

THE EFFECT OF ENDODONTIC REGENERATION MEDICAMENTS ON
MECHANICAL PROPERTIES OF RADICULAR DENTIN

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DEDICATION

To my dear mom for her prayers, love, support and encouragement. To my elder sister “Alaa Yassen” for her support and prayers. Last, I dedicate this degree to my late father “Hamdon Yassen”, who always supported my education.

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ABSTRACT

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THE EFFECT OF ENDODONTIC REGENERATION MEDICAMENTS ON MECHANICAL PROPERTIES OF RADICULAR DENTIN

Endodontic regeneration treatment of necrotic immature teeth has gained popularity in recent years. The approach suggests a biological alternative to induce a continuous root development. In this project, three *in vitro* experiments were conducted to investigate the effect of three medicaments used in endodontic regeneration on mechanical properties and chemical structure of radicular dentin. In the first experiment, we investigated longitudinally the effect of medicaments on the indentation properties of the root canal surface of immature teeth using a novel BioDent reference point indenter. A significant difference in the majority of indentation parameters between all groups was found after one-week and one-month application of medicaments ($p < 0.0001$): triple antibiotic paste (TAP) > double antibiotic paste (DAP) > control > calcium hydroxide [Ca(OH)₂]. The four-week exposure of dentin to TAP and DAP caused 43% and 31% increase in total indentation distance outcome, respectively.

In the second experiment, we investigated longitudinally the effect of medicaments on the chemical structure of immature radicular dentin by measuring the phosphate/amide I ratios of dentin using Attenuated Total Reflection Fourier Transform Infrared Spectroscopy. Phosphate/amide I ratios were significantly different between the four groups after one week, two weeks and four week application of medicaments

($p < 0.0001$): Ca(OH)_2 -treated dentin > untreated dentin > DAP-treated dentin > TAP-treated dentin.

In the third experiment, we investigated longitudinally the effect of medicaments on root fracture resistance and microhardness of radicular dentin. For the microhardness, the two-way interaction between group and time was significant ($p < 0.001$). TAP and DAP caused a significant and continuous decrease in dentin microhardness after one and three month application, respectively. The three-month intracanal application of Ca(OH)_2 significantly increased the microhardness of root dentin. The time factor had a significant effect on fracture resistance ($p < 0.001$). All medicaments caused significant decrease in fracture resistance ranging between 19%-30% after three month application compared to one week application. The three medicaments used in endodontic regeneration caused significant change in the chemical integrity of the superficial radicular dentin and significantly affected the indentation properties of the root canal surface. Furthermore, the three month intracanal application of medicaments significantly reduced the fracture resistance of roots.

Jeffrey A. Platt, D.D.S., M.S., Chair

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INTRODUCTION

The challenge of treating necrotic immature teeth

Pulp necrosis is a common complication after dental trauma of immature permanent teeth. The successful endodontic treatment of traumatized immature permanent teeth with necrotic pulps presents multiple challenges due to thin and divergent walls of root canals and wide open apices. These challenges make the endodontic debridement and obturation rather difficult. Furthermore, sufficient widening of the root coronal segment to make its diameter greater than that of the apical portion would significantly weaken the root and increase the risk of fracture. A recent four-year retrospective study found that necrotic immature teeth were the most common type of traumatic dental injuries referred by general dentists (Yassen et al. 2013).

The oldest approach suggested to treat necrotic immature teeth was periapical surgical intervention (apicoectomy) (Friend 1967). The disadvantages of apical surgery include the difficulty of obtaining the required apical seal in the immature tooth with its thin, fragile and irregular walls at the root apex. These thin walls may shatter during preparation of the retro-cavity or condensation of the filling material. Additionally, the wide apical foramen results in a large volume of filling material and a compromised seal. Apicoectomy further reduces the length of the root leading to a very unfavorable crown root ratio (Rafter 2005). The limited success of apicoectomy resulted in significant interest in the phenomenon of continued apical development or establishment of an apical barrier (apexification).

Calcium hydroxide apexification

Calcium hydroxide [Ca(OH)₂] apexification treatment of necrotic immature permanent teeth was first introduced in the 1960s (Frank 1966). The high pH and antibacterial property have made non-setting Ca(OH)₂ the traditional material of choice in the endodontic treatment of immature teeth to stimulate the formation of mineralized fibrous tissue in the apical part of the root canal (Vojinovic 1974; Kontakiotis et al. 1995). However, Ca(OH)₂ apexification technique is associated with some disadvantages such as the long time required for the completion of treatment, the need for patient compliance, chance of coronal leakage and re-infection of the canal system after loss of temporary restorations during treatment and the chance of unsuccessful treatment because of undiagnosed root fracture of the traumatized tooth. Additionally, the relatively thin tooth structure after apexification treatment of immature teeth increases the risk of root fracture or loss of final coronal restoration. Furthermore, it has been suggested that the use of Ca(OH)₂ adversely affects the mechanical properties of radicular dentin, such as fracture resistance (Hatibovic-Kofman et al. 2008; Tuna et al. 2011), microtensile fracture strength (Rosenberg et al. 2007), elastic modulus (Kawamoto et al. 2008) and flexural strength (Grigoratos et al. 2001).

Theoretically, the alkalinity of the Ca(OH)₂ paste is postulated to denature the dentin collagen fibrils, leaving the radicular dentin more prone to catastrophic fracture (Kawamoto et al. 2008). However, the effect of Ca(OH)₂ on the dentin collagen has not been fully understood.

Mineral trioxide aggregate apexification

Mineral trioxide aggregate (MTA) has been used by clinicians as an alternative to long term use of $\text{Ca}(\text{OH})_2$ in order to create an artificial apical barrier after short term disinfection of the canal with $\text{Ca}(\text{OH})_2$ followed by compaction of obturating material and application of a coronal restoration (Erdem & Sepet 2008; Mente et al. 2009; Moore et al. 2011). MTA has been proposed to have antimicrobial properties (Torabinejad et al. 1995), low cytotoxicity (Osorio et al. 1998), excellent biocompatibility (Mitchell et al. 1999) and low microleakage (Yildirim et al. 2009). However, some drawbacks have been associated with the use of MTA such as poor handling properties, a long setting time, tooth discoloration and high cost (Parirokh & Torabinejad 2010). Unintentional MTA extrusion into the periradicular tissue during apical barrier treatment has been recently reported in several cases (Tahan et al. 2010; Tezel et al. 2010; Comin Chiaramonti & Cavalleri 2011) and might require endodontic surgical intervention (Nosrat et al. 2012). Finally, the use of MTA as an apical plug in apexification does not reinforce the remaining root structure. Therefore, MTA treated immature necrotic teeth remains susceptible to fracture (Neha et al. 2011).

Endodontic regeneration

The first attempt of endodontic regeneration was suggested in the early 1970s (Nygaard-Ostby & Hjortdal 1971). However, endodontic regeneration techniques for the treatment of cases with necrotic immature permanent teeth have gained popularity in the last 10 years (Iwaya et al. 2001; Banchs & Trope 2004; Thibodeau & Trope 2007). These approaches suggest a biological alternative to induce a continuous root development and reduce the risk of fracture associated with traditional apexification procedures where the

root remains thin and weak (Trope 2010). The endodontic regeneration therapy includes the initial irrigation of the root canal with a root canal irrigant; followed by disinfection of the canal using an antibacterial medicament. After washing out the medicament at a subsequent visit, a blood clot is evoked in the canal by irritating the periapical tissues and finally sealing the canal with MTA and a composite restoration. However, endodontic regeneration is still an unpredictable technique with a moderate success rate (Ding et al. 2009). Therefore, it has been suggested that if after three months no signs of endodontic regeneration are present, the more traditional apexification treatment methods should be initiated (Banchs & Trope 2004; Trope 2010).

Medicaments used in endodontic regeneration

One of the essential elements for a successful endodontic regeneration protocol is the creation of a bacteria-free biological environment inside the root canal space through the use of intracanal antibacterial medicaments. In addition to the traditional use of Ca(OH)_2 as a long-term intracanal medicament to induce apexification in immature permanent teeth (Yassen et al. 2012), recent publications suggest the short-term application of Ca(OH)_2 as an intracanal disinfectant in endodontic regeneration (Bose et al. 2009; Cehreli et al. 2011). However, the most widely used intracanal medicament in endodontic regeneration is the triple antibiotic paste (TAP) (Hoshino et al. 1996), which is a mixture of metronidazole, ciprofloxacin, and minocycline (Lovelace et al. 2011; Miller et al. 2012). TAP was found to be effective against root canal bacterial pathogens both *in vitro* and *in vivo* (Sato et al. 1996; Windley et al. 2005). Double antibiotic paste (DAP), which is a combination of only metronidazole and ciprofloxacin, has been also

used successfully in endodontic regeneration (Iwaya et al. 2001) and was suggested as a substitute for TAP to avoid the discoloration effect of minocycline (Trope 2010).

Drawbacks of endodontic regeneration

The disadvantages of the endodontic regeneration approach are not yet well known. However, potential biological and clinical complications might be associated with endodontic regeneration. The majority of histological endodontic regenerative studies that have been performed on animal models showed that the tissues formed inside the root canal during endodontic regeneration are not pulp tissues (Thibodeau et al. 2007; da Silva et al. 2010; Wang et al. 2010; Yamauchi et al. 2011a; Yamauchi et al. 2011b). The formed tissues inside the canal were periodontal ligament-like tissues, cementum-like tissue, dentin-like tissue or bone-like tissue.

The outcome of regenerative endodontic treatments reported in some studies was questionable. This includes a lack of increase in root length (Petrino et al. 2010; Chen et al. 2012; Nosrat et al. 2012) or lack of increase in root wall thickness (Lenzi & Trope 2012; Nosrat et al. 2012). Additionally, the increase in root wall thickness during endodontic regeneration treatment was found to be limited to mid and/or apical root structures in the majority of reported endodontic regeneration cases (Bose et al. 2009; Hargreaves & Law 2010; Jeeruphan et al. 2012; Lenzi & Trope 2012; Nosrat et al. 2012) rather than the cervical part of the root, which is the area prone to fracture in treated necrotic immature teeth (Cvek 1992).

Crown discoloration of anterior teeth has been associated with TAP during the endodontic regeneration technique due to the presence of minocycline (Kim et al. 2010; Petrino et al. 2010). Therefore, some authors suggest sealing the dentinal tubules within

the pulp chamber before the TAP application (Reynolds et al. 2009). Furthermore, development of resistant bacterial strains (Eickholz et al. 2002; Slots 2002) and allergic reaction to the antibiotic medications (de Paz et al. 1999; Hausermann et al. 2005; Madsen et al. 2007) are some other concerns that have been raised (Reynolds et al. 2009). A recent study suggested that TAP and DAP concentrations currently used in regenerative endodontic had a detrimental effect on the survival of human stem cells of the apical papilla (Ruparel et al. 2012).

Acids are commonly added to some antibiotics to maintain chemical stability, control tonicity or to ensure physiological compatibility. However, long term exposure of dental hard tissues to acidic antibiotics might cause demineralization and negatively affect their mechanical properties. Minocycline, a component in the TAP, has been found to chelate calcium and demineralize dental hard tissues (Minabe et al. 1994; Maruyama et al. 2008). Furthermore, *in vitro* enamel exposure to aqueous tetracycline solutions for one and 25 hours caused dramatic and continuous reduction in microhardness (Bjorvatn & Olsen 1982). The relatively long application time of TAP reported in some cases of endodontic regeneration, which may reach up to 11 weeks (Thibodeau & Trope 2007; Thibodeau 2009) and the limited or no increase of dentin thickness in the cervical part of the root may render some teeth that are treated with regenerative technique more susceptible to tooth fracture. However, no previous studies have investigated the effect of antibiotic medicaments on the mechanical properties of root dentin.

Root mechanical testing in endodontics

One of the major concerns regarding any new root canal medicament/irrigant is its adverse effect on the mechanical properties of radicular dentin, which may render the

endodontically treated tooth more susceptible to fracture. Endodontic irrigation solutions such as chlorhexidine, sodium hypochlorite, hydrogen peroxide, EDTA and EDTAC have been found to significantly decrease the microhardness values of root dentin (Slutzky-Goldberg et al. 2004; De-Deus et al. 2006; Cruz-Filho et al. 2011; Patil & Uppin 2011). Furthermore, root canal irrigant solutions were found to have various effects on the structural integrity of radicular dentin. Sodium hypochlorite was found to adversely affect the organic structure of dentin and lead to superficial collagen degradation (Hu et al. 2010; Zhang et al. 2010). On the other hand, EDTA was suggested to decalcify the inorganic part of radicular dentin by chelating calcium and phosphate (Verdelis et al. 1999). Additionally, hydrogen peroxide was found to affect both inorganic and organic components of radicular dentin (Jiang et al. 2007).

