Abstract: We investigated the direct and indirect (residual) antibacterial effects of various concentrations of triple antibiotic paste (TAP) loaded into a methylcellulose system. *Enterococcus faecalis* (*E.* faecalis) was grown on sterilized dentin blocks (*n* = 60) and treated with clinically used TAP (1,000 mg/mL), low concentrations of methylcellulose-based TAP (100, 10, and 1 mg/mL), placebo paste, or 1.5% *NaOCl* (*n* = 10). The pastes were then removed, and biofilm disruption assays were performed. Additional dentin blocks (*n* = 120) were pretreated with the same experimental groups (*n* = 20). The pastes were rinsed off, and the samples were immersed independently in phosphate-buffered saline for 2 and 4 weeks (*n* = 10). *E. faecalis* was then grown on the dentin blocks, and biofilm disruption assays were performed. Fisher’s Exact and Wilcoxon rank sum tests were used for statistical analyses. With regard to direct antibacterial effects, all treatment groups demonstrated complete eradication of biofilms in comparison to placebo paste, while 10 mg/mL of TAP or higher provided substantial residual antibacterial effects. However, dentin treated with 1 mg/mL of TAP or 1.5% *NaOCl* did not provide substantial residual antibacterial effects. Dentin pretreated with 10 mg/mL of TAP or higher exhibited extended residual antibacterial effects and can thus be used during endodontic regeneration. (J Oral Sci 58, 575-582, 2016)

Keywords: bacterial biofilm; endodontic regeneration; *Enterococcus faecalis*; triple antibiotic paste; sodium hypochlorite.

Introduction

Traditional apexification techniques utilizing long-term calcium hydroxide dressings (1) or mineral trioxide aggregate artificial apical barriers (2) have been used for decades for the management of immature necrotic teeth with blunderbuss apices. Although apexification procedures facilitate obturation process and completion of root canal therapy, the roots of the treated teeth remain fragile with limited development. Regenerative endodontic therapy has emerged as a contemporary treatment option for necrotic immature teeth. Numerous studies have reported the success of this technique with regard to resolution of infection and continuous development of the immature roots (3,4). Root canal disinfection is imperative for successful endodontic regeneration procedures, and various antimicrobials have been used for this, including irrigation solutions such as sodium hypochlorite (*NaOCl*) (5) and chlorohexidine (6), inter-appointment medicaments such as calcium hydroxide (7), or various antibiotic mixtures (3). A recent systemic review found that 80% of clinical endodontic regeneration studies and
case reports used antibiotic combinations as intracanal medicaments (8). Triple antibiotic paste (TAP, a mixture of ciprofloxacin, metronidazole, and minocycline) is the most commonly used inter-appointment paste in endodontic regeneration (9).

There are various challenges associated with the disinfection protocol in endodontic regeneration. As this protocol is expected to eliminate established root canal infection in the absence of root canal instrumentation (10), and sustain the aseptic environment within the root canal system for an extended period of time in an attempt to stabilize the newly developed tissue (11). Furthermore, this protocol is expected to create a biologically friendly environment to preserve stem cells and endogenous growth factors within the root canal system and the periapical area (12). Numerous antimicrobial studies have recommended the use of calcium hydroxide or low concentrations of combinations of antibiotics (13,14) and NaOCl (15) to minimize the cytotoxic effects of these antimicrobials during endodontic regeneration.

Both calcium hydroxide and low concentrations of TAP in liquid form have been suggested to have a direct antibacterial effect against established bacterial biofilms (16). Additionally, dentin treated with TAP was proposed to have extended residual antibacterial effects for 2 weeks following TAP removal (17). However, liquid dilutions of TAP cannot be used as inter-appointment medicament, and need to be integrated into a stem cell friendly delivery system that can maintain their antibacterial properties. Therefore, the aims of current study were as follows: to investigate the direct antibacterial effect of various concentrations of TAP incorporated into the methycellulose system and to evaluate the residual antibacterial effects of dentin treated with different concentrations of TAP.

