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Effects of two combinations of triple antibiotic paste used in endodontic regeneration on root microhardness and chemical structure of radicular dentine

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Abstract: We investigated the effects of triple antibiotic paste (TAP) and modified triple antibiotic paste (MTAP) concentrations on the microhardness and chemical structure of radicular dentine. Human root cylinders were instrumented and randomized into four treatment groups and an untreated control group. Two treatment groups received 1 g/mL TAP or MTAP, and the other two treatment groups received 1 mg/mL methylcellulose-based TAP or MTAP. Cylinders were stored at 100% relative humidity for 4 weeks. Each root cylinder was subjected to a microhardness test before and after treatment. Different sets of radicular dentine specimens were treated as mentioned previously, and were examined using attenuated total reflection Fourier transform infrared spectroscopy. All treatment groups showed significant reductions in microhardness of roots when compared to untreated control roots at 1,000 and/or 500 μm from the pulp-dentine interface. However, 1 mg/mL methylcellulose-based antibiotics caused significantly less reduction in microhardness when compared to 1 g/mL antibiotics. In addition, 1 g/mL TAP and DAP caused significantly lower phosphate/amide I ratios when compared to other groups. The use of 1 mg/mL

methylcellulose-based TAP and MTAP may minimize the reduction in microhardness of roots compared with the currently used 1 g/mL concentration of these antibiotics. (J Oral Sci 56, 245-251, 2014)

Keywords: endodontic regeneration; FTIR; microhardness; modified triple antibiotic paste; triple antibiotic paste.

Introduction

Case reports and clinical studies have suggested the use of endodontic regeneration procedures to treat immature teeth with infected necrotic pulps (1). These procedures are proposed to induce continuous root development and reduce the risk of fracture associated with traditional treatments, such as calcium hydroxide apexification (2). Necrotic immature teeth treated with an endodontic regeneration approach were found to have significant increases in root length and thickness when compared to cases treated with calcium hydroxide apexification or MTA (3). Root canal disinfection using intracanal medicaments is an essential requirement for endodontic regeneration (4). Triple antibiotic paste (TAP), a mixture of metronidazole, ciprofloxacin, and minocycline, is the most widely used intracanal medicament in endodontic regeneration (1). However, crown discoloration has been associated with TAP (5-8). Therefore, recent studies have suggested substituting minocycline with another antibiotic (1,9). Clindamycin has been found to be effective against various endodontic pathogens (10,11). A modified triple

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antibiotic paste (MTAP) composed of metronidazole, ciprofloxacin, and clindamycin was successfully used as an intracanal medicament to disinfect necrotic immature teeth during an endodontic regeneration procedure (9).

A pasty consistency for antibiotic medicaments, which occurs at 1 g/mL, is commonly used in endodontic regeneration (12,13). However, this high concentration is suggested to have detrimental direct and indirect effects on human stem cells of the apical papilla (12,14) and human dental pulp cells (15). Therefore, recent studies have recommended the use of lower concentrations of these antibiotic medicaments ranging from 0.1-2 mg/mL to overcome their negative cytotoxic effects (12,14,15). The clinically applied concentration of various antibiotic medicaments was also found to negatively affect the mechanical (16,17) and chemical properties (18) of radicular dentine. A recent *in vitro* study reported a reduction in microhardness and root resistance to fracture after one and three months of application of intracanal medicaments, respectively (16). The aim of this study was to investigate the effects of two concentrations of TAP and MTAP on the microhardness and chemical structure of radicular dentine.

Materials and Methods

Microhardness experiment

Sample preparation and baseline measurement

Intact human single-rooted mature premolars ($n = 85$) were selected after obtaining local Institutional Review Board (IRB) approval to use the teeth (#1301010364, 2013). Teeth with caries, restorations, hypoplasia, or cracks were excluded and replaced. Teeth were stored at 4°C in 0.1% thymol solution and were used within 6 months after extraction. A cervical 5-mm root cylinder was obtained from each tooth. The canal of each root cylinder was mechanically prepared using EndoSequence 0.04 taper rotary instruments (Brasseler, Savannah, GA, USA) with a master apical size 80 file. One milliliter of 5.25% sodium hypochlorite was used for irrigation between subsequent filings and each canal was finally rinsed with 5 mL of sterile water. Instrumented root cylinders were embedded in clear acrylic blocks (Fastry; Harry J Bosworth, Skokie, IL, USA). The coronal side of each cylinder was leveled with the surface of the acrylic block in order to maintain easy access to the root canal. The resulting blocks were ground flat and polished with water-cooled abrasive discs (500-, 1,200-, 2,400-, and 4,000-grit Al₂O₃ papers; MD-Fuga, Struers, Cleveland, OH, USA) and a polishing cloth with a diamond suspension (1 mm; Struers). Blocks were then ultra-sonically cleansed for 3 min, and pre-treatment microhardness