Root resistance to fracture is one of the most common *in vitro* mechanical tests in endodontics to predict the effect of various endodontic materials on root fracture (Andreasen et al. 2002; Andreasen et al. 2006; Rosenberg et al. 2007; Hatibovic-Kofman et al. 2008; Sahebi et al. 2010; Uzunoglu et al. 2012). The main advantage of a fracture resistance test is that the roots are treated as in a clinical situation before they are subjected to the mechanical test. However, the difficulty in standardizing the dimensions of the tested samples given the anatomical variations between roots is the main disadvantage of fracture resistance test. Therefore, there is great heterogeneity in the literature regarding the size and dimensions of root specimens and the direction of applied force during fracture resistance tests. The microhardness test is another common mechanical test in endodontics (Slutzky-Goldberg et al. 2004; De-Deus et al. 2006; Cruz-Filho et al. 2011), which measures the resistance of the dentin to deformation caused by

penetration of an indenting stylus. Microhardness testing is very useful for the small, thin specimens typical of studies of mineralized tissues (Kinney et al. 2003). Dental manuscripts have reported that hardness depends on mineral concentration (Featherstone et al. 1983; Ogawa et al. 1983). The disadvantages of the microhardness test are the need for a highly polished and flat test sample and the required time to finish the test operation (Sakaguchi & Powers 2011). Hardness testing is not the best way to predict root fracture (Yassen & Platt 2013). Nevertheless, hardness might be related to other mechanical properties such as tensile strength, compressive strength and modulus of elasticity (Kinney et al. 2003).

The use of various indentation techniques to determine the mechanical properties of radicular dentin after exposure to various root canal irrigants and medicaments is a common approach in endodontic research (De-Deus et al. 2006; Twati et al. 2009). Previous studies have used segments of polished radicular dentin because of the difficulties of performing a standardized indentation test on the actual root canal surface. However, trying to study the mechanical properties of the root canal surface would be more accurate and representative of the actual clinical situation. Recently, a novel BioDent reference point indentation (RPI) instrument has been introduced to directly measure bone resistance to indentation both *in vitro* and *in vivo* (Randall et al. 2009; Diez-Perez et al. 2010). In the endodontic regeneration procedure, there is minimal root canal instrumentation, and the intracanal medicament is applied in direct contact with the root canal surface (Thibodeau 2009). Therefore, the use of BioDent RPI to directly characterize the indentation properties of slightly curved root canal surfaces in

regenerative endodontics without the need for any intensive and complicated polishing protocols might be more clinically relevant.

Specific aims

The overall objective of this study was to investigate the effects of medicaments used in endodontic regeneration techniques (TAP, DAP, and Ca(OH)₂ paste) on mechanical properties, chemical structure and surface morphology of radicular dentin.

To accomplish this, the following three specific aims were investigated:

Specific aim 1: To investigate longitudinally the effect of the three suggested endodontic regeneration medicaments on the indentation properties of root canal surface of immature human single rooted premolars *in vitro*. Our null hypothesis stated that the three root canal medicaments used in the endodontic regeneration technique have no significant effect on root canal surface indentation properties measured with BioDent at various time points.

Specific aim 2: To investigate longitudinally the effects of the three suggested intracanal medicaments used during endodontic regeneration on the chemical structure of human immature radicular dentin by measuring the phosphate/amide I ratios of the radicular dentin using Attenuated Total Reflection Fourier Transform Infrared Spectroscopy (ATR-FTIR) *in vitro*. Our null hypothesis stated that the three intracanal medicaments used in endodontic regeneration have no significant effect on the chemical structure of immature radicular dentin measured by ATR-FTIR at various time points.

Specific aim 3: To investigate longitudinally the effect of the three suggested endodontic regeneration medicaments on root resistance to fracture and microhardness of radicular dentin *in vitro*. Our null hypothesis stated that the three root canal medicaments used in

the endodontic regeneration technique have no significant effect on radicular dentin microhardness and root fracture resistance at all time points.

MATERIALS AND METHODS

A novel approach to evaluate the effect of medicaments used in endodontic regeneration on root canal surface indentation

Tooth selection and specimen preparation

Twenty two intact immature human mandibular premolars were used within six months of extraction after obtaining local IRB approval (IRB number; 1108006606). To be included in the study, the bucco-lingual diameter of the premolar root had to be 6.8 ± 0.5 mm as measured from cemento-enamel junction. Each tooth was decoronated horizontally at the cemento-enamel junction, and two 4-mm root dentin cylinders were obtained using a water-cooled low-speed diamond saw (Buehler, Lake Bluff, IL). The pulp tissues were removed with a barbed broach. Then, each cylinder was sectioned into two specimens longitudinally across the maximum diameter of the root canal without touching the root canal surface. Thus, four specimens were obtained from each root. Any specimen with a damaged root canal surface was excluded, and the four specimens obtained from that root were replaced.

Treatment procedure

A TAP was prepared by mixing USP grade antibiotic powders compounded of equal portions of metronidazole, ciprofloxacin, and minocycline (Champs Pharmacy, San Antonio, TX) with de-ionized water (powder/liquid ratio of 3:1). A DAP was prepared by mixing USP grade antibiotic powders compounded of equal portions of metronidazole and ciprofloxacin (Champs Pharmacy) with de-ionized water (powder/liquid ratio of 2.5:1). Ca(OH)_2 paste was prepared by mixing Ca(OH)_2 powder (Dentonics, Monroe, NC) with de-ionized water (powder/liquid ratio of 2:1). The four specimens obtained

from each root were randomly assigned to three treatment groups (TAP, DAP, and Ca(OH)₂) and one control group (de-ionized water). Each specimen was placed in a 2 mL conical sample cup (Fisher Scientific, Florence, KY) containing 0.15 mL of one of the treatment pastes or de-ionized water. The amount of paste selected was just enough to cover the root canal surface of each specimen. The containers were stored at 37°C for one or four weeks. The two time points were selected to represent the intra-canal application time of medicaments as reported in some cases of pulp regeneration (Ding et al., 2009; Nosrat et al., 2012). For the four-week groups, each specimen was hydrated with 0.07 mL of de-ionized water weekly. After each time interval, 44 specimens from 11 teeth were taken out and rinsed thoroughly with de-ionized water until no visible paste was observed.

BioDent RPI testing

The indentation properties of superficial root canal dentin of each specimen were measured using a BioDent H reference point indentation apparatus (RPI) (Active Life Scientific Inc., Santa Barbara, CA). The main part of the BioDent RPI is a probe assembly, which consists of a reference probe (a custom sharpened 22 gauge hypodermic needle) and a test probe (a 375 µm diameter rod with a 90° V-shaped end and a sphere tip of 2.5 µm radius) that slides through the inside of the reference probe. The probe assembly can be rested on the tested hard tissue, and the distance that the test probe is indented into the tissue can be measured relative to the position of the reference probe (Hansma et al., 2008). The BioDent probe assembly can operate effectively perpendicular to the probe-sample contact point with an angulation of $\pm 7.5^\circ$ (Diez-Perez et al., 2010).

Each root specimen was mounted on an acrylic block and the probe assembly was rested with full weight on the center of the root canal surface of the specimens according to the manufacturer's instruction (Figure 1 and Figure 2). The measurement was performed using the following measurement Protocol: indentation force = 5N; indentation frequency = 2Hz; Indentations per measurement = 10 Cycles. Three indentation measurements were taken and averaged from each dentin specimen with at least 1 mm distance between each measurement. Each test probe used in this study was calibrated after every 30 dentin indentations by making three indentations on PMMA block (Auburn Plastics and Rubbers, Inc, Indianapolis, IN) to ensure the integrity of the test probe tip and the consistency of the indentation measurements. Each root specimen's resistance to indentation was quantified by measuring the following BioDent RPI parameters: indentation distance increase (IDI), first cycle indentation distance (ID), total indentation distance (total ID), and creep indentation distance (CID) (Figure 3).

The hardness values of dentin specimens from all groups were calculated by simply dividing the applied force over the estimated conical indentation area created by the test probe according to the following equation:

$$\text{Hardness} = P / (\pi \times r \times \sqrt{r^2 \times h^2})$$

Where P is the constant load applied= 5 Newton; r and h are the first cycle ID values obtained from BioDent A.

Scanning electron microscopy (SEM)

One root specimen was randomly selected from each group after each time point for SEM analysis to detect the shape of indentations and observe morphological changes in root canal dentin. Thus, six BioDent indentations were viewed from each group. The

selected dentin specimens were sputter coated for 3 minutes with gold/palladium using a sputter-coater (Polaron, Agawam, MA), and images were taken with a JEOL 6390LV scanning electron microscope (Peabody, MA) in SEI imaging mode. All images were taken from the root canal surface area of the specimens.

Statistical analysis

Data were checked for normality using the Shapiro-Wilk test. The effects of group and time on indentation measurements and hardness values were examined using two-way ANOVA followed by Fisher's Protected Least Significant Differences. To satisfy the ANOVA assumptions the analyses were performed using the natural-log transformed microhardness data. A 5% level of statistical significance was applied.

Effect of medicaments used in endodontic regeneration technique on the chemical structure of human immature radicular dentin

Tooth selection and specimen preparation

Eighteen intact immature human mandibular premolars were used within six months of extraction after obtaining local IRB approval. Each tooth was decoronated horizontally at the cemento-enamel junction and two 4-mm root dentin cylinders obtained using a water-cooled low-speed diamond saw (Buehler, Lake Bluff, IL). Then, each cylinder was sectioned longitudinally across the maximum diameter of the root canal resulting in two specimens. Thus, four specimens were obtained from each root. The pulpal side of each specimen was leveled off under continuous water-cooling using 600 SiC papers (Buehler) so that the pulpal surface was flat. The convex side of each specimen was leveled using 600 SiC papers. The specimens were rinsed and ultrasonicated for five minutes under de-ionized water to remove the smear layer.

Treatment procedure

The four specimens obtained from each root were randomly assigned to three treatment groups (TAP, DAP, and Ca(OH)₂) and one control group (de-ionized water). TAP, DAP and Ca(OH)₂ paste were prepared as described in the first study. Each specimen was placed in a small 2 mL conical sample cup (Fisher Scientific, Florence, KY) containing 0.1 mL of the treatment pastes or de-ionized water. The amount of paste selected was just enough to cover the pulpal surface of each specimen. The containers were stored at 37°C for one, two, or four weeks. For the two- and four-week groups, each specimen was hydrated with 0.05 mL of de-ionized water weekly. After each time interval, 24 specimens from six teeth were taken out, rinsed thoroughly with de-ionized

water until no visible paste was observed, ultrasonicated for 15 minutes under de-ionized water, and completely air-dried.

ATR-FTIR Spectroscopy

A 4100 FTIR spectrophotometer (Jasco Inc., Tokyo, Japan) with a ZnSe ATR accessory was used to obtain infrared spectra for analysis of dentin specimens. Three randomly selected spots were marked on the non-pulpal surface of each specimen using a Sharpie marker. The pulpal surface of the specimens was then positioned on a standard FTIR sample holder with a 5 mm diameter opening and spectra were obtained. The ATR-FTIR spectra of air were collected and automatically subtracted by the Spectra Manager CFR software (Jasco Inc.). Each spectrum was then processed by smoothing, baseline correction and normalization to the amide I peak. The effect of various treatments on collagen and apatite composition of surface dentin was evaluated using the mineral matrix ratio; the ratio of integrated areas of the phosphate ν_1 , ν_3 contour to the amide I peak (Figure 14). The mean of the phosphate/amide I ratios derived from the spectra obtained from each group was used quantitatively for statistical evaluation. Larger ratios corresponded to a greater dentin collagen deproteinization, while smaller ratios corresponded to a greater dentin demineralization.

Scanning electron microscopy (SEM)

One dentin specimen was randomly selected from each group after each time point for SEM analysis. The selected dentin specimens were sputter coated for 3 minutes with gold/palladium using a sputter-coater (Polaron, Agawam, MA) and imaged with a JEOL 6390LV scanning electron microscope (Peabody, MA). All images were taken from the leveled root canal area at the center of the specimens.

pH measurements

Because it is challenging to measure the pH of a paste, saturated solutions of the triple antibiotic, double antibiotic, and Ca(OH)₂ powder were used to measure the pH. The solutions were prepared in triplicates and the pH of each solution was measured as described in a previous study (Hiraishi et al. 2010). In summary, each solution was freshly made by dissolving excess amounts of triple antibiotics, double antibiotics, or Ca(OH)₂ powder in de-ionized water with agitation for 24 hours. The solutions were then centrifuged (2,000 rpm, 10 minutes) and filtered using a 0.22 µm MCE filter unit (Fishers Scientific, Ireland). The pH of each solution was measured in triplicate using a digital pH meter (ATI Orion Model 330, Boston, MA). The meter was calibrated using pH standards of 7 and 4 before use. The solutions to be tested were placed in direct contact with the pH electrode and left in place for at least 15 seconds or until a stable pH reading was obtained (maximum of 30 seconds). The electrode was rinsed with de-ionized water and wiped dry between readings.