**Materials and Methods**

**Specimen preparation**

After obtaining local Institutional Review Board approval (IRB# 1408889870, 2014), 180 de-identified intact human permanent teeth were collected and stored in 0.1% thymol solution at 4°C. The roots of all teeth were utilized to prepare 180 standardized dentin samples (4 × 4 × 1.5 mm³) using diamond saws (IsoMet; Buehler, Lake Bluff, IL, USA) under continuous irrigation with deionized water. The non-pulpal sides of the samples were flattened using 1,200 grit abrasive papers (Struers, Cleveland, OH, USA) and an automatic polishing unit (Rotoforce 4, Struers). The pulpal sides of dentin samples were flattened and polished with abrasive papers (1,200-4,000 grit; Struers). To remove the smear layer, all dentin samples were sonicated with 1.5% NaOCl (Value Bleach; Kroger, Cincinnati, OH, USA), 17% EDTA (VISTA, Racine, WI, USA) and sterile water (4 min each). The samples were then wrapped individually with cotton gauze saturated with sterile water, inserted in Whirl-pak bags (Sigma-Aldrich, St Louis, MO, USA), gas sterilized with ethylene oxide, stored at 4°C, and used within 4 weeks.

**Preparation of antimicrobials used in the study**

This study had six experimental groups, as follows: clinically used concentrations of TAP (1,000 mg/mL), three lower concentrations of TAP (1, 10, and 100 mg/mL), placebo paste with no TAP, and 1.5% NaOCl. A creamy mix of TAP that is commonly used clinically was prepared by mixing 1 mL of sterile water with 1,000 mg of equal portions of metronidazole, ciprofloxacin, and minocycline (Champs Pharmacy, San Antonio, TX, USA) (13). A low concentration of TAP with a creamy consistency that can be injected into root canals as inter-appointment medicaments (18) was prepared, as described in recent reports (19,20). Briefly, 2,500, 250, and 25 mg of TAP were dissolved independently in 25 mL of sterile water. Thereafter, 2 g of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich) was gradually added to each TAP solution over 2 h under continuous stirring to obtain a homogenous creamy consistency with 1, 10, or 100 mg/mL of TAP. An aqueous methylcellulose-based paste with no TAP (placebo) was also prepared and used in the current study. All methylcellulose-based pastes were prepared at room temperature under strict aseptic conditions. For the NaOCl group, 1.5% NaOCl was prepared immediately before treatment by diluting 3% stock solutions of NaOCl (Value Bleach; Kroger, Cincinnati, OH, USA) in sterile water.

**Bacterial strain and media**

*Enterococcus faecalis* (*E. faecalis*; ATCC strain 29212, American Type Culture Collection, Manassas, VA, USA) was grown anaerobically on blood agar plates (Bio-Merieux, Durham, NC, USA). Colonies of *E. faecalis* were then inoculated in a sterile broth of brain heart infusion (BHI) supplemented with 5 g of yeast extract/L (BHI-YE) and incubated for 24 h at 37°C and 5% CO₂ atmosphere.

**The direct antibacterial effect of antimicrobials against established biofilms**

Dentin samples (*n = 60*) were independently inserted into the wells of sterile 96-well microtiter plates (FisherBrand, Fischer Scientific). The pulpal side of each sample was
oriented upward to receive the bacterial culture. Each dentin sample received a mixture of 10 µL of overnight *E. faecalis* culture (approximately 10^7 CFU/mL) and 190 µL of fresh BHI-YE. Samples were then incubated at 37°C in 5% CO2 atmosphere for 3 weeks, and the growth media was replaced every 3 days. A pilot study was conducted to confirm the ability of 3-week-old *E. faecalis* biofilms to penetrate the tubules of the dentin samples prepared for this study. Briefly, four additional dentin samples were inoculated with *E. faecalis* for 3 weeks and the formed biofilms were scraped off. Two samples were obliquely fractured to expose the dentin tubules and processed for confocal laser scanning microscopy (CLSM) to confirm the presence of *E. faecalis* within the dentin tubules (Fig. 1). The remaining two samples were subjected to biofilm disruption assays (described later) to further confirm the ability of the protocol to retrieve *E. faecalis* from the dentin tubules (mean log10 = 5 ± 0.2 CFU/mL).

