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Inhibitory effect of gels loaded with a low concentration of antibiotics against biofilm formation by *Enterococcus faecalis* and *Porphyromonas gingivalis*

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Abstract: We explored longitudinally the inhibitory effect of gels loaded with 1 mg/mL modified triple antibiotic paste (MTAP) or double antibiotic paste (DAP) against biofilm formation by *Enterococcus faecalis* and *Porphyromonas gingivalis*. Methylcellulose-based antibiotic gels of MTAP (ciprofloxacin, metronidazole and clindamycin) and DAP (ciprofloxacin and metronidazole) were prepared at a concentration of 1 mg/mL. Individually cultured *E. faecalis* and *P. gingivalis* bacterial suspensions were treated with MTAP, DAP, or placebo (vehicle only) gels at different dilutions and allowed to grow in 96-well microtiter plates. Untreated bacterial suspensions served as a negative control. Crystal violet assays were used to evaluate biofilm formation after 48 h. The ability of the gels to inhibit biofilm formation was determined immediately, and at 1 month and 3 months after the gels had been prepared. Data were analyzed using a mixed-model ANOVA. The MTAP and DAP gels significantly reduced biofilm formation by both bacterial species at all time points, regardless of the tested dilution. No significant differences in biofilm-inhibitory effects between MTAP and DAP gels were observed at the majority of the tested dilutions through various time

points. Gels loaded with 1 mg/mL MTAP and DAP demonstrated a significant antibiofilm effect against *E. faecalis* and *P. gingivalis*. (J Oral Sci 57, 213-218, 2015)

Keywords: double antibiotic paste; endodontic regeneration; *Enterococcus faecalis*; modified triple antibiotic paste; *Porphyromonas gingivalis*.

Introduction

Endodontic regeneration is a recent approach for management of immature permanent teeth with necrotic pulps (1). To achieve successful endodontic regeneration, it is crucial to eliminate root canal infection throughout the process using various chemical treatments (2). Triple antibiotic paste (TAP) composed of minocycline, metronidazole and ciprofloxacin is the most popular intracanal medicament used during endodontic regeneration (3). However, tooth discoloration has been associated with the minocycline present in TAP (4-6). Double antibiotic paste (DAP), a combination of metronidazole and ciprofloxacin, has been considered to possess antibacterial properties comparable to those of TAP (7) and to minimize discoloration during endodontic regeneration (8). Other studies have suggested substituting minocycline in TAP with a narrow-spectrum antibiotic such as clindamycin (9) or other broad-spectrum antibiotics such cefaclor or amoxicillin (3,10). Clindamycin has been found to be active against various anaerobic bacteria isolated from

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primary endodontic infections (11,12). Furthermore, a modified triple antibiotic paste (MTAP) composed of metronidazole, ciprofloxacin and clindamycin, has been used successfully during endodontic regeneration (9).

Recent studies have suggested that antibiotic medications at the concentrations employed clinically have both direct and indirect cytotoxic effects against stem cells and pulp fibroblasts (10,13-15). Finding a balance of antibiotic concentrations that exerts comprehensive activity against endodontic pathogens without having cytotoxic effects on various stem cells might be the key to achieving successful and predictable clinical outcomes for endodontic regeneration. Indeed, various antibiotic combinations at 1 mg/mL have been suggested to exert antibiofilm effects against endodontic pathogens (7) without having any indirect cytotoxic effects against the stem cells of apical papillae (14). Furthermore, this was recently proposed to be the highest antibiotic concentration that can be used to disinfect the root canal during endodontic regeneration (1). However, this low concentration is in liquid form and cannot be used as an inter-appointment medicament during endodontic regeneration. The aim of the present study was to investigate longitudinally the antibacterial ability of antibiotic gels loaded with 1 mg/mL DAP or MTAP against *Enterococcus faecalis* and *Porphyromonas gingivalis* biofilms.

Materials and Methods

Gel preparation and loading with DAP and MTAP

Antibiotic gels were prepared as described previously (16,17). To prepare 1 mg/mL methylcellulose-based MTAP, 50 mg of United States Pharmacopeia grade antibiotic powders compounded of 43% clindamycin, 14% ciprofloxacin, and 43% metronidazole (Skywalk Pharmacy, Wauwatosa, WI, USA) was dissolved in 50 mL of sterile water. Then, 4 g of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St Louis, MO, USA) was added to the mixture and stirred for 2 h at room temperature to obtain a homogeneous antibiotic gel. The gel was left to stand for an additional 2 h to ensure the complete disappearance of all foam from the mixture. To prepare 1 mg/mL methylcellulose-based DAP, 50 mg of United States Pharmacopeia grade antibiotic powders compounded of equal portions of metronidazole and ciprofloxacin (Champs Medical, San Antonio, TX, USA) was used, and the antibiotic gel was prepared as described above. An antibiotic-free placebo gel composed of sterile water and methylcellulose was also prepared utilizing the same method. The viscosity of the prepared gels was selected based on pilot studies that had examined the viscosities of various methylcellulose-based gels. A

