique.

The two foremost treatment strategies employed in the cases of necrotic immature teeth are apexification (*i.e.*, calcium hydroxide) or revascularization/regeneration (*i.e.*, TAP as the intracanal medicament) [26,27]. Most importantly, over the past decade, pulp revascularization has become a very popular approach, especially when considering the success demonstrated in several clinical cases/serial reports that highlighted healing of periapical lesions associated with complete root development provided by the ingrowth of a “pulp-like” tissue [28]. Conversely, the success of regenerative endodontic treatments is very much dependent on the achievement and maintenance of a bacteria-free niche, lately attained through the use of the so-called TAP [26, 29-31]. Regrettably, the TAP has been used in a very high concentration (1 g/mL), which has led to tooth discoloration and cell death [14,32]. Of note, antibiotic-containing nanofibrous-based polymer scaffolds have demonstrated great clinical potential in terms of releasing a low-concentration, yet antimicrobially-effective dose of antibiotics through an initial burst (first 24 h), while sustaining its effects for up to 14 days [16,17,19]. Remarkably, the proposed antibiotic release mechanism may avoid root canal reinfection and residual bacterial proliferation even though these scaffolds are designed to contain ca. 3 mg/mL of antibiotics, or approximately 300-fold less concentrated than the established TAP paste at 1 g/mL [15-17, 19]. These

unique characteristics may contribute to creating conditions inside the root canal system for the subsequent in-growth of a new pulp-like tissue after regenerative endodontic procedures (*i.e.*, evoked bleeding) and, at the same time, prevent stem cell toxicity.

In the present research, the exposure of a young biofilm to a relatively novel TAP-mimic polymer scaffold exhibited a notable elimination of cultivable bacteria similar to the saturated TAP solution. The SEM images of the TAP-mimic scaffold revealed that non-viable bacteria adhered to the dentin surface even after being killed by the antibiotics. This probably occurred because PDS scaffolds may have an attractive potential that makes removal of the dead bacteria difficult [19]. On the other hand, SEM images of the saturated TAP solution group demonstrated the formation of calcium-enriched insoluble agglomerates over the dentin surface, agreeing with previous research studies [19,33]. The antimicrobial effects of both triple and double antibiotic pastes against *E. faecalis* and *P. gingivalis* biofilms have been previously demonstrated [31,34]; however, no study has investigated the effects of a TAP-mimic scaffold on *P. gingivalis*-infected dentin biofilm. Thus far, only one study used the proposed TAP-mimic scaffold against *Actinomyces naeslundii*-infected dentin biofilm, proving significant bacteria elimination through CLSM [19]. Taken together, both previous and current findings reveal that the proposed, low-concentrated TAP-mimic polymer-based scaffold acts not only on monospecies facultative bacteria (*A. naeslundii*) but also on strictly anaerobe bacteria. Worth mentioning, the kinetics of drug release of the TAP-mimic scaffolds was previously evaluated via high performance liquid chromatography (HPLC). It was demonstrated a sustained maintenance of MET and CIP was observed over 4 weeks, but not for MINO [19]. Therefore, considering the drug release profile of the proposed TAP-mimic PDS-based electrospun scaffold, MET and MINO might have the most antimicrobial effects against *P. gingivalis*. Future studies using strictly anaerobe bacteria, including *P. gingivalis* are deemed necessary to confirm through CLSM its antimicrobial efficacy deep into the dentinal tubules. Moreover, multispecies biofilms developed on dentin surfaces should also be tested to provide further insight regarding the antimicrobial action of this scaffold prior to its clinical use.

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Compliance with Ethical Standards

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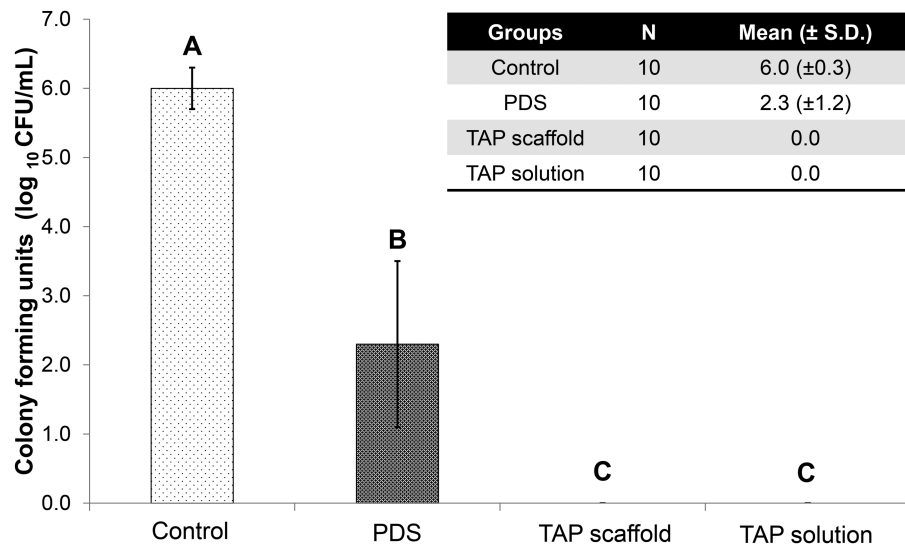


Figure 1.