After the incubation period, the infected dentin samples were randomized into six experimental groups (*n* = 10 per group). Each infected dentin sample in the first experimental group was immersed in 5 mL of 1.5% NaOCl for 5 min. The infected dentin samples in the remaining groups were individually transferred to new wells of sterile 96-well microtiter plates containing 100 µL of fresh BHI-YE, and received 200 µL of 1, 10, 100, 1,000 mg/mL TAP or placebo paste. No untreated control was included since our pilot work as well as another recent study found no differences between the placebo paste and untreated control group (20). Samples were then incubated for 3 weeks at 37°C and 100% humidity.

The application time of TAP and NaOCl and the concentration of NaOCl used in the current study were selected according to the most updated clinical recommendations for regenerative endodontics (21).

After the designated treatments, each sample was immersed for 2 min in 5 mL of sterile water under mild agitation to remove any remaining TAP or NaOCl. One sample was randomly selected from each group and immediately processed for CLSM. The remaining nine samples from each group were subjected to biofilm disruption assays as described in previous studies (17,20). Briefly, each dentin sample was inserted in a plastic test tube containing 2 mL of sterile water, sonicated for 20 s, and vortexed for 30 s to separate the biofilm. The dislodged biofilms were diluted, spiral plated, and incubated anaerobically for 24 h. The CFUs/mL were determined utilizing an automated colony counter (Synbiosis, Inc, Frederick, MD, USA).

**The indirect (residual) antibacterial effect of antimicrobials against established biofilms**

An additional 120 dentin samples were randomized into the same six experimental groups (*n* = 20) and treated with various concentrations of TAP (1,000, 100, 10, or 1 mg/mL), placebo paste, or 1.5% NaOCl, as described earlier. After the assigned treatment, each dentin sample received 3 minutes of irrigation with 10 mL of sterile water, followed by 5 min of irrigation with 10 mL of 17% EDTA to simulate the clinical scenario during regenerative endodontic treatment. Dentin samples from each group were individually immersed in 200 µL of PBS and incubated at 37°C and 100% relative humidity for 2 or 4 weeks (*n* = 10). After each assigned immersion period, a standardized 3-week *E. faecalis* biofilm was grown on the treated pulpal side of each sample, as described earlier. Thereafter, one randomly selected sample from each group at each immersion period was further processed for CLSM, and the other nine specimens were used for biofilm disruption assays, as mentioned earlier.

*E. faecalis*-free dentin samples were also used in direct and indirect testing (*n* = 2 per experiment) as negative control groups. The samples were submerged individually in BHI-YE growth media at 37°C and 100% relative humidity during the period of the experiments, and subjected to biofilm disruption assays to validate the absence of any contamination.

**Visualization of biofilms using CLSM**

CLSM was used to view *E. faecalis* biofilms grown on the randomly chosen dentin samples in both the direct and indirect antibacterial experiments, as described in a recent study (20). In summary, equal volumes of propidium iodide and SYTO 9 dyes (Molecular Probes, Eugene, OR, USA) were diluted and mixed according to the manufacturer’s instructions. Thereafter, 200 µL...
of the prepared mixture was used to stain the biofilm on each dentin sample inside an independent well of sterile 96-well microtiter plates. The samples were then stored in the dark for 20 min and immediately viewed under CLSM (FV1000; Olympus Corp, Center Valley, PA, USA). Three randomly selected biofilm areas from each dentin sample were scanned using FV10-ASW software (Olympus Corp). Furthermore, dedicated software (Imaris 7.7; Bitplane, South Windsor, CT, USA) was utilized for the three-dimensional construction of the biofilms and quantification of live/dead bacteria within each biofilm area.