Statistical analysis

Data were checked for normality using the Shapiro-Wilk test, and a natural logarithm transformation of the phosphate/amide I ratios was performed to satisfy the ANOVA assumptions. The effects of group and time on phosphate/amide I ratios were examined using mixed-model ANOVA followed by Fisher's Protected Least Significant Differences post-hoc comparisons ($\alpha=0.05$).

The effect of medicaments used in endodontic regeneration on root fracture and microhardness of radicular dentin

Tooth selection and endodontic preparation

Extracted human mandibular single rooted premolars (n=180) were selected for this study after obtaining local university IRB approval. The teeth were stored in 0.1% thymol solution at 4°C and used within six months after extraction. The inclusion criteria were absence of caries, root cracks, restorations, and previous endodontic treatments. Furthermore, only teeth with similar mesio-distal and bucco-lingual root dimensions ($\pm 8\%$) were included in the study. An endodontic access cavity was prepared in each tooth using a round bur and a high-speed handpiece. The working length was determined by visualizing the tip of a #15 K-file (Dentsply Maillefer, Ballaigues, Switzerland) extending beyond the apical foramen and subtracting 1 mm from that length of the file.

The root canals were mechanically prepared using EndoSequence 0.06 taper rotary instruments (Brasseler, Savannah, GA) to a master apical #70 file size. Along with instrumentation, 1 mL of 5.25% NaOCl was used as an intracanal irrigant between uses of each succeeding file. Furthermore, the canals were finally rinsed with sterile saline using a 27-gauge needle and 5 mL syringe to remove any dentin debris remaining in the canal after instrumentation. Root canals were then dried with sterile paper points (Hygienic, Akron, Ohio).

Medicament application

The teeth were then randomly assigned to three treatments groups (TAP, DAP, and Ca(OH)₂ paste) and one control group. For the control group (n=45), no medication was applied in the canal. TAP, DAP and Ca(OH)₂ paste were prepared as described in the

first study. For the first treatment group (n=45), TAP was applied to the canal spaces with a sterile lentulo spiral in a slow speed handpiece (Dentsply, Caulk, Milford, DE) and tamped in the canal space to the level of the cemento-enamel junction using various sizes of sterile pluggers. For the second treatment group (n=45), DAP was applied to the canals as described previously. For the third treatment group (n=45), Ca(OH)₂ paste was applied to the canals as described previously. The root canals of the three treatment groups were sealed apically with flowable composite (TPH3, Dentsply, Caulk). Furthermore, the access openings of all teeth were sealed with a thickness of at least 4 mm of Cavit (3MESPE, St. Paul, MN). Teeth in the four groups were kept in normal saline at 37°C for one week, one month, or three months. The solution was changed every week. The three time points were selected to represent the intracanal application time of medicaments suggested clinically, which usually ranges between 1-11 weeks (Ding et al. 2009; Thibodeau 2009; Lenzi & Trope 2012).

Preparation of root specimens

After each storage period, 15 randomly selected teeth were taken from each group and decoronated at the level of 0.5 mm radicular to the facial cemento-enamel junction with a low-speed diamond saw (Buehler Ltd., Lake Bluff, IL, USA) under water cooling. Then, two root cylinders were horizontally sectioned from each root using a water-cooled diamond saw (Buehler Ltd). A cervical 5 mm root cylinder was used for fracture resistance testing and a middle 3 mm root cylinder was used for microhardness testing. The root cylinders from the treatment groups were irrigated with distilled water to remove the medicaments.

Microhardness testing

The 3 mm root cylinders were mounted on special rods and the coronal sides of the specimens were polished using a Struers Rotopol 31/Rotoforce 4 polishing unit (Struers, Cleveland, PA, USA) with 1,200-, 2,400- and 4,000-grit papers (Struers), and finally using a 1 μm diamond polishing suspension (Struers). As a final cleaning step, the polished specimens were sonicated in de-ionized water for 3 minutes. Microhardness measurements were performed using a Knoop Microhardness Tester (Leco, LM247, St. Joseph, MI) on the polished side of each root cylinder at 500 μm , and 1000 μm from the pulp-dentin interface. At each depth, three indentations were made using a 50-g load oriented perpendicular to the indentation surface for 15 s. The indentations were carefully observed in an optical microscope with a digital camera and image analysis software, allowing the precise measurement of their diagonals. The representative hardness value for each specimen at each depth was obtained as the mean of the results for the three indentations.

Fracture resistance testing

Each 5 mm root cylinder was tested for the resistance to fracture using a Universal testing machine (Sintech Renew 1123, MTS, Eden Prairie, MN). The application technique of a vertical loading force to fracture used in this study was modified from that used in a previous study (Sedgley & Messer 1992). A slow-speed carbide bur was used under water coolant to shape the root canal access at the coronal end of each root cylinder to accept the loading fixture. The root cylinders were positioned vertically on the lower fixed platform of the Universal testing machine with the coronal face upward using a double sided adhesive tape. A cylindrical loading fixture with a

spherical tip ($r=1.9$ mm) attached to the upper crosshead was lowered until the spherical tip rested in the prepared coronal root seat (Figure 4). Then, a vertical loading force was applied at a crosshead speed of 0.5 mm per minute until the root cylinder fractured (Figure 5). Fracture was identified in the study when an instantaneous and sharp drop of more than 25% of the applied load was observed (Teixeira et al. 2004), the load at fracture was measured and expressed in Newtons. Furthermore, the energy to yield was also obtained from for all fractured root cylinders.

Scanning electron microscopy (SEM)

One fractured root specimen was randomly selected from each group after each time point for SEM analysis to observe the presence of medicament remnants and/or any morphological changes in root canal dentin. Each selected root specimen was irrigated with 5 mL of distilled water, sonicated in de-ionized water for 3 minutes, and desiccated for 48h. Then, specimens were sputter coated for 3 minutes with gold/palladium using a sputter-coater (Polaron, Agawam, MA), and images were taken with a JEOL 6390LV scanning electron microscope (Peabody, MA) in secondary electron Imaging mode. All images were taken from the root canal surface area of the specimens.

Statistical analysis

Normal distribution of the data was tested using the Shapiro-Wilk test. The effects of type of medicaments and duration of treatment on fracture resistance and microhardness measurements were examined using two-way ANOVA followed by Tukey pairwise comparisons. Furthermore, the effects of group and time on energy to yield were examined using two-way ANOVA followed by pair-wise comparisons using

Fisher's Protected Least Significant Differences. A 5% level of statistical significance was applied for the analyses.

RESULTS

First Study

Tables 1 and 2 show a statistically significant difference in three BioDent RPI parameters (IDI, ID and total ID) between all groups at both time points: TAP > DAP > control > Ca(OH)₂ (p<0.001 for control vs Ca(OH)₂, p<0.0001 for all other group comparisons). For the CID parameter (Table 2), a statistically significant difference between all groups after one-week treatment was observed: TAP > DAP > control > Ca(OH)₂ (p<0.05 for control vs Ca(OH)₂, p<0.01 for TAP vs DAP, p<0.0001 for all other group comparisons). However, the CDI parameter after four-week treatment showed the following order of significance: TAP > DAP > control and Ca(OH)₂ (p>0.05 for control vs Ca(OH)₂, p<0.0001 for all other group comparisons). The four-week exposure of dentin to TAP and DAP caused a 43% and 31% increase in total ID, respectively. Furthermore, the four-week exposure of dentin to TAP and DAP caused a 40% and 30% increase in first cycle ID. The group by time interaction was not significant in the four BioDent RPI parameters (p>0.05). For the IDI, ID, and total ID parameters, one-week treatment was significantly higher than four-week treatment in all groups (p>0.01). For the CDI parameter, the one-week treatment was significantly higher than the four-week treatment for DAP group (p<0.001) and TAP (p<0.01), but there was no significant difference between times for Ca(OH)₂ or control groups (p>0.05).

For the hardness data (Table 3), the group-by-time interaction was not significant (p=0.86). Dentin hardness was significantly higher after four weeks than after one week (p=0.0083). Dentin hardness was significantly higher for Ca(OH)₂ treated group than for

control ($p=0.0013$), DAP ($p<0.0001$), and TAP ($p<0.0001$) treated groups at both time points. Mean hardness of dentin was significantly higher for control group than for DAP ($p<0.0001$) and TAP treated groups ($p<0.0001$) at both time points. Furthermore, hardness was higher for DAP than TAP ($p=0.0004$) at both time points.

SEM images of indentations taken at 1100X magnification showed microcracks created during the repetitive loading cycles applied among the untreated root canal specimens and those treated with various medicaments (Figures 6-9). Furthermore, a few areas of exposed collagen matrix could be identified among TAP treated specimens and DAP-treated specimens at both time points (Figure 10 and Figure 11). However, no morphological changes were observed among untreated control specimens (Figure 12) and $\text{Ca}(\text{OH})_2$ treated specimens (Figure 13) at both time points.

Second Study

The pH of saturated solutions of $\text{Ca}(\text{OH})_2$, double antibiotics, and triple antibiotics were 11.8 ± 0.1 , 3.4 ± 0.1 , and 2.9 ± 0.1 respectively. Figure 14 shows a representative ATR-FTIR spectrum of intact mineralized human dentin from the control group. The FTIR spectra showed a clear weakening of amide III, amide II, and amide I peaks after $\text{Ca}(\text{OH})_2$ paste treatment at all time points. Therefore, the presence of phosphate and carbonate peaks became more prominent. Conversely, the spectra suggested a clear weakening of phosphate and carbonate peaks after TAP and DAP treatments at all time points. Therefore, the presence of amide III, amide II, and amide I peaks became more apparent (Figures 15-17).

A statistically significant difference ($p<0.0001$) in phosphate/amide I ratios between all groups at all time points was observed (Table 4): $\text{Ca}(\text{OH})_2 > \text{untreated} >$

DAP> TAP. The within-group time comparison varied by group. No significant time effect was observed in phosphate/amide I ratios among Ca(OH)₂-treated dentin (p=0.40) or untreated dentin (p=0.97). For DAP, four-week treated dentin had a significantly lower phosphate/amide I ratio compared to one-week (p=0.046) and two-week (p=0.007) treated dentin, however, one-week and two-week treated dentin did not have a significant difference (p=0.46). For TAP, four-week treated dentin had a significantly higher phosphate/amide I ratio than one-week (p=0.0005) and two-week treated dentin (p<0.0001). Furthermore, the phosphate/amide I ratio of one-week treated dentin was significantly higher than two-week treated dentin (p=0.037).

Low magnification SEM images taken at 1500X magnification from untreated dentin and Ca(OH)₂-treated dentin did not show any sign of demineralization at the different time points (Figure 18 and Figure 19). A few areas of exposed collagen matrix could be identified for two-week and four-week DAP-treated dentin, which indicates a demineralization effect (Figure 20). A few areas of exposed collagen matrix were observed for one-week and two-week TAP-treated dentin. However, a strong demineralization with intensive collagen exposure was observed in the four-week treated specimen (Figure 21). Additionally, when the four-week TAP-treated dentin was viewed with 12,000X magnification, the native structure of collagen fibrils was easily recognized (Figure 22).

Third Study

Microhardness

The two-way interaction between group and time was significant (p<0.001) at both 500 and 1000 μm from pulp-dentin interfaces (Tables 5 and 6). Time had a

significant effect on the TAP groups at both depths ($p < 0.001$), with significantly lower microhardness for the three month group than for the one week ($p < 0.001$) and one month groups ($p = 0.02$ at $500 \mu\text{m}$ and $p = 0.01$ at $1000 \mu\text{m}$), and lower microhardness for the one month group than for the one week group ($p = 0.0007$ at $500 \mu\text{m}$ and $p = 0.014$ at $1000 \mu\text{m}$). Time had a significant effect on the DAP groups at both depths ($p < 0.001$), with significantly lower microhardness for the three month group than for the one week ($p < 0.001$ at both depths) and one month groups ($p = 0.04$ at $500 \mu\text{m}$ and $p < 0.001$ at $1000 \mu\text{m}$), and lower microhardness for the one month group than for the one week group ($p < 0.001$ at both depths). Time had a significant effect on the Ca(OH)_2 groups ($p = 0.005$ at $500 \mu\text{m}$ and $p = 0.045$ at $1000 \mu\text{m}$), with significantly higher microhardness for the three month group than for the one week group ($p = 0.0012$ at $500 \mu\text{m}$ and $p = 0.038$ at $1000 \mu\text{m}$). Time did not have a significant effect on the control groups ($p > 0.05$).