measurements were performed using a Vickers microhardness Tester (LM247; Leco, St. Joseph, MI, USA) on the coronal side of each root cylinder at 500 µm and 1,000 µm from the pulp-dentine interface. Three indentations (50 g, for 10 s) were made at each depth and representative hardness values were reported as the mean of the three indentations.

Medicament application and final measurement

In order to prepare 1 g/mL TAP, 1 g of United States Pharmacopeia (USP)-grade antibiotic powders comprising equal portions of metronidazole, ciprofloxacin, and minocycline (Champs Pharmacy, San Antonio, TX, USA) were mixed with 1 mL of sterile water. To prepare 1 g/mL MTAP, 1 g of USP-grade antibiotic powders comprising ciprofloxacin 14%, metronidazole 43% and clindamycin 43% (Skywalk Pharmacy, Wauwatosa, WI, USA) were mixed with 1 mL of sterile water. To prepare a 1 mg/mL solution of TAP or MTAP, 100 mg of each compounded powder mentioned above was dissolved in 100 mL of sterile water. Then, 8 g of methylcellulose powder (Methocel 60 HG; Sigma-Aldrich, St Louis, MO, USA) was added to 100 mL of each 1 mg/mL solution under magnetic stirring for 2 h to obtain a homogenous gel with a 1mg/mL concentration of TAP or MTAP.

Root cylinders were randomly assigned to four treatments groups (1 g/mL TAP, 1 g/mL MTAP, 1 mg/mL TAP, and 1 mg/mL MTAP) and a no treatment control group ($n = 17$ per group). For the control group, sterile water was applied to the canal. For the treatment groups, the exposed coronal surface of each root cylinder was covered with adhesive unplasticized polyvinyl chloride tape (Graphic Tape; Chartpak, Leeds, MA, USA), leaving only the root canal orifice. Each root canal was dried with sterile paper points (Hygienic, Akron, OH, USA) and the antibiotic paste was tamped into the canal space. Acrylic blocks were covered with custom made vinyl material caps (Soft-Try; Ultradent Products, South Jordan, UT, USA) and stored for four weeks at 37°C at an approximately 100% relative humidity. Each root canal was then copiously irrigated with sterile water for 30 s to wash out the treatment paste. Adhesive tape was removed and post-treatment microhardness measurements were taken as described previously. The percentage change in microhardness for each sample was calculated as follows:

$$(\text{Pre-treatment microhardness} - \text{post-treatment microhardness}) \times 100 / \text{pre-treatment microhardness}.$$

Table 1 Mean (SD) Vickers microhardness (VH) for roots treated for four weeks with various endodontic regeneration medicaments and a control group at 500 μm and 1,000 μm from the pulp-dentine interface

Group	500 μm from pulp-dentine interface (VH)			1,000 μm from pulp-dentine interface (VH)		
	Pre-treatment [†]	Post-treatment [†]	% change [‡]	Pre-treatment [†]	Post-treatment [†]	% change [‡]
No treatment	52 (7.5)a	52 (7.7)a	0 (4.6)A	59.1 (7.1)a	60.1 (8)a	-1.6 (3.2)A
1 mg/mL MTAP	52.9 (6.9)a	50.4 (6.9)b	4.6 (5)B	59.9 (6.4)a	59.4 (6.3)a	0.9 (3.5)AB
1 mg/mL TAP	54.6 (6.5)a	50.4 (7.2)b	7.7 (5.9)B	61.3 (5.8)a	58.5 (6.2)b	4.5 (5.8)B
1 g/mL MTAP	53.5 (7.1)a	45.9 (6.6)b	13.9 (7.5)C	60.8 (4.7)a	53.6 (5.4)b	11.8 (6.2)C
1 g/mL TAP	49.7 (6.9)a	39 (5.8)b	21.2 (8.6)D	57.8 (6.6)a	51.4 (5.7)b	10.3 (8)C

[†]At each distance, different lower-case letters indicate significant differences between pre- and post-treatment of the same group.

[‡]At each distance, different upper-case letters indicate significant differences between different treatment groups.