The number of *P. gingivalis* cells in biofilm on exposed dentin in CFU/mL (\log_{10}). TAP solution and TAP-mimic groups significantly reduced bacterial numbers compared to the control and pure PDS. Bacterial numbers of a group with different letters differs statistically from other groups ($p < .05$). There was no difference between TAP solution and TAP-mimic.

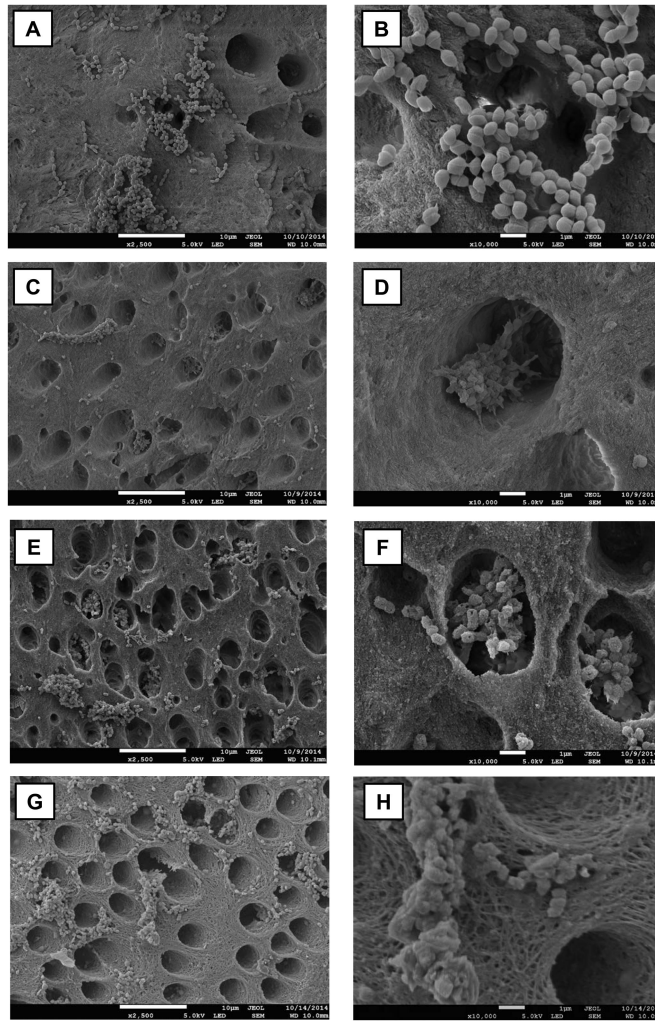


Figure 2. Representative SEM images ($\times 2500$ and $\times 5000$ magnifications) of 7-day *P. gingivalis* biofilm formation, both on the dentin surface and inside the dentinal tubules (A-H). Infected dentin treated with pure PDS demonstrates bacteria inside dentinal tubules and on the dentin surface (C-D). TAP-mimic scaffold (E-F) and TAP solution (G-H) images demonstrate dead cells on the dentin surface.

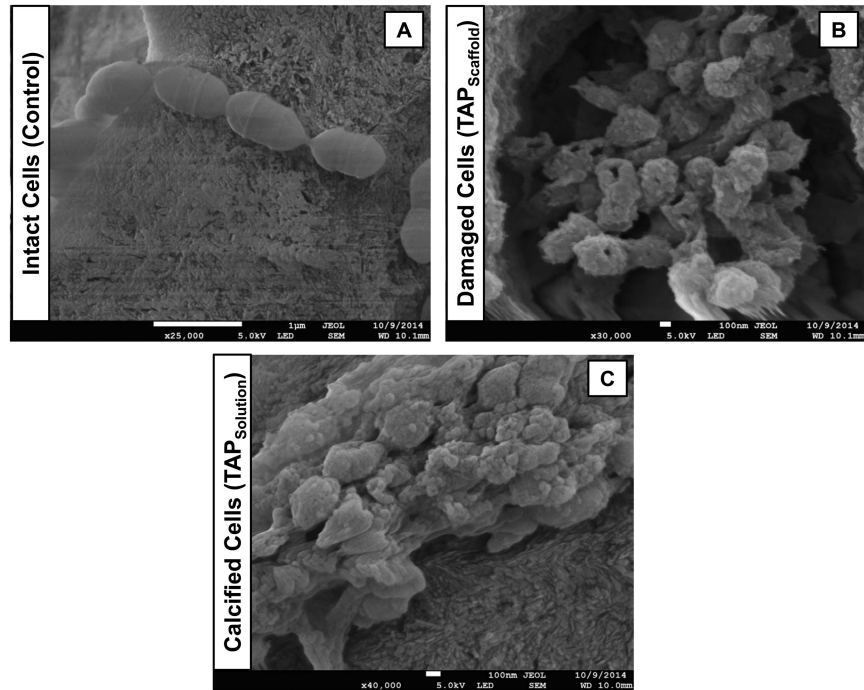


Figure 3. High magnification SEM images comparing intact (A), damaged (B), and (C) calcified cells found in the different groups, according to the external cell morphology.