The groups' comparisons at each time point are also shown in Tables 5 and 6. No significant difference was found after one week ($p > 0.05$) at both depths. However, after one month, the TAP group and the DAP group had significantly lower microhardness than the Ca(OH)_2 and control groups ($p < 0.001$ at $500 \mu\text{m}$ and $p < 0.0005$ at $1000 \mu\text{m}$). After three months, the TAP and DAP groups had significantly lower microhardness than the Ca(OH)_2 and control groups at both depths ($p < 0.001$). Furthermore, the control group had significantly lower microhardness than the Ca(OH)_2 group ($p = 0.0003$ at $500 \mu\text{m}$ and $p = 0.015$ at $1000 \mu\text{m}$).

Fracture resistance

The time factor had a significant effect on root fracture resistance ($p < 0.001$). Time had a significant effect on the TAP and DAP groups (Table 7), with significantly

lower fracture resistance for the three month group than for the one week group ($p=0.017$ for TAP and $p=0.008$ for DAP). Time had a significant effect on the $\text{Ca}(\text{OH})_2$ groups, with lower fracture resistance for the three month group than for the one week ($p<0.001$) and one month ($p=0.007$) groups. The three month percentage decrease in fracture resistance of the TAP, DAP and $\text{Ca}(\text{OH})_2$ treatment groups compared to one week groups was 19%, 21%, and 30%, respectively. No significant difference was found between the control groups at all time points ($p>0.05$). The overall effect of type of medicaments used on fracture resistance did not reach a significant difference ($p=0.055$) (Table 7). No significant difference was found between all groups after one week or one month ($p>0.05$). However, a significantly lower fracture resistance was found in the $\text{Ca}(\text{OH})_2$ ($p=0.007$) and DAP ($p=0.042$) groups compared to the control group after three months.

Energy to yield

The group-by-time interaction was not significant ($p=0.34$) (Table 8). The mean energy to yield was significantly higher after one month than after one week ($p=0.018$) and three months ($p<0.0001$), and was significantly higher after one week than after three months ($p=0.012$). Untreated control root cylinders required significantly higher energy to yield than $\text{Ca}(\text{OH})_2$ ($p=0.026$), DAP ($p=0.0100$), and TAP ($p=0.016$). However, the energy to yield of DAP, TAP, and $\text{Ca}(\text{OH})_2$ treated roots were not significantly different from each other ($p>0.71$).

SEM

SEM images taken at 1500X magnification showed that TAP and DAP treated root canal dentin were depleted of the smear layer with open dentin tubules at all time points (Figure 23 and Figure 24). Furthermore, no TAP remnants were observed at all

time points. However, few DAP remnants were observed at all time points. SEM images of Ca(OH)_2 treated root canals showed firmly attached Ca(OH)_2 deposits at all time points (Figure 25). The root canal dentin in the control group was covered with a smear layer at all time points (Figure 26).

DISCUSSION

BioDent RPI parameters

The high density of dentin tubules and/or the less mineralized intertubular dentin matrix of the inner radicular dentin near the pulp may alter the mechanical property measurements for root canal surface dentin (Kinney et al., 1996; Pashley et al., 1985). However, measuring the mechanical properties of the superficial dentin is challenging due to the need for standardized specimen preparation to perform traditional mechanical testing methodologies. Furthermore, the micro-flaws that might be created during the machining and polishing of radicular dentin specimens before any traditional mechanical testing could negatively affect the outcomes of studies dealing with mechanical properties.

Our study suggests a novel approach to directly measure the degree of root canal surface indentation under repetitive constant loading cycles without any surface modification. Previous studies in bone have shown a correlation between IDI BioDent parameter and fracture resistance (Hansma et al., 2008), fracture toughness (Rasoulian, 2011), and crack growth toughness (Diez-Perez et al., 2010). However, the exact mechanical properties that are represented through BioDent parameters remain to be clearly determined. In addition to the indentation parameters, hardness values were also obtained from first cycle ID in this study. However, the significance trends between hardness values and first cycle ID at both time points were the same. Therefore, first cycle ID may be sufficient to rank hardness between various groups in future studies without the need of hardness calculations.

The significantly lower IDI, ID, total ID, and creep ID (for one-week treatment) values reported among Ca(OH)₂ treated surface dentin compared to all other groups could be explained by the increased indentation resistance of the modified surface dentin after exposure to Ca(OH)₂. Previous studies hypothesized that the small molecular weight (56.1 Da) and high alkaline pH of Ca(OH)₂ might enhance its penetration through the apatite-encapsulated collagen matrix and lead to denaturation of the collagen organic matrix (Andreasen et al., 2002; Leiendecker et al., 2012). Thus, the indentations created using BioDent RPI on the collagen deficient surface dentin after exposure to Ca(OH)₂ might be harder to initiate, yet easier to propagate during cyclic stresses. Therefore, Ca(OH)₂-treated radicular dentin might be more susceptible to catastrophic fracture, which was reported in previous studies (Andreasen et al., 2002; Rosenberg et al., 2007).

The significantly higher IDI, ID, total ID, and creep ID values reported among DAP- and TAP-treated surface dentin compared to untreated and Ca(OH)₂-treated dentin at both time points could be due to the demineralization effect of these acidic antibiotic pastes. Exposure of the collagen matrix on the surfaces of the root canal dentin treated with TAP and DAP was confirmed by the SEM images (Figure 10 and Figure 11). Furthermore, the ability of minocycline, one of the antibiotics in TAP, to chelate calcium and demineralize dental hard tissues (Maruyama et al., 2008; Minabe et al., 1994) might explain the significantly higher indentation parameters reported among TAP-treated dentin compared to DAP-treated dentin.

The demineralization effect of TAP and DAP might play a significant role in pulp regeneration by enhancing the attachment and growth of host stem cells on root canal surfaces through the exposure of embedded collagen fibers and various growth factors.

However, the mineral component in dentin contributes to the strength of the tooth structure. Therefore, long-term exposure of radicular dentin to antibiotic pastes might negatively affect the mechanical properties of dentin and increase the susceptibility to root fracture. Taking into consideration the unpredictable success rate of the pulp regeneration technique (Chen et al., 2012; Ding et al., 2009) and the relative long application time of disinfectant medications reported in some cases (Thibodeau and Trope, 2007; Thibodeau, 2009), further studies are needed to investigate the effect of various medicaments used in pulp regeneration on root fracture and to optimize the application time of these medicaments.

The significantly higher IDI, ID, and total ID parameters of one-week treated dentin compared to four-week treated dentin among all treatment groups reported in this study could be due to the baseline difference between the mechanical properties of radicular dentin of the immature teeth selected for the two time points. This may be supported by the fact that the IDI, ID, and total ID parameters of the one-week untreated dentin were significantly higher than that of the four-week untreated dentin. The null hypothesis that the medicaments used in the endodontic regeneration technique have no significant effect on root canal surface indentation properties was rejected.

FTIR measurement

It is recommended that clinicians apply various antibacterial root canal medicaments such as antibiotic or Ca(OH)_2 pastes from one week up to 11 weeks during pulp regeneration protocols. Thus, radicular dentin specimens were exposed to three commonly used intracanal medicaments for one, two, or four weeks in this experiment to reflect various clinical situations. The depth of penetration of IR radiation in the ATR-

FTIR technique is limited to a few microns. Therefore, the spectra data and the phosphate/amide I ratios used in this study only represent the superficial subsurface characteristics of the dentin specimens. In order to decrease the baseline structural variability between dentin specimens at each time point, four specimens were obtained from each immature premolar root and randomized to one of the treatment groups or the control group. A previous study showed no significant difference in phosphate/amide I ratios between FTIR spectra obtained from cervical, middle, and apical root portions of immature premolars (Verdelis et al. 1999). No significant difference in phosphate/amide I ratios was found in this study between untreated control groups used at different time points, which justifies and validates the comparison of each treatment group at different time points in this study.

Our results demonstrated significantly higher phosphate/amide I ratios in Ca(OH)_2 -treated radicular dentin compared to untreated dentin after all time points. These results support the proposed denaturation effect of highly alkaline Ca(OH)_2 (pH=11.8) on dentin organic matrix (Andreasen et al. 2002; White et al. 2002). The relatively small molecular weights (56.1 Da) of Ca(OH)_2 may facilitate its penetration through the apatite-encapsulated collagen matrix and lead to a change in the 3-dimensional conformation of tropocollagen (Leiendecker et al. 2012).

The significant reduction in phosphate/amide I ratio in TAP- and DAP-treated dentin indicates a demineralization effect of these pastes and formation of a collagen-rich matrix on the surface of the radicular dentin, which was confirmed by the SEM images (Figure 20 and Figure 21). The acidity of the double (pH=3.4) and triple (pH=2.9) antibiotic mixtures used in this study further supports their demineralization effect. The

significant reduction in phosphate/amide I ratio in TAP-treated dentin compared to DAP-treated dentin could be explained by the lower pH of TAP and the ability of minocycline present in TAP to chelate calcium and demineralize dental hard tissues (Minabe et al. 1994; Maruyama et al. 2008). For the DAP-treated dentin, the phosphate/amide I ratio was significantly lower in four-week treated dentin compared to one-week and two-week treated dentin, which indicates a gradual increase in demineralization effect of DAP with time. For TAP-treated dentin, the phosphate/amide I ratio was significantly lower in two-week treated dentin compared to one-week treated dentin, which also indicates a gradual increase in the demineralization effect of TAP with time. However, the phosphate/amide I ratio of four-week TAP-treated dentin was significantly higher than that of one-week and two-week treated dentin. This could be due to the partial collapse of a superficial apatite-sparse collagen layer, which was clearly detected on the SEM image (Figure 22). This agrees with previous studies that reported a relative decrease in the intensity of organic amide I peaks of dentin spectra after long time exposure to various acidic solutions (Eliades et al. 1997; Di Renzo et al. 2001).

In addition to the antibacterial role of antibiotic pastes, the demineralization effect of TAP and DAP suggested in this study might play a significant role in endodontic regeneration by enhancing the attachment and growth of host stem cells on dentin through the exposure of embedded collagen fibers and various growth factors. A recent *in vivo* study suggested that the conditioning of the dentin surface with EDTA may enhance the adherence and differentiation of dental pulp stem cells during pulp regeneration (Galler et al. 2011). On the other hand, long-term exposure of radicular dentin to Ca(OH)_2 or antibiotic pastes might negatively affect the mechanical properties of dentin

and increase the susceptibility to root fracture either due to collagen degradation in the case of $\text{Ca}(\text{OH})_2$ or excessive demineralization in the case of antibiotic pastes. The null hypothesis that the three intracanal medicaments have no significant effect on the chemical structure of radicular dentin was rejected.

Microhardness measurement

The significant decrease in root microhardness at both depths after one and three month treatment with TAP or DAP could be explained by the strong demineralization effect of these medicaments since dentin hardness has been correlated to mineral concentration (Kinney et al. 2003). On the other hand, $\text{Ca}(\text{OH})_2$ caused a gradual increase in root microhardness with time, and this increase was significant at both depths after three months. This might be explained by the denaturation of the collagen matrix caused by the low molecular weight and highly alkaline pH of $\text{Ca}(\text{OH})_2$ as hypothesized in previous studies (Andreasen et al. 2002; Leindecker et al. 2012). It is well-known that the collagen component is responsible for toughness of the hard tissues (Wang et al. 2001). Therefore, the compromised collagen matrix in the more mineralized dentin could lead to a more brittle and less tough, even though harder, substrate. This could explain an accelerated fatigue crack propagation during cyclic stresses and an increase in the susceptibility of root fracture in $\text{Ca}(\text{OH})_2$ -treated root canals (Andreasen et al. 2006). Increased susceptibility to fracture was obvious in this study's three-month fracture resistance data. Further studies are required to optimize the application time of pulp regeneration medicaments and explore the advantage and disadvantages of the demineralization effect of TAP and DAP suggested in this study on endodontic regeneration techniques.

Fracture resistance

The importance of exploring the effect of medicaments used in pulp regeneration on root fracture should not be overlooked since the application time of intracanal medicaments reported in some cases of pulp regeneration is relatively long, which may reach up to 11 weeks (Thibodeau & Trope 2007; Thibodeau 2009). Additionally, the increase in root wall thickness was found to be limited to mid and/or apical root structures in the majority of reported pulp regeneration cases (Bose et al. 2009; Hargreaves & Law 2010; Jeeruphan et al. 2012; Lenzi & Trope 2012; Nosrat et al. 2012) rather than the cervical part of the root, which is the area prone to fracture in treated necrotic immature teeth (Cvek 1992). Therefore, in cases where root thickening in the cervical area is not achieved through regeneration, the potential of further weakening the root structure through long term use of medicaments should be avoided.