Fourier transform infrared spectroscopy (FTIR) experiment

Sample preparation

An additional 18 intact mature single-rooted premolars were selected according to the same criteria described in the microhardness experiment. A cervical 4-mm root cylinder from each tooth was obtained and sectioned longitudinally across the maximum diameter of the root canal resulting in two specimens. Both sides of each specimen were ground flat to a uniform thickness with 1,200 grit silicon carbide grinding paper (Buehler Ltd., Lake Bluff, IL, USA) under continuous water-cooling. Specimens were ultrasonicated for 5 min under sterile water to remove the smear layer.

Medicament application and FTIR measurement

Dentine specimens were randomly assigned to the same four treatment groups and a control group ($n=7$ per group) as described in the microhardness experiment. Each dentine specimen was placed in a 2-mL conical sample cup (Fisher Scientific, Florence, KY, USA) containing 0.15 mL of one of the treatment pastes or sterile water (control). The amount of paste selected was sufficient to cover the pulpal surface of each specimen with a 0.2-mm layer of treatment paste. The containers were stored for four weeks at 37°C with approximately 100% relative humidity. Specimens were then rinsed thoroughly with sterile water until no visible paste remained, followed by ultrasonication for 15 min and air-drying.

The chemical structure of treated dentine specimens was analyzed using a 4100 FTIR spectrophotometer (JASCO Inc., Tokyo, Japan) with a diamond ATR setup. The pulpal surface of each dentine specimen was placed on a standard FTIR sample holder with a 2.5-mm diameter opening and spectra were collected in triplicate from each treated dentine specimen between 800 and 2,000 cm^{-1} at 4 cm^{-1} resolution using 100 scans. Each obtained

spectrum was then processed by smoothing, baseline correction, and normalization against the amide I peak using dedicated Spectra Manager CFR software (JASCO Inc.). The effects of various antibiotic medicaments on collagen and apatite composition of surface dentin were evaluated using the mineral matrix ratio (ratio of integrated areas of phosphate ν_1 and ν_3 peaks to the amide I peak). The final ratio assigned for each dentine specimen represented the average of the ratios obtained from the three spectra.

Scanning electron microscopy (SEM)

Two root cylinders from the microhardness experiment were randomly selected from each group for SEM analysis in order to observe any morphological changes in root canal dentine. Each selected root cylinder was sectioned longitudinally without touching the root canal surface. Then, each half of the root cylinder was irrigated with 5 mL of de-ionized water, sonicated in de-ionized water for 5 min, and desiccated for 48 h. Specimens were sputter coated for 70 s with gold/palladium using a sputter coater (Polaron, Agawam, MA, USA) and images were taken from the treated root canal surface area of the specimens with a JEOL 7800F scanning electron microscope (JEOL, Peabody, MA, USA) in secondary electron imaging mode.

Statistical analyses

All data were checked for normality using the Shapiro-Wilk test and normality assumptions were satisfied. The paired t -test was used to compare between the pre- and post-treatment Vickers microhardness in each group. The effects of medicament type on percentage change in microhardness and phosphate/amide I ratios was examined using one-way ANOVA followed by Fisher's Least Significant Difference. A 5% level of statistical significance was used for all analyses.

Results

Microhardness measurements at 500 μm from pulp-dentine interface

Table 1 shows that the post-treatment microhardness was significantly lower than the pre-treatment microhardness for all treatment groups ($P < 0.0001$). The percentage reduction in microhardness was significantly higher for all treated groups when compared with the untreated control group ($P < 0.0001$ for 1 g/mL TAP and MTAP; $P = 0.0008$ for 1 mg/mL TAP; and $P = 0.039$ for 1 mg/mL MTAP). The percentage reduction in microhardness was significantly higher for 1 g/mL TAP-treated dentine when compared with 1 g/mL MTAP-treated ($P = 0.0016$) and 1 mg/mL TAP- and MTAP-treated ($P < 0.0001$) dentine. In addition, the percentage reduction in microhardness was significantly higher for 1 g/mL MTAP when compared with 1 mg/mL TAP- ($P = 0.0069$) and MTAP-treated ($P < 0.0001$) roots.