Statistical analyses
Multiple experimental groups demonstrated complete eradication of biofilms. Therefore, Fisher’s Exact tests were utilized to evaluate differences in the presence or absence of biofilm growth (α = 0.05). Additionally, Wilcoxon rank sum tests were used to compare different experimental groups that exhibited bacterial growth (α = 0.05).

Results
Direct antibacterial effects
All TAP concentrations as well as 1.5% NaOCl demonstrated significant anti-biofilm effects and complete eradication of biofilm in comparison to the placebo paste (P < 0.00001). No biofilms were observed using CLSM on dentin treated with various concentrations of TAP or 1.5% NaOCl. However, dentin treated with placebo paste demonstrated a uniform biofilm structure. The mean log_{10} of biofilms on dentin treated with the placebo paste was 6.2 ± 0.3 CFU/mL, and the mean percentage of live cells (green) in the biofilm detected using CLSM was 77 ± 6% (Fig. 2).

Indirect (residual) antibacterial effects
For experimental groups immersed in PBS for 2 weeks after treatment (Fig. 3), dentin treated with 10, 100, or 1,000 mg/mL of TAP exhibited significantly higher residual antibacterial effects and complete eradication of biofilm in comparison to dentin treated with 1 mg/mL TAP, 1.5% NaOCl, or placebo paste (P = 0.0001). Furthermore, no significant differences in residual antibacterial effects were observed between dentin treated with 1 mg/mL TAP, 1.5% NaOCl, or placebo paste.

For experimental groups immersed in PBS for 4 weeks after treatment (Fig. 3), dentin treated with 100 mg/mL of TAP exhibited a significantly higher residual antibacterial effect as well as complete eradication of biofilm in comparison to all other groups (P < 0.01). Dentin treated with 1,000 mg/mL TAP demonstrated a significantly higher residual antibacterial effect in comparison to dentin treated with 10 mg/mL TAP, 1 mg/mL TAP, 1.5% NaOCl, or placebo paste (P < 0.05). Dentin treated with 10 mg/mL TAP demonstrated a significantly higher residual antibacterial effect in comparison to that treated with 1 mg/mL TAP, 1.5% NaOCl, or placebo paste (P < 0.05). Dentin treated with 1 mg/mL TAP demonstrated significant but limited residual antibacterial effect in comparison to dentin treated with 1.5% NaOCl or placebo paste (P < 0.05). No significant difference in residual antibacterial effect was observed between dentin treated with 1.5% NaOCl or placebo paste.

The amount of biofilm formed on treated dentin samples was significantly higher after 4 weeks immersion in PBS compared to 2 weeks immersion for dentin treated with 10 mg/mL of TAP (P = 0.0001), 1,000 mg/mL of TAP (P = 0.0335), placebo paste (P = 0.0273), or 1.5% NaOCl (P = 0.003). No significant differences
in biofilms formed between the two immersion periods were observed between dentin treated with 1 or 100 mg/mL of TAP (Fig. 3).

Overall, the images from CLSM were consistent with the results obtained from biofilm disruption assays. The live cell percentage within the biofilm mass on dentin immersed for 2 weeks in PBS after treatment with 1 mg/mL of TAP, 1.5% NaOCl, and placebo paste were 67 ± 10%, 60 ± 4%, and 66 ± 13%, respectively (Fig. 4). Furthermore, no biofilm was detected on dentin treated with 10 mg/mL or higher TAP concentrations and immersed for 2 weeks in PBS. The percentage of live cells within biofilms on dentin immersed in PBS for 4 weeks after treatment with 1 mg/mL of TAP, 1.5% NaOCl, and placebo paste were 76 ± 5%, 55 ± 10%, and 75 ± 6%, respectively (Fig. 5). Additionally, limited live bacteria were observed on dentin treated with 10 or 1,000 mg/mL of TAP and immersed for 4 weeks in PBS (Fig. 5). No live bacteria were noticed on dentin immersed in PBS for 4 weeks after treatment with 100 mg/mL of TAP.