Three month application of pulp regeneration medicaments caused a significant reduction in fracture resistance of root specimens ranging between 19%-30% when compared to one week application. The significant reduction in fracture resistance after Ca(OH)_2 application for three months agrees with previous studies that found a significant reduction in root fracture strength after 84 and 100 days of Ca(OH)_2 application, respectively (Andreasen et al. 2006; Rosenberg et al. 2007). However, the reduction in fracture resistance after one month application of Ca(OH)_2 did not reach a significant difference in this study. A recent systematic review of the literature found inconclusive data regarding the effect of Ca(OH)_2 exposure for one month or shorter on the mechanical properties of radicular dentin (Yassen & Platt 2013). The significant reduction in fracture resistance of root specimens after three month application of TAP

and DAP compared to one week application might be explained by the demineralization effect of these acidic pastes on radicular dentin, which was confirmed by the microhardness data.

The mineral component in dentin contributes to the strength of the tooth structure, and the relatively long-term exposure of radicular dentin to antibiotic pastes might be the reason for the significant reduction in root resistance to fracture observed in this study. The effect of intracanal medication on root fracture might be more detrimental *in vivo* due to the very wide immature root canals that may lead to a higher amount of medicament per canal wall surface area compared to this *in vitro* study. Furthermore, the irrigation protocols suggested during endodontic regeneration techniques might cause further reduction in root strength. EDTA, which is usually recommended during endodontic regeneration (Galler et al. 2011; Miller et al. 2012), was found to significantly reduce the radicular dentin microhardness (De-Deus et al. 2006) and root resistance to fracture (Uzunoglu et al. 2012).

The SEM images showed heavy Ca(OH)_2 deposits on Ca(OH)_2 -treated root canal dentin at all time points. On the other hand, TAP and DAP treated dentin showed open tubules with no evidence of a smear layer, which may further support the suggested demineralization effect of these pastes. In addition to the well known antibacterial role of TAP and DAP, the demineralization effect of these antibiotic pastes, as suggested in this study, might play an additional significant role in the creation of an environment conducive to attachment of host stem cells on root canal surfaces and exposure of collagen fibers and various growth factors during endodontic regeneration. A relatively recent retrospective study found that pulp regeneration cases treated with TAP had

significantly thicker root walls compared to regeneration cases treated with Ca(OH)_2 (Bose et al. 2009), which might be explained by the root canal surface conditioning effect of TAP observed in this study. If Ca(OH)_2 is to be used as a disinfectant during pulp regeneration, the use of an intensive irrigation protocol should be recommended to remove Ca(OH)_2 remnants and create a conditioned root canal surface. On the other hand, the need for such an extensive irrigation protocol should be reconsidered when TAP or DAP is used for pulp regeneration. However, further *in vivo* studies are needed to substantiate these suggestions.

It is noteworthy to mention that pulp regeneration cases usually require minimal or no instrumentation. However, root canal instrumentation was performed in this study in order to standardize the internal dimensions of roots before fracture resistance testing. The null hypothesis stated that the three root canal medicaments used in endodontic regeneration techniques have no significant effect on radicular dentin microhardness and root fracture resistance at all time points was rejected.

CONCLUSION

1. Our data showed a significant difference in the majority of BioDent indentation parameters of the root canal surfaces treated with various endodontic regeneration medicaments for one and four weeks.
2. The results suggest a superficial collagen degradation caused by $\text{Ca}(\text{OH})_2$ and superficial dentin demineralization caused by antibiotic pastes after one, two, or four weeks of dentin exposure to the medicaments.
3. TAP and DAP caused a surface conditioning effect on radicular dentin and exposure of collagen fibrils.
4. TAP and DAP caused significant and continuous decreases in microhardness of root dentin after one and three month intracanal application. Additionally, $\text{Ca}(\text{OH})_2$ caused significant increase in microhardness of root dentin after three month intracanal application.
5. The three month application of TAP, DAP, and $\text{Ca}(\text{OH})_2$ medicaments significantly reduced the root fracture resistance of mandibular premolars compared to a one week application.

CLINICAL EXTRAPOLATION

Within the limitation of the *in vitro* studies conducted, the following clinical points can be extrapolated:

1. The clinical protocols suggesting long term application of various endodontic regeneration medicaments should be reconsidered to avoid increasing the susceptibility of root fracture of the immature necrotic tooth, specifically at the wide and weak coronal third of roots.
2. In addition to their antibacterial effect, the demineralizing effect of TAP and DAP may play an important role in the endodontic regeneration process either directly by conditioning the root canal surface and enhancing the attachment of stem cells to root canal walls or indirectly by exposing and mobilizing various proteins and growth factors within the dentin organic matrix.
3. Collectively, our data showed a superficial collagen denaturation effect of Ca(OH)_2 on radicular dentin, firmly attached Ca(OH)_2 remnants to root canal surface dentin and a 30% decrease in root resistance to fracture after three month intracanal application of Ca(OH)_2 . Therefore, the use of Ca(OH)_2 during endodontic regeneration techniques should be avoided.
4. If Ca(OH)_2 is to be used in endodontic regeneration, an intensive irrigation protocol should be recommended to remove Ca(OH)_2 remnants and condition the root canal surface.
5. Our data demonstrated that DAP and TAP caused complete removal of the smear layer and a strong surface conditioning effect. Therefore, the current endodontic

regeneration technique suggesting long and extensive irrigation protocols after removal of antibiotic medicaments might not be needed.

Table 1. Mean (SE) of First cycle ID and IDI BioDent parameters of specimens treated with various endodontic regeneration medicaments and a no treatment control group.

Type of treatment	First cycle ID (μm)		IDI (μm)	
	One-week	Four-week	One-week	Four-week
TAP	70 (1)Aa*	66 (1)Ba	8.8 (0.2)Aa	8 (0.3)Ba
DAP	64 (1)Ab	61 (1)Bb	7.9 (0.2)Ab	7.5 (0.2)Bb
Control	48 (1)Ac	47 (1)Bc	5.1 (0.1)Ac	4.8 (0.2)Bc
Ca(OH)₂	43 (2)Ad	42 (1)Bd	4.4 (0.2)Ad	4.1 (0.1)Bd

* Different upper-case letters indicate a significant difference between the two time points of each parameter.

* Different lower-case letters indicate a significant difference between various groups within single time point.

Table 2. Mean (SE) of Total ID and Creep ID BioDent parameters of specimens treated with various endodontic regeneration medicaments and a no treatment control group.

Type of treatment	Total ID (μm)		Creep ID (μm)	
	One-week	Four-week	One-week	Four-week
TAP	74 (1)Aa	70 (2)Ba	5.4 (0.2)Aa	4.8 (0.2)Ba
DAP	69 (1)Ab	64 (2)Bb	4.7 (0.1)Ab	4 (0.2)Bb
Control	51 (1)Ac	49 (1)Bc	3.1 (0.1)Ac	2.9 (0.2)Ac
Ca(OH)₂	46 (2)Ad	45 (1)Bd	2.7 (0.2)Ad	2.8 (0.1)Ac

* Different upper-case letters indicate a significant difference between the two time points of each parameter.

* Different lower-case letters indicate a significant difference between various groups within single time point.

Table 3. Mean (SE) of hardness (MPa) of specimens treated with various endodontic regeneration medicaments and a no treatment control group.

Type of treatment	Hardness (MPa)	
	One-week	Four-week
TAP	0.23 (0.01) Ad	0.26 (0.01) Bd
DAP	0.28 (0.01) Ac	0.31 (0.02) Bc
Control	0.49 (0.02) Ab	0.52 (0.02) Bb
Ca(OH)₂	0.58 (0.03) Aa	0.61 (0.03) Ba

* Different upper-case letters indicate a significant difference between the two time points.

* Different lower-case letters indicate a significant difference between various groups within single time point.

Table 4. Mean (SE) of the phosphate/amide I ratios derived from FTIR for the three treatment groups and the control group.

Type of treatment*	One week	Two weeks	Four weeks
Ca(OH)₂	9.9 (1.1)Aa	9.5 (0.7)Aa	11.8 (1.1)Aa
Control	6.6 (0.6)Ab	6.3 (0.5)Ab	6.5 (0.5)Ab
DAP	4.0 (0.5)Ac	4.5 (0.4)Ac	2.8 (0.2)Bc
TAP	1.0 (0.2)Bd	0.7 (0.1)Cd	1.7 (0.1)Ad

* Different upper-case letter indicate a significant difference between the three time points of each treatment.

* Different lower-case letters indicate a significant difference between various treatments within a single time point.

Table 5. Mean (SD) of Knoop microhardness (KHN) for roots treated with endodontic regeneration medicaments and a control group for one week, one month and three months at 500 μm from the pulp-dentin interface.

Group	500 μm from pulp-dentin interface*		
	One week	One month	3 months
Ca(OH)₂	52 (5) Ba	54 (5) ABa	57 (4) Aa
Control	53 (4) Aa	53(4) Aa	51(4) Ab
DAP	53 (5) Aa	46 (4) Bb	43 (5) Cc
TAP	52 (4) Aa	47 (3) Bb	43 (6) Cc

* Different upper-case letters indicate a significant difference between the three time points of each treatment.

* Different lower-case letters indicate a significant difference between various treatments within a single time point.

Table 6. Mean (SD) of Knoop microhardness (KHN) for roots treated with endodontic regeneration medicaments and a control group for one week, one month and three months at 1000 μm from the pulp-dentin interface.

Group	1000 μm from pulp-dentin interface*		
	One week	One month	3 months
Ca(OH)₂	58 (5) Ba	61 (5) ABa	62 (4) Aa
Control	60 (4) Aa	61 (4) Aa	58 (4) Ab
DAP	59 (5) Aa	55 (4) Bb	51 (5) Cc
TAP	59 (5) Aa	55 (4) Bb	51 (6) Cc

* Different upper-case letters indicate a significant difference between the three time points of each treatment.

* Different lower-case letters indicate a significant difference between various treatments within a single time point.

Table 7. Mean (SD) of load at fracture (Newton) for premolar roots treated with endodontic regeneration medicaments and untreated control group for one week, one month and three months.

Group	One week*	One month*	3 months*
Control	843 (128) Aa	814 (167) Aa	788 (138) Aa
Ca(OH)₂	867 (121) Aa	777 (222) Aa	607 (175) Bb
DAP	807 (172) Aa	740 (143) ABa	641 (131) Bb
TAP	829 (130) Aa	806 (155) ABa	676 (113) Ba

* Different upper-case letters indicate a significant difference between the three time points of each treatment.

* Different lower-case letters indicate a significant difference between various treatments within a single time point.

Table 8. Mean (SE) of energy to yield (N*mm) for premolar roots treated with endodontic regeneration medicaments and untreated control group for one week, one month and three months.

Group	One week*	One month*	3 months*
Control	127 (9) Ba	137(13) Aa	122 (6) Ca
Ca(OH)₂	119 (8) Bb	136(14) Ab	78 (10) Cb
DAP	107(11) Bb	120 (10) Ab	96 (9) Cb
TAP	105(10) Bb	131 (11) Ab	91 (9) Cb

* Different upper-case letters indicate a significant difference between the three time points of each treatment.

* Different lower-case letters indicate a significant difference between various treatments within a single time point.

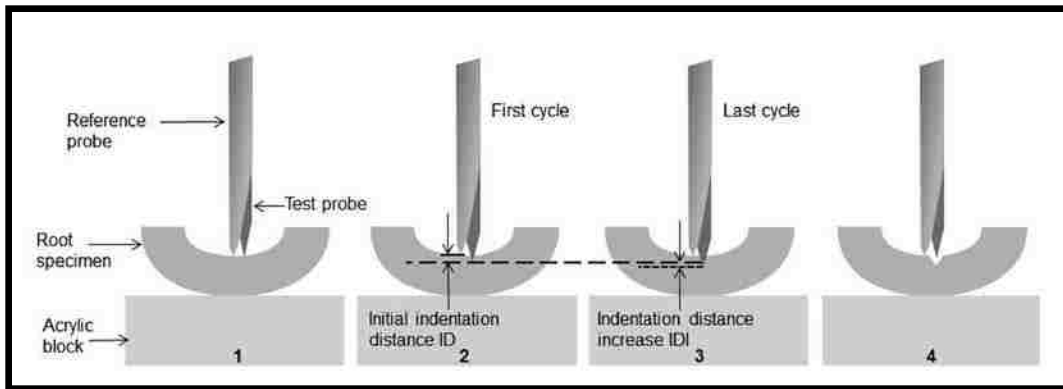


Figure 1. Illustration of the method for obtaining indentation measurements of root canal surface dentin *in vitro* using the BioDent H. 1) Application of the test probe assembly. 2) First-cycle indentation. 3) Last-cycle indentation, which determines the IDI with respect to the first cycle. 4) End of the procedure.