Microhardness measurements at 1,000 μm from pulp-dentine interface

Table 1 shows that the post-treatment microhardness was significantly lower than the pre-treatment microhardness for 1 g/mL TAP-, 1 g/mL MTAP- ($P < 0.0001$), and 1 mg/mL TAP-treated ($P = 0.005$) roots. However, no significant differences were observed between pre- and post-treatment microhardness for roots treated with 1 mg/mL MTAP and untreated control roots. The percentage reduction in microhardness was significantly higher for 1 g/mL TAP- ($P < 0.0001$), 1 g/mL MTAP- ($P < 0.0001$), and 1 mg/mL TAP-treated ($P = 0.002$) roots when compared with the untreated control group. However, the percentage reduction in microhardness of 1 mg/mL MTAP-treated roots was not significantly different from the untreated control group ($P = 0.2$). The percentage reduction in microhardness was significantly higher for 1 g/mL TAP- and MTAP-treated roots when compared to 1 mg/mL TAP- ($P = 0.0036$ or $P = 0.0003$, respectively) and MTAP-treated ($P < 0.0001$) roots.

ATR-FTIR Spectroscopy measurements

Representative spectra obtained from untreated control dentine and various dentine treatments are shown in Figs. 1A and 1B. Figure 1C shows that the phosphate/amide I ratio in the 1 g/mL TAP-treated group was significantly lower than in all other treatment groups and in untreated control dentine ($P < 0.0001$). The phosphate/amide I ratio with 1 g/mL MTAP was significantly lower than with 1 mg/mL TAP ($P = 0.0004$) and 1 mg/mL MTAP ($P < 0.0001$), and in untreated control dentine ($P = 0.044$). Furthermore, phosphate/amide I ratios in 1 mg/mL

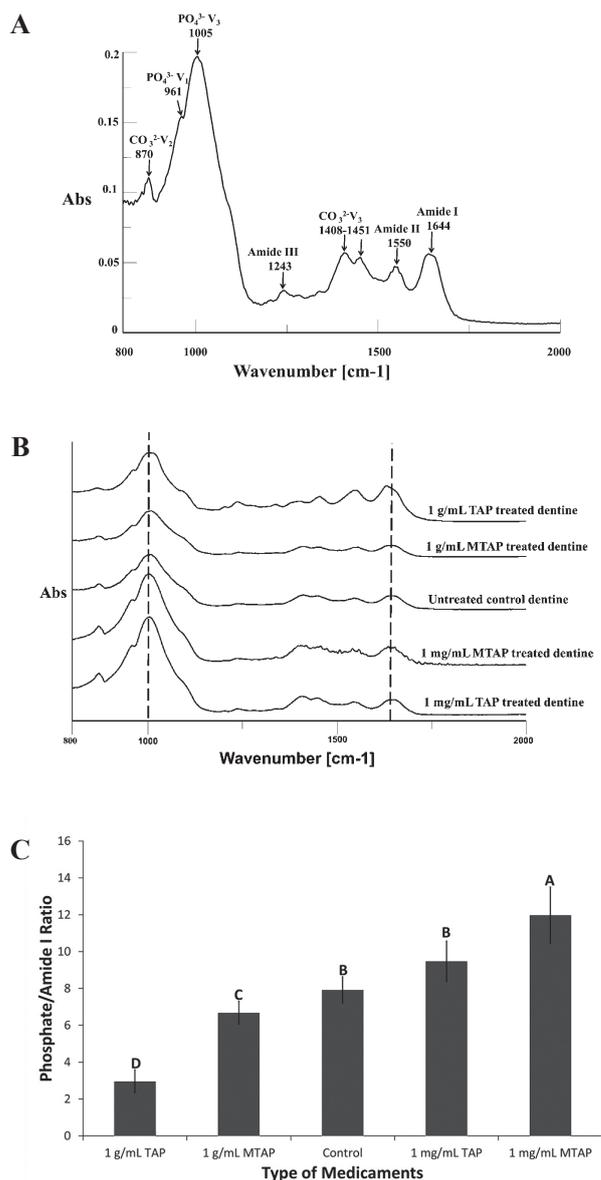


Fig. 1 Representative ATR spectrum of intact radicular dentine from untreated control group (A). Representative ATR spectra of radicular dentine specimens after exposure to 1 g/mL TAP, 1 g/mL MTAP, sterile water, 1 mg/mL TAP, and 1 mg/mL MTAP for 4 weeks (B). Mean (SD) phosphate/amide I ratios of radicular dentine treated for four weeks with various endodontic regeneration medicaments and a control group (C). Different upper-case letters indicates significant differences.

MTAP-treated dentine were significantly higher than in all other groups ($P < 0.0001$).