### Discussion

Excessive amounts of TAP powder are usually required when mixing with water in order to achieve a creamy consistency that can be clinically applied into the canal as an inter-appointment medicament (1,000 mg/mL). This high concentration was suggested to have unfavorable effects on stem cells from apical papillae (14) and dental pulp stem cells (22), as well as on the chemical (23), physical (19), and mechanical properties of dentin (24). Therefore, the current study investigated the ability of a creamy consistency of low concentrations of TAP loaded into a methylcellulose system in maintaining the antibacterial properties of the original antibiotic mixture. This would be helpful in developing a balanced disinfection protocol that can eliminate infection without compromising the biological environment within the root canal system and the structural integrity of radicular dentin.

The current study demonstrated that a 3-week application of all tested TAP concentrations as well as 5 min application of 1.5% NaOCl was able to eradicate an established *E. faecalis* biofilm. On the other hand, no antibacterial effect was observed with TAP-free placebo paste. This indicates that TAP concentrations as low as 1 mg/mL were successfully loaded into the aqueous methylcellulose system and could efficiently be used as an intra-canal medicament during endodontic regeneration. This generally agrees with a recent study suggesting an antibacterial effect of 1 mg/mL of TAP in a liquid form (25). It is also worth noting that 1 mg/mL of TAP solution was suggested to have no cytotoxic effects against stem cells from apical papillae (14). Furthermore, 1 mg/mL of TAP loaded into a methylcellulose system was found to

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**Fig. 4** CLSM 3D images showing live (green) and dead (red) cells of 3-week-old *E. faecalis* biofilms formed on dentin samples previously treated with different concentrations of TAP followed by immersion in PBS for 2 weeks (a total of 5 weeks): Placebo paste (A1), 1.5% NaOCL (A2), 1 mg/mL TAP (A3), 10 mg/mL TAP (A4), 100 mg/mL TAP (A5), and 1,000 mg/mL TAP (A6).
have minimal negative effects on the structural integrity and mechanical properties of dentin in comparison to the clinically used concentration of TAP (26).

In the current study, dentin treated with 10 mg/mL or higher of TAP completely prevented the colonization of *E. faecalis* up to 5 weeks (2 weeks immersion in PBS plus a 3-week period for biofilm colonization) after TAP removal. Additionally, dentin treated with 10 mg/mL of TAP or higher was able to exert a significant and substantial residual antibacterial effect (more than 4 log₁₀ reduction in CFU/mL) up to 7 weeks after TAP removal. On the other hand, dentin treated with 1 mg/mL of TAP did not demonstrate a substantial residual antibacterial effect. A recent study suggested that dentin treated with 1 mg/mL of TAP solution exerted a residual antibacterial effect up to 1 week after TAP removal (17). The disagreement between the current study and the previous one (17) could be explained by the longer time interval between removing the medicament and investigating the residual antibacterial effects, which was 5-7 weeks in the current study and only 1 week in the previous study.

The residual antibacterial effect of dentin treated with TAP reported in the current study could be explained by the ability of TAP to bind to dentin and be gradually released in an active form. The minimum bactericidal concentration of TAP against *E. faecalis* was found to be 0.3 mg/mL (16). Therefore, it is expected that dentin previously treated with TAP should be able to release at least 0.3 mg/mL of TAP to maintain a substantial antibacterial effect at a specific time point. A recent study suggested that 85% of radiolabeled TAP was retained within radicular dentin after various irrigation methods were used (27). It is well documented that various tetracycline derivatives have the ability to bind to dentinal collagen and exert residual antibacterial effects (28,29). Consequently, the presence of minocycline in TAP might be helpful in explaining the residual antibacterial properties of dentin previously treated with TAP. However, a recent study demonstrated that dentin treated with a minocycline-free antibiotic combination, namely double antibiotic paste (equal portion of ciprofloxacin and metronidazole), can also exert residual antibacterial effects (17). Therefore, it may be logical to assume that more than a single antibiotic component in TAP can be helpful in charging the dentin with extended antibacterial properties.