gel viscosity that had sufficient consistency to be used as an intracanal medicament and applied to root canals using commercially available endodontic syringe tips (NaviTips, Ultradent, South Jordan, UT, USA) was selected. The pH of the prepared gels was measured in triplicate during the pilot study, and the values for DAP, MTAP, and placebo gels were 7.2, 7.6, and 7.7, respectively.

Bacterial strains and culture conditions

Enterococcus faecalis (ATCC 29212) and *Porphyromonas gingivalis* (ATCC 33277) strains were used in this study. *E. faecalis* and *P. gingivalis* were selected as representative common endodontic pathogens that are present in various types of endodontic infections. *E. faecalis* is a gram-positive facultative anaerobe that has been detected in 67-77% of cases of secondary root canal infection (18,19). On the other hand, *P. gingivalis* is a gram-negative obligatory anaerobe that has been detected in 44-48% of cases of primary root canal infection (20,21). Each bacterial strain was initially grown on anaerobic blood agar plates (CDC, BioMerieux, Durham, NC, USA), and then grown and maintained as described previously (7,22,23) utilizing sterile Brain Heart Infusion broth supplemented with 5 g of yeast extract/L (BHI-YE; Becton Dickinson Co., Franklin Lakes, NJ, USA) containing 5% v/v vitamin K (0.5 mg/mL) and hemin (50 mg/mL) (Remel, Lenexa, KS, USA). Both test bacteria were grown in an anaerobic environment created using gas-generating sachets (Gas-Pak EZ; Becton) and incubated for 48 h in an incubator at 37°C. Bacterial growth was confirmed by changes in turbidity at 48 h. The number of colony-forming units/mL for *E. faecalis* and *P. gingivalis* after 48 h was 1.78×10^8 (optical density = 0.6 at 600 nm) and 3.6×10^8 (optical density = 0.67 at 600 nm), respectively.

Determination of biofilm inhibition

The ability of the prepared gels to inhibit biofilm formation by *E. faecalis* and *P. gingivalis* was tested as described previously (7). In summary, 250 μ L of two-day-old cultures of *E. faecalis* and *P. gingivalis* broth were treated with 5 mL of 1:10, 1:20, 1:40, 1:80, and 1:160 dilutions of freshly prepared MTAP, DAP, or placebo gels in BHI-YE. Inoculated *E. faecalis* and *P. gingivalis* broth without gels served as negative controls. The treated and untreated bacterial media were incubated anaerobically at 37°C for 48 h in 96-well microtiter plates (200 μ L per well). The culture fluid was carefully withdrawn without touching the formed biofilms using a multichannel pipette to remove planktonic bacteria. Then, the biofilm in each

well was gently washed twice with sterile 0.9% saline, fixed for 30 min with 10% formaldehyde, washed two additional times with 0.9% sterile saline, and stained for 30 min with 0.5% crystal violet. The biofilm in each well was washed three more times with sterile 0.9% saline to remove any unbound crystal violet, and the crystal violet bound to the biofilm was then extracted by adding 200 μ L of 2-propanol for 1 h. The extract was diluted 1:5 with 2-propanol and the optical absorbance was measured at 490 nm using a microplate spectrophotometer (SpectraMax 190; Molecular Devices, Sunnyvale, CA, USA). 2-Propanol was used as a blank control. The same batches of the prepared gels were stored at 4°C and the microtiter plate antibiofilm test was repeated after the gels had been aged for 1 and 3 months to verify the antibacterial stability of the prepared gels over time.

Statistical analysis

Each experiment was conducted two separate times using two independent batches of the prepared gels, and three readings were obtained for each dilution in each experiment. The percentage of biofilm formation after various treatments was calculated using the equation: Biofilm formation (%) = (experimental absorbance value) / (untreated negative control absorbance value) \times 100. The percentages of biofilm formation in the presence or absence of the treatment gels were analyzed statistically using a mixed-model ANOVA followed by Fisher's least significant difference test for pairwise comparisons. A random effect to correlate the data within each experiment was also included. The significance level was set at 0.05.