SEM

SEM images showed the presence of a smear layer in instrumented root canals of untreated control dentine (Fig. 2A) and 1 mg/mL MTAP-treated dentine (Fig. 2B). No visible smear layer was observed in other treatment

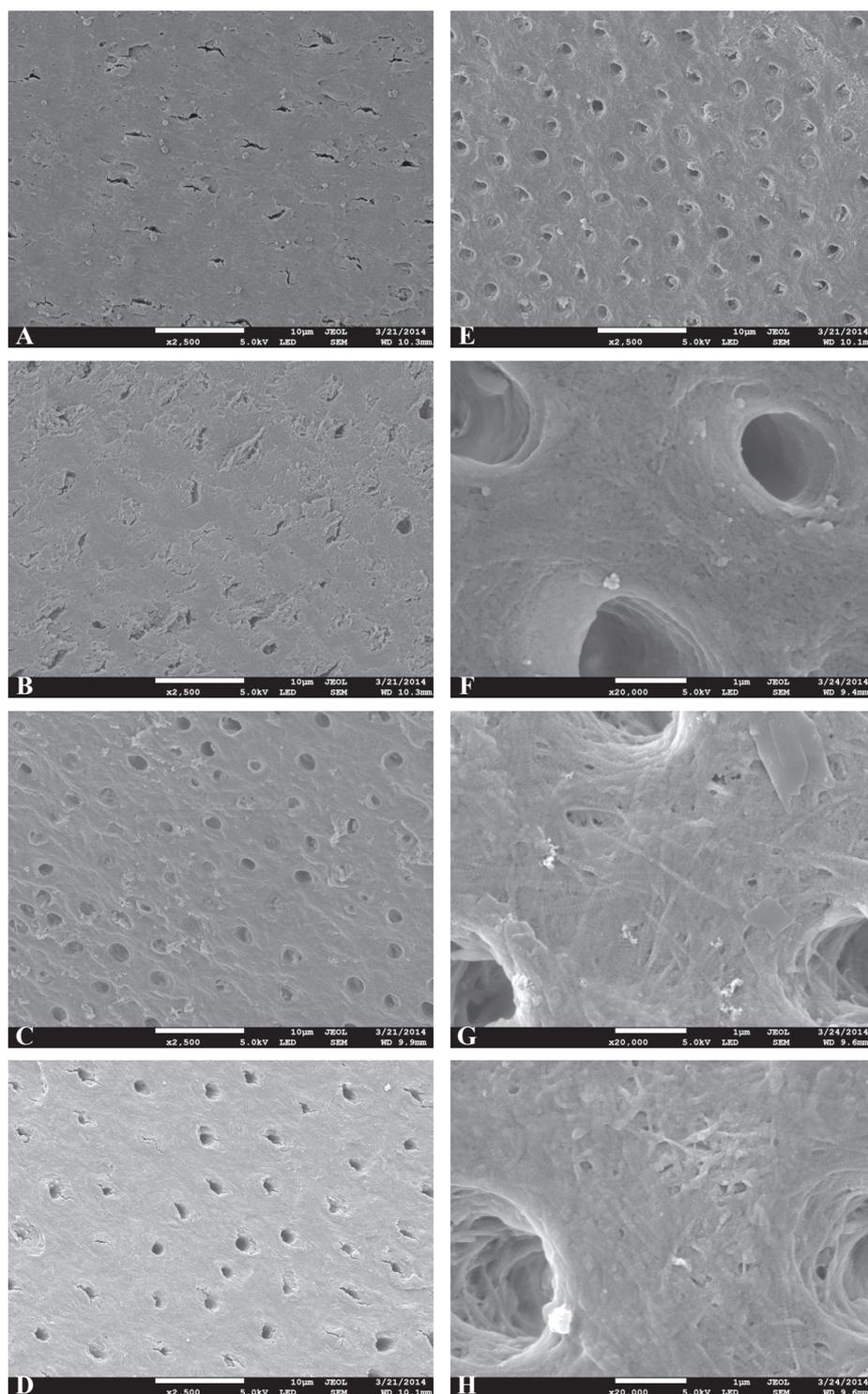


Fig. 2 Representative SEM images from root canal surface of roots treated with various endodontic regeneration medicaments and untreated control group. Untreated control root (A). 1 mg/mL MTAP-treated root canal (B). 1 mg/mL TAP-treated root canal (C). 1 g/mL MTAP-treated root canal (D). 1 g/mL TAP-treated root canal (E). 1 mg/mL TAP-treated root canal under high magnification (F). 1 g/mL MTAP-treated root canal under high magnification (G). 1 g/mL TAP-treated root canal under high magnification (H).

groups (Figs. 2C-E). In addition, the native structure of collagen fibrils was identified under higher magnification in root canals treated with 1 mg/mL TAP, 1 g/mL MTAP, and 1 g/mL TAP (Figs. 2F-H).