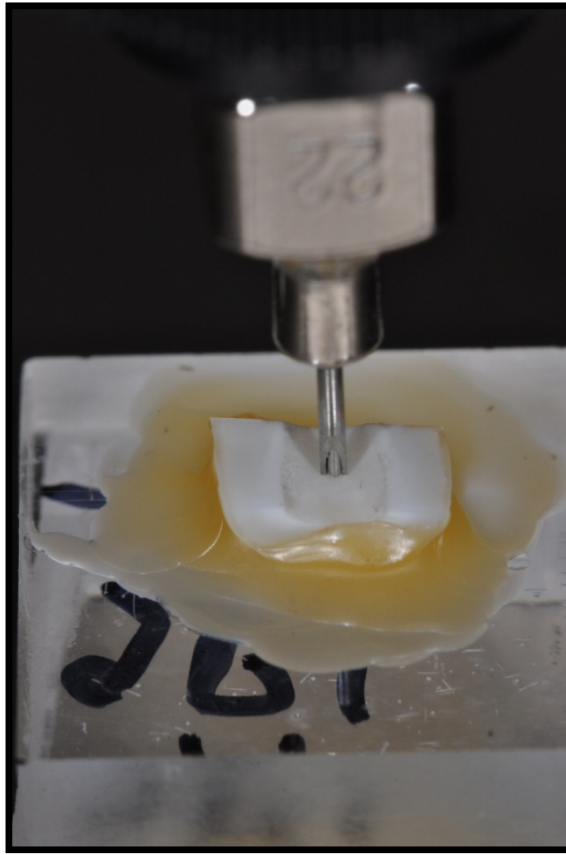


Figure 2. BioDent probe assembly rested on the center of a root canal surface.

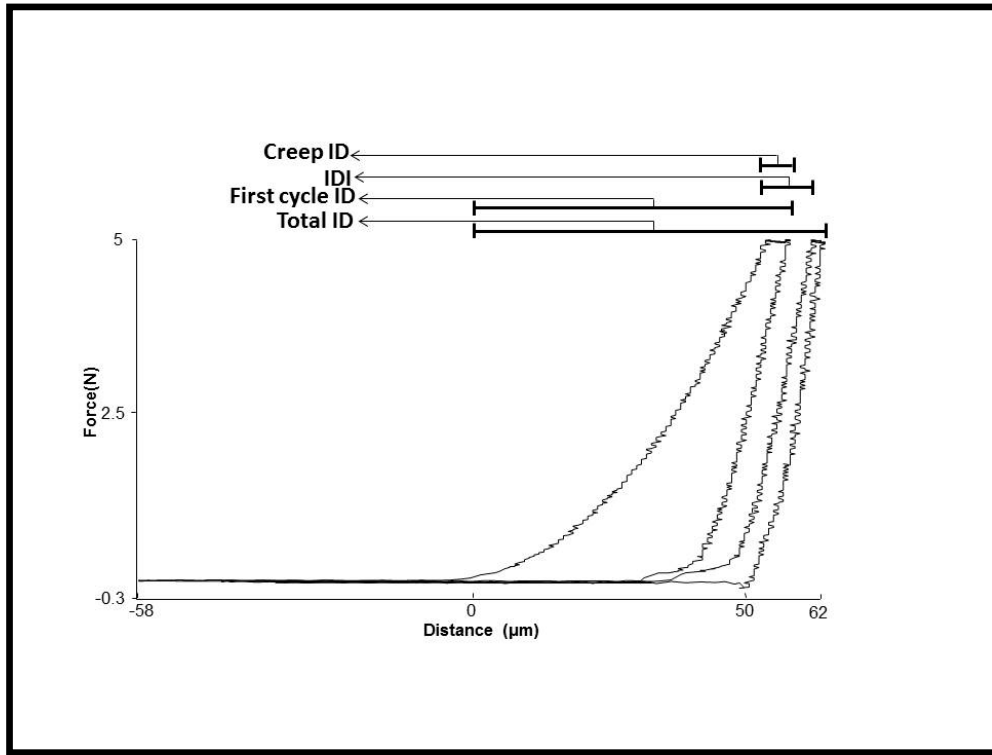


Figure 3. A typical load-displacement curve obtained from one of the DAP treated specimens illustrating the way RPI instrument parameters are calculated (only two cycles were included in the curve for clarity).

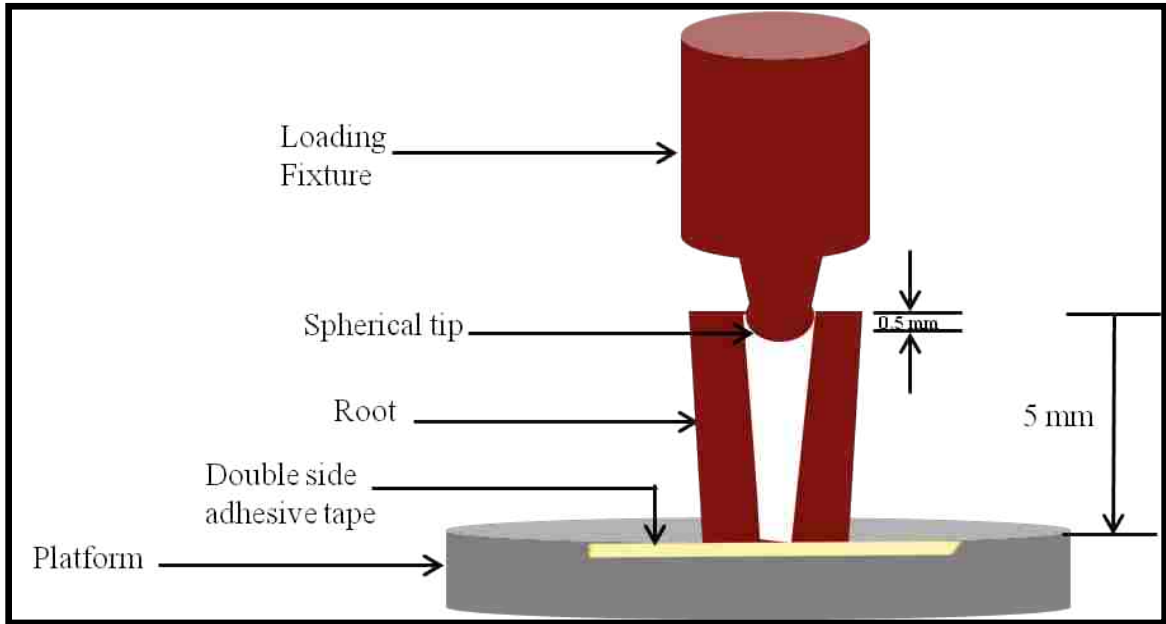


Figure 4. Illustration of fracture resistance test performed in the study showing a root cylinder against loading fixture with its spherical tip ($r = 1.9$ mm) aligned with the center of the canal opening of the cylinder.

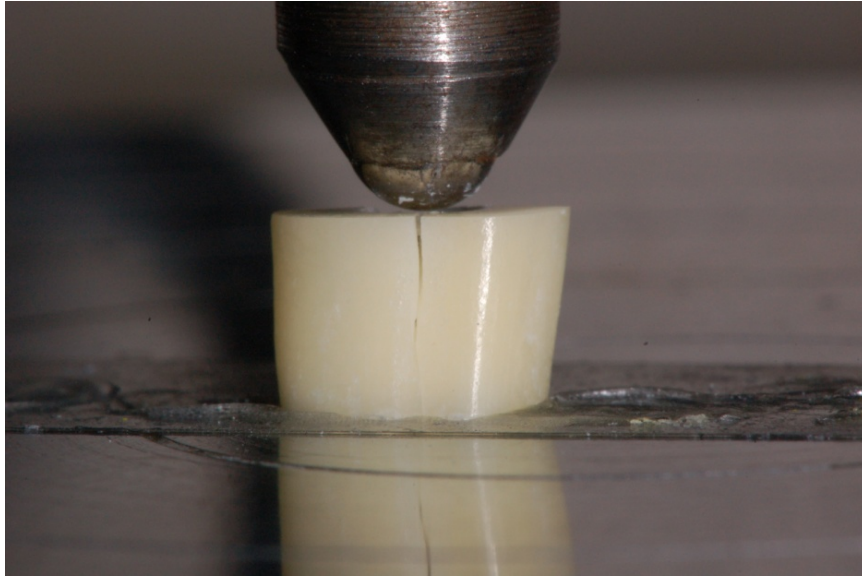


Figure 5. Image of the loading fixture and fractured root cylinder after fracture resistance test.

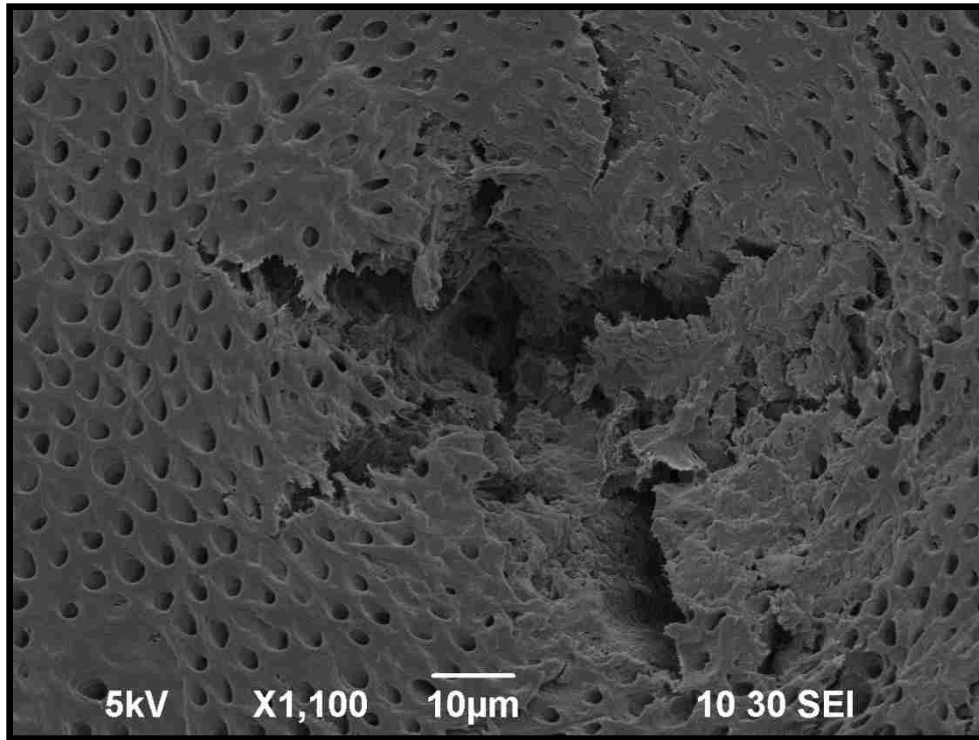


Figure 6. Representative SEM image of BioDent indentation shows the microcracks created during the repetitive loading cycles from one-month TAP-treated specimen.

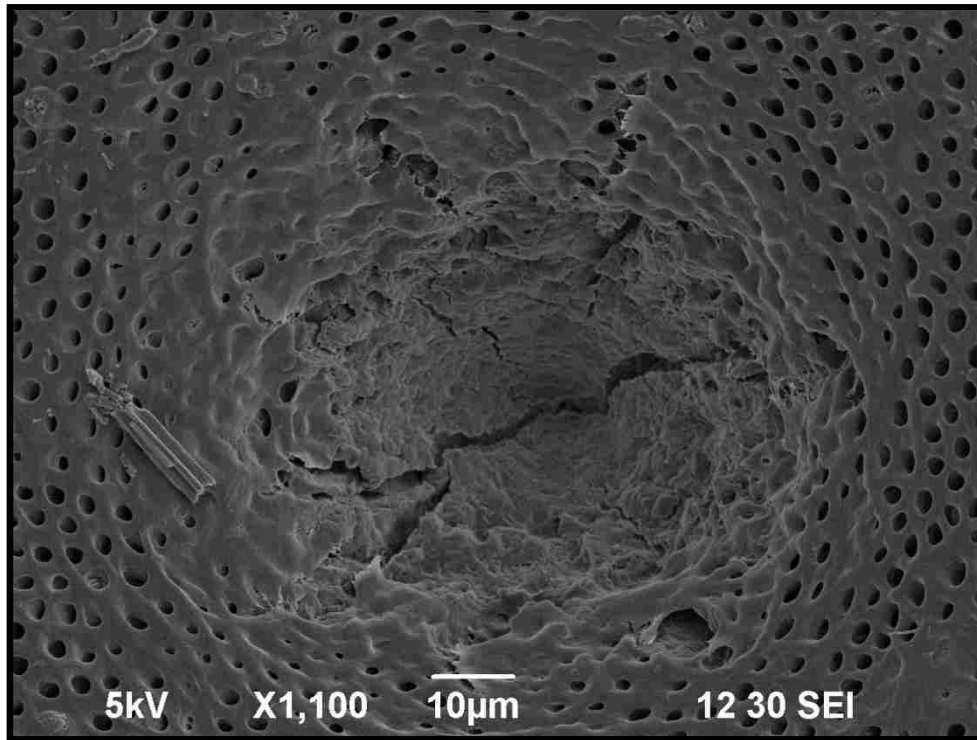


Figure 7. Representative SEM image of BioDent indentation shows the microcracks created during the repetitive loading cycles from one-month DAP-treated specimen.