Results

Antibiofilm effect against *E. faecalis*

Figures 1 A-C indicate that the MTAP and DAP gels caused significant reduction of biofilm formation relative to the untreated negative control bacteria at all tested dilutions through all time points ($P < 0.00001$). Furthermore, both the DAP and MTAP gels significantly reduced biofilm formation relative to the placebo gel at all dilutions regardless of the tested time points ($P < 0.0001$) except for 1:160 dilution of the gels tested at the baseline. At the baseline (Fig. 1A), there was no significant difference between the MTAP and DAP gels at any of the tested dilutions, except for 1:80 where DAP demonstrated significantly higher biofilm-inhibitory activity than did MTAP ($P = 0.031$). After 1 and 3 months (Figs. 1 B and C), the MTAP gel provided a significantly higher biofilm-inhibitory effect than the DAP gel at the majority of lower dilutions over the range 1:10-1:40 ($P = 0.01 - P$

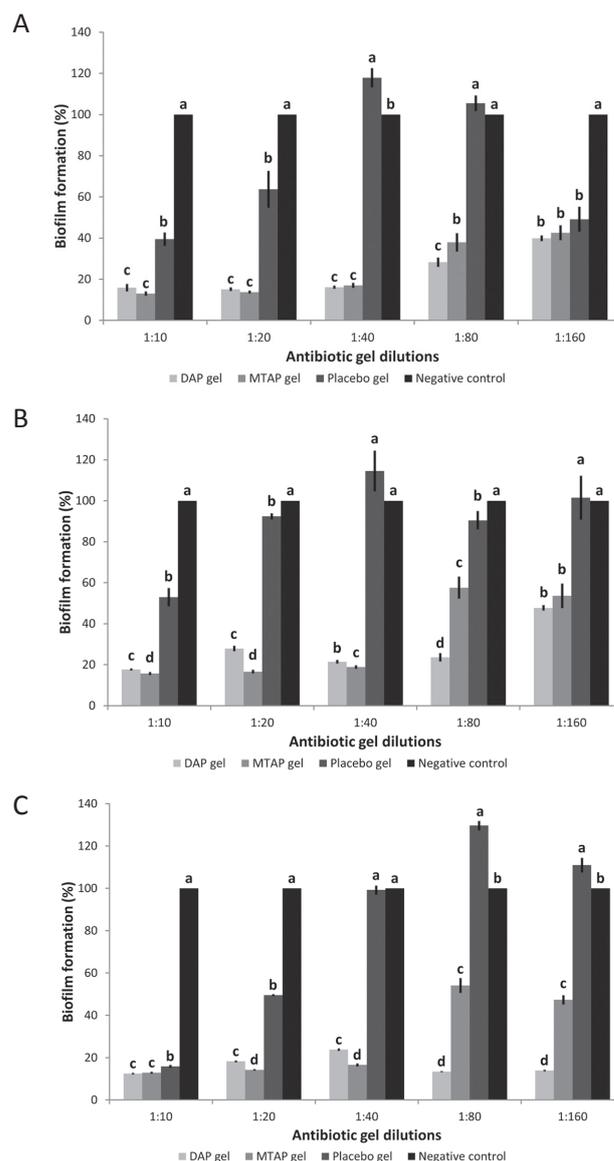


Fig. 1 Mean (SE) percentage of biofilm formation by *E. faecalis* treated with various dilutions of gels and an untreated negative control (set at 100%) at the baseline (immediately after gel preparation) (A), at 1 month after gel preparation (B), and at 3 months after gel preparation (C). Within each dilution, different lower case letters indicate statistically significant differences.

< 0.00001). On the other hand, the DAP gel exhibited a significantly stronger biofilm-inhibitory effect than did the MTAP gel at dilutions of 1:80 and 1:160 ($P < 0.00001$). The placebo gel demonstrated a significant biofilm-inhibitory effect at lower dilutions (1:10, 1:20) relative to the negative control ($P < 0.001 - P < 0.00001$), regardless of the time point at which the gels were tested. However, no significant differences were found between the placebo gel and the negative control at the majority of higher dilutions over the range 1:40-1:160 through all of the tested time points. Furthermore, the placebo gel

allowed significantly higher degrees of biofilm formation relative to the negative control at the baseline for the 1:40 dilution ($P = 0.0002$), as well as at 3 months for the 1:80 and 1:160 dilutions ($P < 0.0001$ and $P = 0.0011$, respectively).