For experimental groups examined 7 weeks after TAP removal, dentin treated with 100 mg/mL of methylcellulose-based TAP demonstrated significantly higher residual antibacterial effects in comparison to dentin treated with 1,000 mg/mL of TAP (clinically used concentration prepared without methylcellulose). This indicates that the use of a methylcellulose system may be helpful in extending the antibacterial properties of TAP in the oral environment by slowing down the antibiotic degradation process. Previous studies have also demonstrated that the incorporation of Ca(OH)₂ into an aqueous
methylcellulose system resulted in significantly better antibacterial effects against various endodontic pathogens in comparison to higher concentrations of Ca(OH)\(_2\). Pastes prepared without methylcellulose (30,31). It is also worth noting that methylcellulose is frequently used as culture media for stem cell growth and differentiation due to its noncytotoxic nature (32). Indeed, various concentrations of aqueous methylcellulose paste were proposed to improve the proliferation of dental pulp stem cells in a recent study (Sabrah AH. Diluted antibiotics for treating traumatized immature teeth. Dissertation, Indiana University School of Dentistry, 2014). Therefore, methylcellulose loaded with specific low concentrations of antibiotics may be deliberately used as a stem cell-friendly inter-appointment antimicrobial medication during endodontic regeneration. However, future studies are warranted to investigate the cytotoxic nature of various concentrations of TAP loaded into aqueous methylcellulose systems.

The current study indicated that 5 min application of 1.5% NaOCl was able to completely eradicate established \textit{E. faecalis}. This finding is consistent with recent studies that found that 5 min of irrigation with 1-1.5% NaOCl eliminated bacterial biofilm (20,33). On the other hand, dentin treated with 1.5% of NaOCl did not induce significant residual antibacterial effects in the current study. However, a trend of lower percentages of live cells within the biofilm was observed in dentin treated with 1.5% NaOCl, which may indicate a limited residual antibacterial effect. Previous studies have also suggested limited residual antibacterial properties of NaOCl (29,34). Few endodontic regeneration case reports have demonstrated successful root canal disinfection using irrigation solutions without supplementary inter-appointment medicaments (5,6). However, the use of antibiotic intra-canal medicaments such as TAP in addition to irrigation solutions can be extremely helpful when an extended residual antibacterial effect is required (10). This may be essential in endodontic regeneration cases with established bacterial infections that clinically present with necrotic pulp, acute or chronic abscesses, and/or a radiographically visible periapical lesions (11). One limitation of this study is the use of a monospecies \textit{E. faecalis} biofilm, a multispecies biofilm would be more representative of the actual clinical situation. However, recent studies suggested equal behavior of 3-week-old multispecies biofilms isolated from human dental plaque and monospecies \textit{E. faecalis} biofilm in terms of resistance to endodontic disinfectants (35,36).

The current study demonstrated the ability of low concentrations of TAP loaded into a methylcellulose system to maintain their antibacterial properties to a greater extent in comparison to the clinically used higher concentration (1,000 mg/mL). Furthermore, 1 mg/mL or higher of TAP loaded into a methylcellulose system was able to eliminate established \textit{E. faecalis} biofilms. However, at least 10 mg/mL of methylcellulose-based TAP should be used to obtain extended residual antibacterial effects. Further studies are warranted to investigate the cytotoxic potentials of various concentrations of methylcellulose-based TAP.

**Conflict of interest**
The authors declare that they have no conflict of interest.

**References**