Discussion

In endodontic regeneration procedures, little or no root canal instrumentation is recommended in order to avoid further weakening of already thin immature root.

Therefore, effective chemical challenge using irrigation solutions and intracanal medicaments is required to eradicate endodontic pathogens and improve the biological environment for endodontic regeneration. However, these chemical agents may negatively affect the chemical, physical, and mechanical properties of radicular dentine (19). Clinical studies have found that the increase in root wall thickness of immature teeth after endodontic regeneration is limited to the mid- and/or apical root rather than the cervical region (3,20), which is the area prone to fracture in treated immature teeth (21). Therefore, it is essential to minimize the negative effects of intracanal medicaments on the weak cervical area of necrotic immature teeth.

In this study, the concentrations of TAP and MTAP currently used in endodontic regeneration (1 g/mL) caused significant reductions in microhardness at both measured depths compared to other groups. This could be explained by the demineralizing effects of these acidic antibiotic mixtures when used at higher concentrations (18). A recently published study has also found significant microhardness reductions in dentine in one-month TAP-treated root canals, as compared to untreated control roots (16). The present study also showed that 1 g/mL TAP treatment caused significantly higher reduction in microhardness at 500 μm from the pulp-dentine interface when compared to MTAP treatment at the same concentration. This could be explained by the ability of the minocycline present in TAP to chelate calcium and demineralize radicular dentine (22). It is also noteworthy that calcium hydroxide has been used as an intracanal medicament during endodontic regeneration procedures and was found to have negative effects on various mechanical properties of radicular dentine (23).

The present study showed that 1 mg/mL methylcellulose-based TAP and MTAP caused a significantly lower reduction in microhardness when compared with 1 g/mL concentrations of the same antibiotics. Low concentrations of TAP and MTAP (1 mg/mL) were included in this study in an attempt to minimize the expected unwanted effects of high concentrations of these medicaments on the chemical and mechanical properties of radicular dentine. This low concentration of TAP was suggested to be efficient against various endodontic pathogens (24). In addition, 1 mg/mL for various antibiotic mixtures was found to have no indirect cytotoxic effects against human stem cells of the apical papilla (14). However, a methylcellulose vehicle was added to this low concentration in order to create a clinically applicable consistency. Methylcellulose is the most commonly used vehicle in commercial calcium hydroxide based intracanal medica-

ments such as UltraCal and Pulpdent.

In an attempt to further understand the effects of antibiotic medicaments on radicular dentine, the changes in chemical integrity of superficial radicular dentine after TAP and MTAP applications were also explored in this study using ATR-FTIR. The significant reduction in phosphate/amide I ratio after 1 g/mL TAP and MTAP treatment when compared to untreated dentine indicates a demineralization effect by these pastes. The presence of an apatite-depleted collagen layer in the root canals treated with 1 g/mL TAP and MTAP was confirmed by the SEM images. Dentine treated with 1 g/mL TAP had a significantly lower phosphate/amide I ratio when compared to dentine treated with the same concentration of MTAP. The significant dentine demineralization effect of 1 g/mL TAP reported by ATR-FTIR may validate the significantly higher reduction in microhardness of roots treated with 1 g/mL TAP when compared to root treated with the same concentration of MTAP at 500 μm from the pulp-dentine interface reported in this study. Our study showed a trend of higher phosphate/amide I ratios in 1 mg/mL TAP- and MTAP-treated dentine when compared to untreated dentine. This could be explained by chemical overlapping in the measured spectra between the residues of organic C-O-C peak from methylcellulose-based antibiotic mixtures and the inorganic phosphate peaks of radicular dentine. Methylcellulose-based calcium hydroxide intracanal medicament was found to have significantly higher retention capacity in radicular dentine when compared with pure calcium hydroxide (25).

Collectively, our study showed that the two concentrations of TAP and MTAP cause significant reductions in the microhardness of root when compared to untreated controls. However, 1 mg/mL methylcellulose-based TAP and MTAP caused significantly less reduction in microhardness when compared to 1 g/mL TAP and MTAP. ATR-FTIR measurements indicated that 1 g/mL TAP and MTAP caused significant demineralization when compared to other treatment groups and untreated control dentine. The use of 1 mg/mL TAP and MTAP can significantly minimize the reduction in root microhardness when compared to the currently used concentration of antibiotic medicaments.

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