Antibiofilm effect against *P. gingivalis*

Figures 2 A-C show that the MTAP and DAP gels reduced biofilm formation significantly relative to the untreated negative control bacteria at all time points, regardless of the tested dilution ($P < 0.00001$). Additionally, both the DAP and MTAP gels significantly reduced biofilm formation relative to the placebo gel at all dilutions through all time points ($P = 0.042 - P < 0.00001$) except for the 1:10 dilution of DAP gel at 1 month, the 1-month MTAP gel at a 1:40 dilution and the 3-month MTAP gel at 1:40 dilutions. No significant differences in biofilm formation were observed between the MTAP and DAP gels at all time points, regardless of the tested dilutions, except for the baseline 1:40 dilution, where MTAP demonstrated a significantly stronger biofilm-inhibitory effect than DAP ($P = 0.007$). At the baseline (Fig. 2A), the placebo gel provided no significant reduction of biofilm formation relative to the untreated negative control bacteria at all dilutions except for 1:10 ($P < 0.0006$). One month after gel preparation, the placebo gel demonstrated a significant reduction in biofilm formation relative to the untreated negative control bacteria at all dilutions ($P < 0.0001$) except 1:80 and 1:160 (Fig. 2B). Three months after preparation of the gel (Fig. 2C), the placebo gel exhibited a significant reduction in biofilm formation relative to the untreated negative control bacteria at all dilutions ($P = 0.027 - P < 0.0001$).

Discussion

Topical application of antibiotics to control endodontic infections has been employed for decades (24). However, this practice has gained popularity in recent years with the introduction of endodontic regeneration procedures (3). DAP was used in the first reported case of pulp revascularization (25). Furthermore, endodontic regeneration cases disinfected with antibiotic medicament were suggested to show better endodontic regeneration outcomes than cases treated with calcium hydroxide or formocresol (26). In the present study, we explored the biofilm-inhibitory ability of two antibiotic gels loaded with DAP or MTAP at only 1 mg/mL in an attempt to introduce an effective antibiotic intracanal medicament with minimal cytotoxic properties.

Our results indicated that methylcellulose-based DAP and MTAP gels significantly reduced biofilm formation

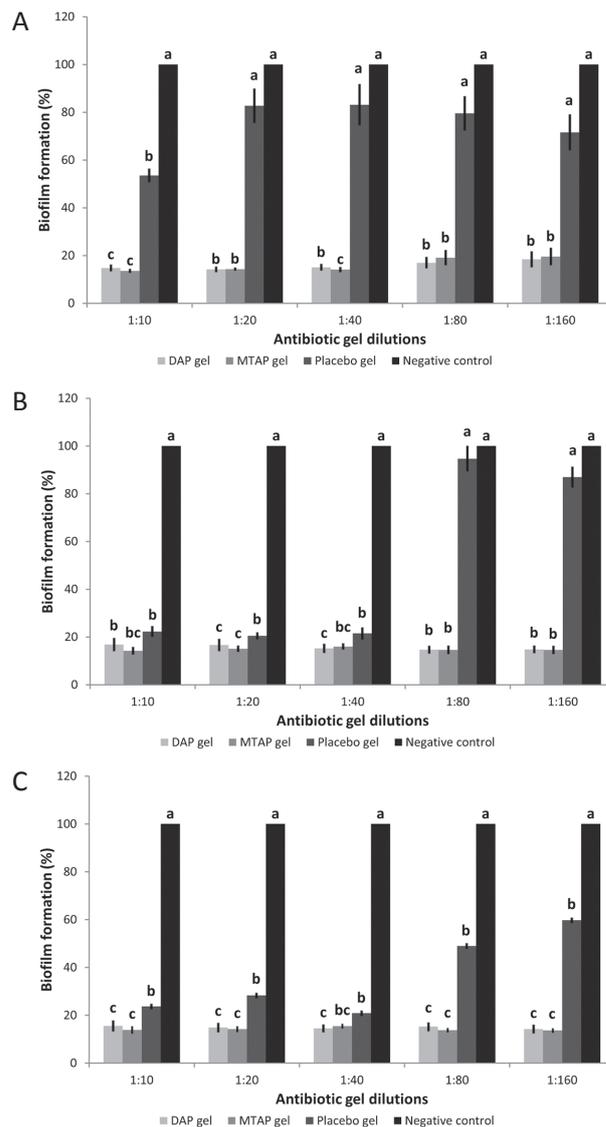


Fig. 2 Mean (SE) percentage of biofilm formation by *P. gingivalis* treated with various dilutions of gels and an untreated negative control (set at 100%) at the baseline (immediately after gels preparation) (A), at 1 month after gel preparation (B), and at 3 months after gel preparation. Within each dilution, different lower case letters indicate statistically significant differences.