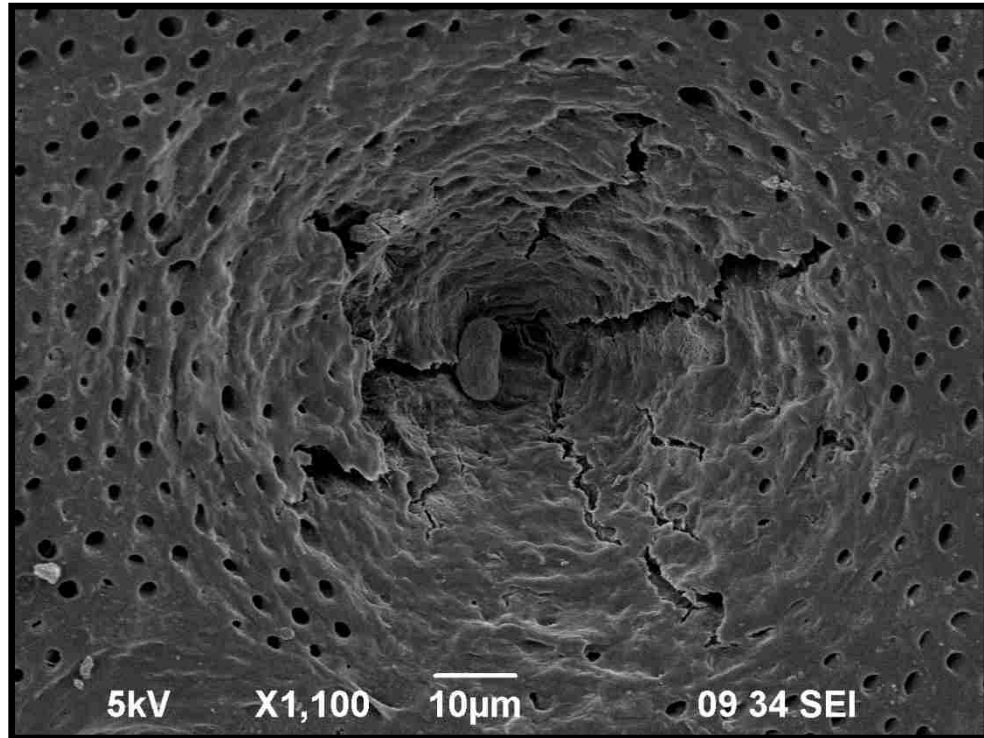


Figure 8. Representative SEM image of BioDent indentation shows the microcracks created during the repetitive loading cycles from untreated control specimen.

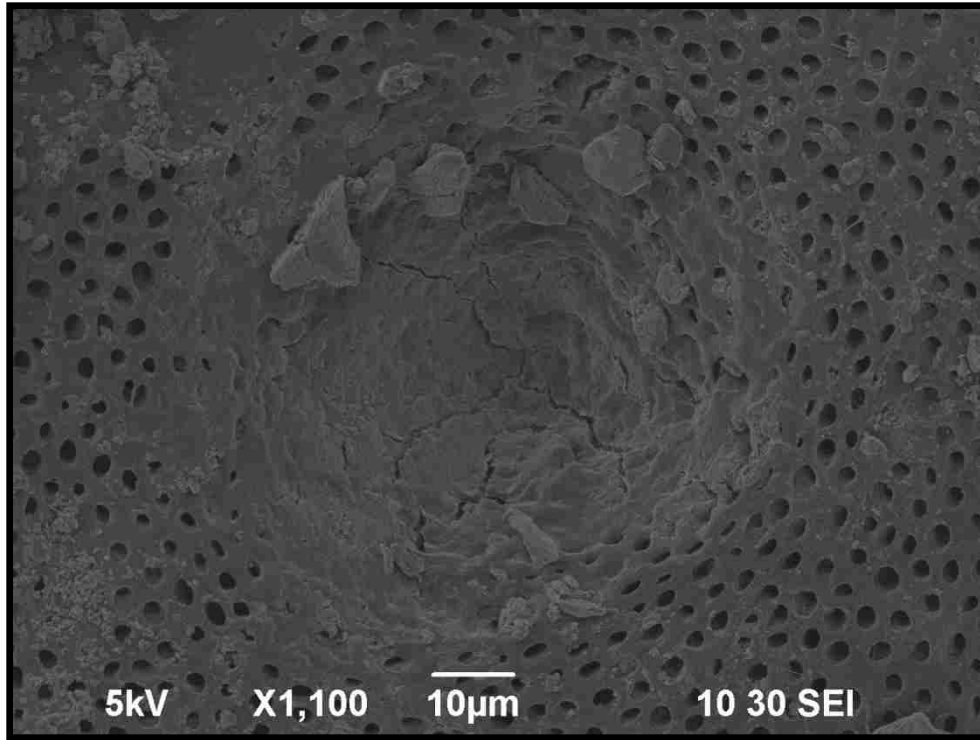


Figure 9. Representative SEM image of BioDent indentation shows the microcracks created during the repetitive loading cycles from one-month Ca(OH)_2 -treated specimen.

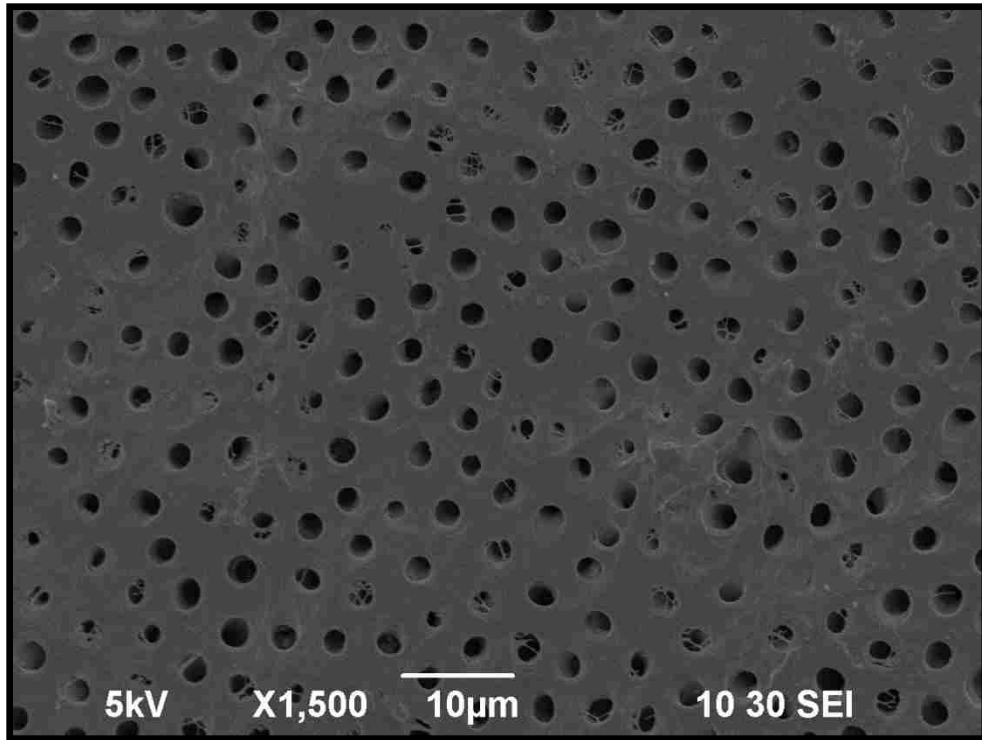


Figure 10. Representative SEM images from root canal surface of one-month TAP-treated specimen.

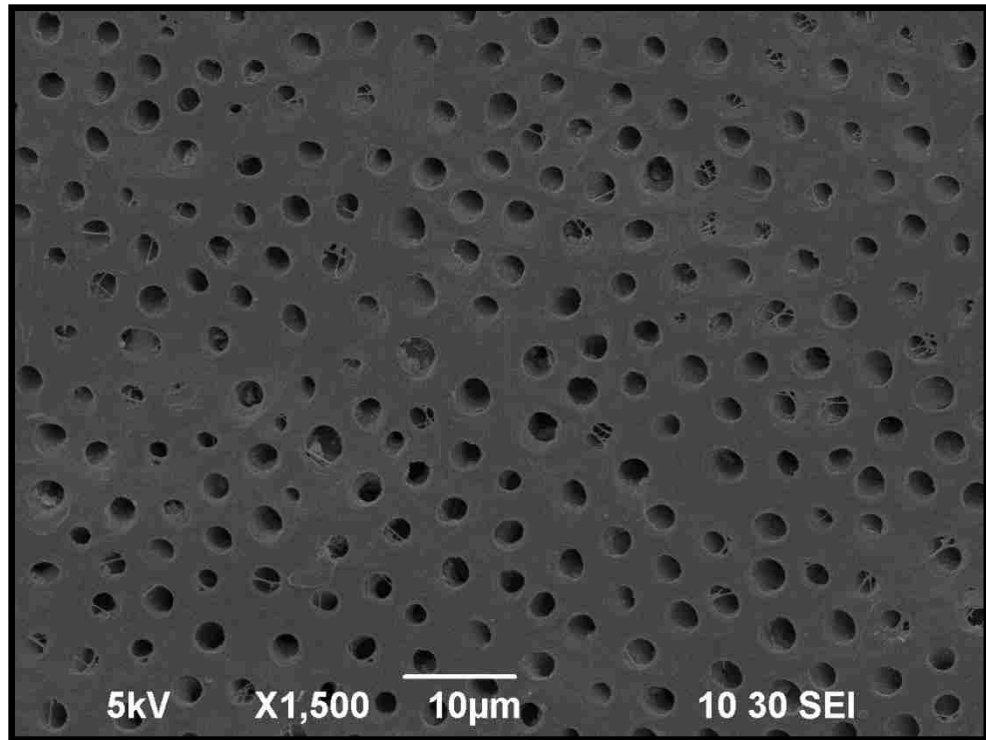


Figure 11. Representative SEM images from root canal surface of one-month DAP-treated specimen.

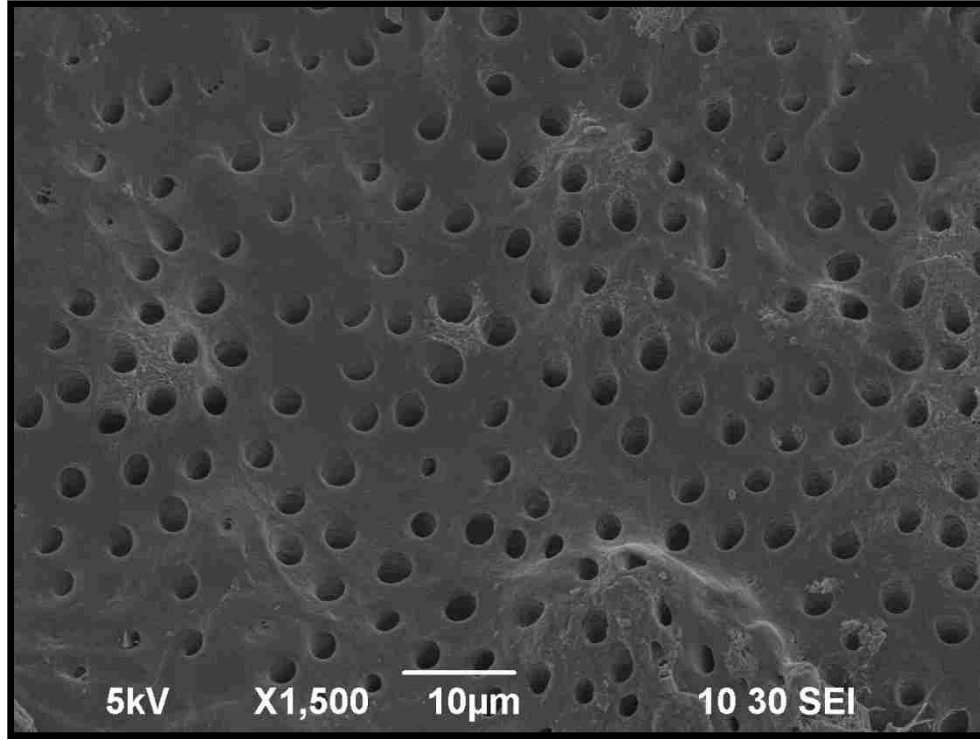


Figure 12. Representative SEM images from root canal surface of one-month untreated specimen.

