Antibacterial Effects of Antimicrobials Used in Regenerative Endodontics against Biofilm Bacteria Obtained from Mature and Immature Teeth with Necrotic Pulps

Jordon C. Jacobs, DDS,[*] Alex Troxel, DDS,[*] Ygal Ehrlich, DMD,[*] Kenneth Spolnik, DDS, MS,[*] Josef S. Bringas, DMD, DDS, MS,[*] Richard L. Gregory, PhD,[†] and Ghaeth H. Yassen, BDS, MSD, PhD[†]

Abstract

Introduction: We investigated the direct and residual antibacterial effects of intracanal antimicrobials against bacterial biofilms obtained from infected mature and immature teeth with necrotic pulps. **Methods:** Sterile dentin slabs (n = 100) were inoculated with bacterial biofilms obtained from root canals of an immature or a mature tooth with pulpal necrosis and incubated anaerobically for 3 weeks (n = 50 per biofilm). Dentin infected with each type of biofilm received 1 week of treatment with 1 or 5 mg/mL double antibiotic paste (DAP) in methylcellulose hydrogels, calcium hydroxide, or placebo paste or received no treatment (n = 10). The pastes were removed, and biofilm disruption assays were performed. Additional dentin slabs (n = 100) were pretreated with the same treatments (n = 20). The pastes were rinsed off, and the samples were immersed in phosphate-buffered saline for 1 week. Thereafter, samples from the treatment groups were infected with bacterial biofilm from both clinical sources mentioned earlier (n = 10 per biofilm) and incubated anaerobically for 3 weeks before conducting biofilm disruption assays. Uninfected dentin slabs were used for both antibacterial experiments as negative control groups (n = 20). **Results:** All antimicrobials showed significant direct antibacterial effects regardless of the biofilm source. Dentin pretreated with 5 mg/mL DAP provided significantly higher residual antibacterial effects in comparison with all other groups regardless of the source of biofilm. Dentin pretreated with calcium hydroxide did not show any residual antibacterial effects. **Conclusions:** Tested antimicrobials showed significant direct antibacterial effects. Only 5 mg/mL DAP exhibited significant residual antibacterial effects against bacterial biofilms from an infected root canal of an immature tooth. *(J Endod 2017;■:1–5)*

Key Words

Biofilms, calcium hydroxide, double antibiotic paste, endodontic regeneration, immature tooth, mature tooth

Significance

Both $Ca(OH)_2$ and 1 mg/mL DAP exhibited significant direct antibacterial effects. However, only 5 mg/mL DAP was able to provide a significant extended residual antibacterial effect against bacterial biofilms from an immature tooth with pulpal necrosis.

Root canal disinfection during endodontic regeneration procedures has gained great attention in recent years. Multiple modifications have been proposed to the commonly used irrigation solutions and intracanal medicaments in an attempt to introduce a disinfection protocol that can promote a healthy biological environment within the root canal system and enhance clinical outcomes of regenerative endodontics (1, 2). One of these suggested modifications is the use of lower concentrations of antibiotic intracanal medicaments (0.1–1 mg/mL) such as triple antibiotic paste (TAP) or double antibiotic paste (DAP) rather than the high concentration (1000 mg/mL) commonly used (3–5). The lower concentrations of antibiotic mixtures were proposed to have direct antibiofilm effects that are comparable with that of calcium hydroxide ($Ca[OH]_2$) (6).

The minimal root canal instrumentation and absence of a hermetic root canal seal after endodontic regeneration procedures may facilitate the establishment of a new bacterial biofilm that can originate from residual pathogens or new microbial contamination. Therefore, aggressive disinfection protocols that can guarantee an extended level of disinfection have been recommended during regenerative endodontics (7, 8). Within this context, both low and high concentrations of antibiotic combinations have been proposed to offer extended residual antibiofilm effects unlike the clinically used formula of $Ca(OH)_2$ (9, 10).

Low concentrations of antibiotic medicaments are yet to be used clinically because they are in a liquid form and cannot be used as an interappointment dressing during regenerative endodontics. Recently, multiple studies suggested a translational approach to load these low concentrations of antibiotic combinations into a biocompatible Food and Drug Administration–approved water-based methylcellulose hydrogel in an attempt

From the Departments of *Endodontics and [†]Biomedical and Applied Sciences, Indiana University School of Dentistry, Indianapolis, Indiana.
Address requests for reprints to Dr Ghaeth H. Yassen, Department of Biomedical and Applied Sciences, Indiana University School of Dentistry, 1121 W Michigan St, Indianapolis, IN 46202. E-mail address: gyassen@iupui.edu
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to introduce an antimicrobial paste with controlled antibiotic concentrations (11, 12). This approach claimed to maintain the antimicrobial effects of these antibiotics (6, 13), minimize the cytotoxic potential of these medications (5), and reduce the notorious effects of these medicaments on physiomechanical properties of radicular dentin (11, 12, 14).

Endodontic studies that explored the antimicrobial potential of low antibiotic concentrations have mainly used standard species of endodontic pathogens that are usually obtained from a nondental origin (6, 15, 16). One novel translational step that might be helpful in understanding the antimicrobial potential of these antibiotic mixtures is to test them against clinical isolates obtained from infected root canals. The majority of clinical cases of endodontic regeneration have been performed in teeth with necrotic pulps and immature roots among younger patients ranging in age from 7–17 years (17, 18). Therefore, it is essential to investigate the antimicrobials effects of intracanal medicaments against bacterial biofilm obtained from immature teeth with necrotic pulps and understand if these biofilms are more resistant to antimicrobials used during regenerative endodontics. The aim of this study was to investigate the direct and residual antibacterial effects of antimicrobials used in endodontic regeneration against biofilm bacteria obtained from immature and mature teeth with pulpal necrosis.