by both species of tested bacteria at all dilutions, regardless of the length of gel aging time. Additionally, the DAP and MTAP gels demonstrated significant reduction of biofilm formation by both of the tested bacterial species relative to the placebo gel at the vast majority of tested dilutions at all of the tested time points. Furthermore, we found that various dilutions of both antibiotic gels (1:10-1:160) reduced biofilm formation significantly. Our results generally agree with a recent study in which the minimum bactericidal concentrations of DAP and TAP against both *E. faecalis* and *P. gingivalis* were 0.14 and 0.3 mg/mL, respectively (7). However, the

minimum bactericidal concentrations of DAP and TAP reported previously were obtained from polyantibiotic solutions rather than medicaments with higher viscosity. The DAP and MTAP gels used in this study were loaded with an antibiotic concentration higher than the previously reported minimum bactericidal concentrations to ensure sufficient availability and diffusion of the antibiotics within the viscous environment of the gels. No significant differences in biofilm formation between the DAP and MTAP gels were observed in the majority of tested dilutions at all time points. Additionally, the significant differences between the two antibiotic gels observed in the present study did not indicate any clear tendency for one antibiotic gel to have a better biofilm-inhibitory effect than the other. The small variability (standard deviations) in biofilm formation may justify the statistically significant differences observed between the two antibiotic gels at some dilutions. Generally, the results from the present study indicate that there is no need to include clindamycin in the intracanal antibiotic medication, and that a combination of metronidazole and ciprofloxacin may be sufficient to achieve the required disinfection during endodontic regeneration procedures.

Various antibiotic medicaments such as DAP and TAP are used clinically at a concentration of 1 g/mL (10), at which they are acidic (pH \approx 3) (27). This acidic nature of such antibiotics may be helpful for maintaining their chemical stability and physiological compatibility (28). However, the antibiotic gels tested in this study had neutral pH due to the low concentration of their active antibiotic ingredients (1 mg/mL). Therefore, the biofilm-inhibitory abilities of DAP and MTAP gels after 1 and 3 months of aging were also tested to explore the stability of their antibacterial properties over time. Generally, both the DAP and MTAP gels were effective in reducing biofilm formation by both of the tested bacterial species at all time points. Furthermore, it is worth mentioning that 1 mg/mL methylcellulose-based MTAP reportedly has minimal adverse effects on the microhardness and chemical structure of radicular dentin in comparison with the concentration of 1 g/mL used clinically (16).

Methylcellulose was used in this study as a vehicle for producing clinically applicable intracanal antibiotic medicaments. Methylcellulose is one of the most common vehicles used in drug delivery, particularly in commercial intracanal medicaments (e.g., UltraCal and Pulpdent). Methylcellulose is considered to increase the duration of therapeutic drug release, thus prolonging the effects (29). Furthermore, the non-cytotoxic nature of methylcellulose makes it one of the most commonly used culture media for stem cell growth and differen-

tiation (30), which might be helpful during endodontic regeneration procedures. The present study demonstrated that the placebo methylcellulose gel provided a significant reduction of biofilm formation relative to the negative control at some of the tested dilutions, primarily the lower dilutions. The viscous nature of the prepared gels, including the placebo, may interfere with bacterial attachment and affect biofilm formation at low dilutions in the microtiter plate model used in this study. Furthermore, various placebo gels (vehicles) have been shown to exert significant antibacterial effects against *P. gingivalis* (31,32).

Various substrates, such as dentin, polystyrene microtiter plates, hydroxyapatite disks, and nitrocellulose membrane filters, can be used to determine the antibiofilm effects of endodontic materials (33). A crystal violet biofilm assay using polystyrene microtiter plates, which has been widely reported in the endodontic literature (7,34,35), was used in the present study. This is a standardized assay that allows rapid retrieval and quantification of bacterial biofilms (33,34). However, the use of a dentin substrate for biofilm formation is more representative of the actual clinical situation. Therefore, the antibiofilm effect of the antibiotic gels tested in this study will need to be confirmed using a dentin biofilm model.

Within the limitations of this in vitro study, it appears that DAP and MTAP gels at 1 mg/mL facilitate significant reduction of biofilm formation by *E. faecalis* and *P. gingivalis* at all tested dilutions, even after aging of the gel preparations for 1 and 3 months. Furthermore, no single antibiotic gel showed a clear tendency to have a biofilm-inhibitory effect that was superior to the other. These antibiotic gels can be considered as potential intracanal medicaments during endodontic regeneration procedures.

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