Materials and Methods

Collection of Clinical Isolates

This study was approved by the local institutional review board (#1510640949). Two subjects without any systemic disease who had not received antibiotics in the last 6 months were selected for the study and signed the informed consent and assent forms before collection of the bacterial isolates from their infected root canals. One of the subjects had an immature tooth with an infected root canal and a periapical lesion that was indicated for endodontic regeneration treatment. The other subject had a mature tooth with an infected root canal and a periapical lesion that was indicated for conventional root canal therapy. The clinical isolates were collected as described in a previous protocol (19). Briefly, the selected tooth was isolated with a rubber dam, and the operative field was cleansed with 3% hydrogen peroxide solution and disinfected with 6% sodium hypochlorite (NaOCl). The coronal root canal access was then performed using a sterile round bur. After that, the pulp chamber was disinfected using a swab soaked in 6% NaOCl, and the residual NaOCl was inactivated with sterile 5% sodium thiosulfate. The bacterial isolate was collected from the infected root canal using a sterile #15 file with the handle cut off. The file was introduced 1 mm short of the apical foramen, and a filing motion was performed for 30 seconds before taken out. After that, 3 sterile paper points were inserted into the root canal at the same working length and left inside for 1 minute to absorb the tissue fluid. Both the file and paper points were then placed into 5 mL brain-heart infusion broth supplemented with 5 g/L yeast extract and 5% (v/v) vitamin k and hemin (BHI-YE), vortexed to elute the bacteria, incubated anaerobically at 37°C for 48 hours, and frozen at −80°C until used.

Dentin Sample Preparation

Intact permanent human teeth were collected, stored in 0.1% thymol solution, and used within 6 months. Standardized radicular dentin slabs (N = 224) were obtained from the collected teeth as described in previous studies (6, 10). Briefly, $4 \times 4 \times 2$ mm^3 slabs were obtained from each root using a low-speed diamond saw under continuous water irrigation. Both sides of each slab were polished using a Roto Pol 31 polishing unit (Struers, Cleveland, OH). The samples were sonicated using 1.5% NaOCl, distilled water, and 17% EDTA for 4 minutes to remove the smear layer. Samples were independently sterilized with ethylene oxide, stored at 4°C, and used within 4 weeks.

Preparation of Intracanal Medicaments

Low concentrations of DAP (1 and 5 mg/mL) loaded into methylcellulose hydrogels were prepared as described in recent publications (5, 6). Briefly, 10 and 50 mg of equal portions of metronidazole and ciprofloxacin (Champs Pharmacy, San Antonio, TX) were dissolved in 10 mL sterile water to form 1 and 5 mg/mL DAP solutions, respectively. Then, 0.8 g methylcellulose powder (Methocel 60 HG; Sigma-Aldrich, St Louis, MO) was gradually incorporated into each DAP solution over 90 minutes under vigorous stirring to obtain a creamy injectable consistency of 1 and 5 mg/mL DAP intracanal medicament. A DAP-free methylcellulose-based hydrogel and a commercial $Ca(OH)_2$ paste (UltraCal XS; Ultradent, South Jordan, UT) were also used as additional treatment groups in this study.

Direct Antibacterial Effect of Antimicrobials

Sterile dentin slabs (n = 104) were independently placed into wells of sterile 96-well microtiter plates (FisherBrand; Fisher Scientific, Hampton, NH) with the pulpal sides oriented outward. Half of the dentin slabs received a mixture of 190 μL fresh BHI-YE and 10 μL overnight culture (approximately 1×10^5 colony-forming unit [CFU]/mL) of biofilm bacteria obtained from an immature tooth with pulpal necrosis (n = 52). The other half of the dentin slabs received a mixture of 190 μL fresh BHI-YE and 10 μL overnight culture (approximately 1×10^5 CFU/mL) of biofilm bacteria obtained from the mature tooth with pulpal necrosis (n = 52). All infected dentin slabs were incubated anaerobically at 37°C for 3 weeks, and the growth medium was replaced every 7 days. Weekly replacement of growth medium was selected to limit the nutritional supply during the 3-week period of *in vitro* biofilm formation to maintain the original taxa of clinical isolates as much as possible (20, 21). Uninfected sterile dentin slabs (negative control) were used in this experiment to confirm the absence of any external bacterial contamination though the course of the study (n = 10). The negative control dentin slabs received fresh BHI-YE (200 μL per sample) and were incubated anaerobically for 3 weeks as described earlier with weekly replacement of BHI-YE.

After the 3-week incubation period, 2 random dentin slabs were selected from each type of biofilm and processed for scanning electron microscopy (JEOL 7800F; JEOL, Peabody, MA) as described in a previous study (6). The rest of the dentin slabs infected with each type of biofilm were transferred to new wells of sterile 96-well microtiter plates containing 50 μL fresh BHI-YE and randomized into 5 experimental groups (n = 10 per group). Infected slabs received 100 μL 1 mg/mL DAP, 5 mg/mL DAP, placebo paste, $Ca(OH)_2$, or no treatment (positive control group) (n = 10 for each type of biofilm). The uninfected dentin slabs in the negative control group received no treatment as well. All slabs were then incubated for 1 week at 37°C and 100% humidity. After that, samples were independently exposed to 5 mL sterile water for 1 minute under mild agitation to wash off the treatment pastes. All dentin slabs were subjected to biofilm disruption assays as detailed in recent studies (9, 10). In summary, dentin slabs were independently placed into sterile test tubes containing 2 mL sterile water, sonicated for 20 seconds, and vortexed for 30 seconds to dislodge the biofilms. The detached biofilms were diluted, spiral plated on blood agar plates, incubated anaerobically for 24 hours, and quantified using an automated colony counter (Synbiosis Inc, Frederick, MD).

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