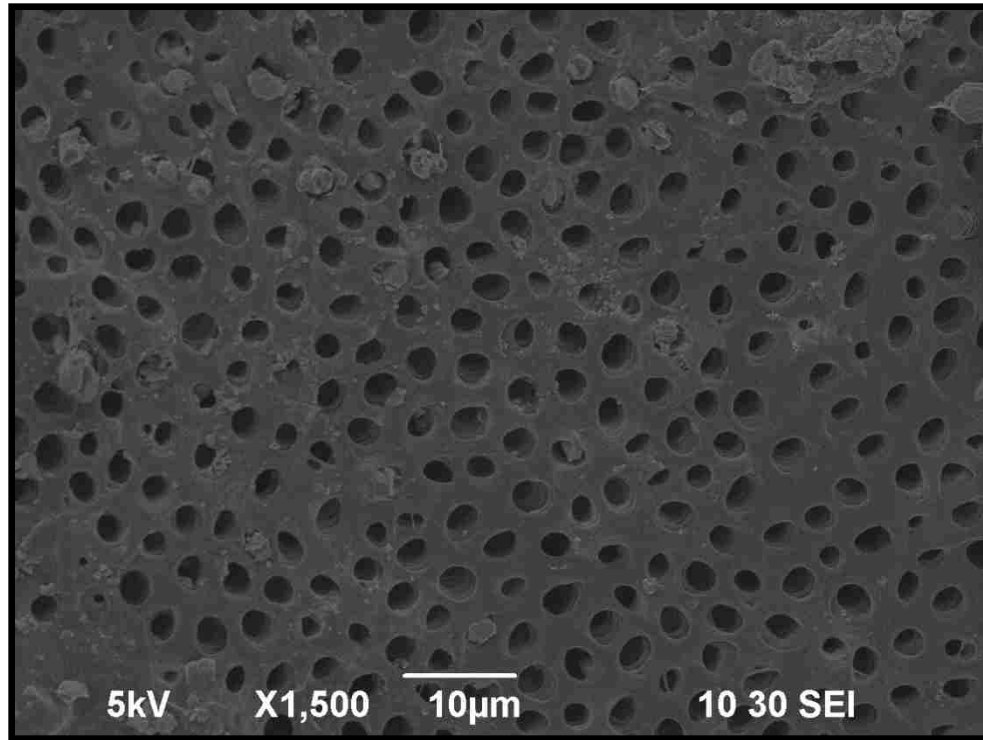


Figure 13. Representative SEM images from root canal surface of one-month $\text{Ca}(\text{OH})_2$ -treated specimen.

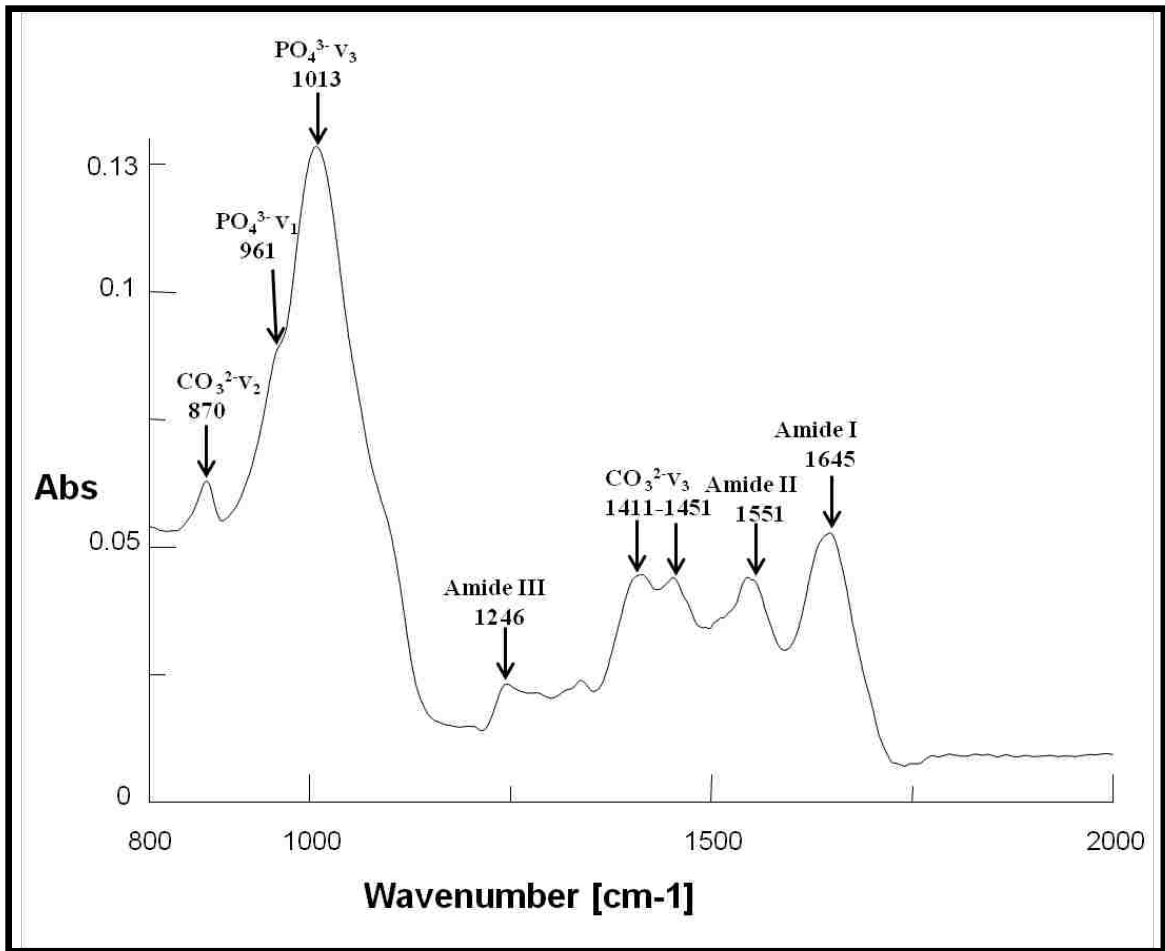


Figure 14. Representative ATR spectrum of intact radicular dentin from the untreated control group

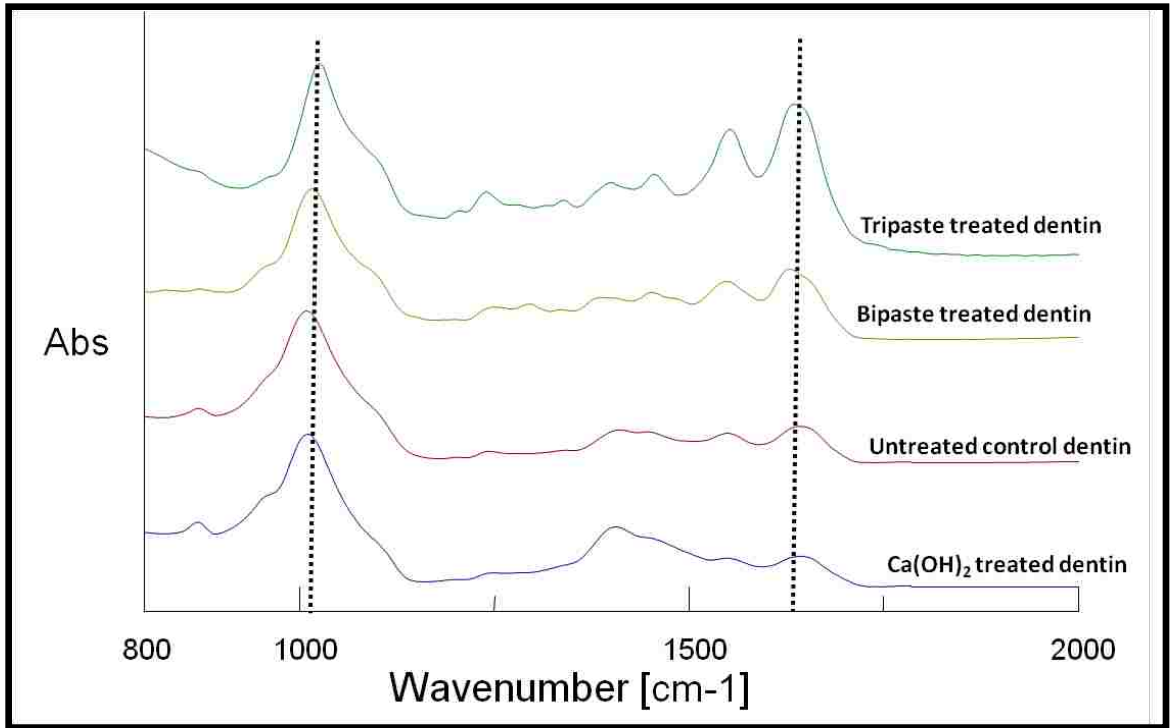


Figure 15. Representative ATR spectra of radicular dentin specimens after exposure to Ca(OH)₂, DAP, TAP, and de-ionized water (control) for one week.

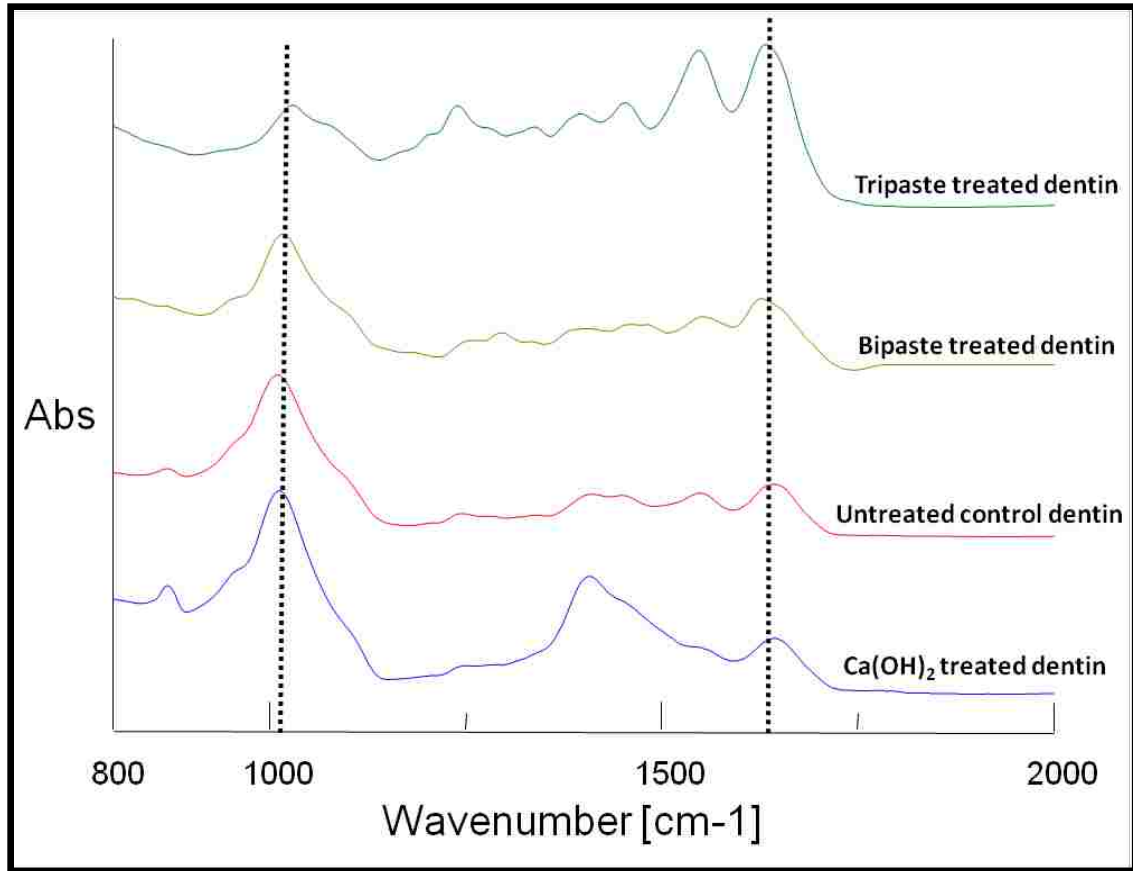


Figure 16. Representative ATR spectra of radicular dentin specimens after exposure to Ca(OH)₂, DAP, TAP, and de-ionized water (control) for two weeks.

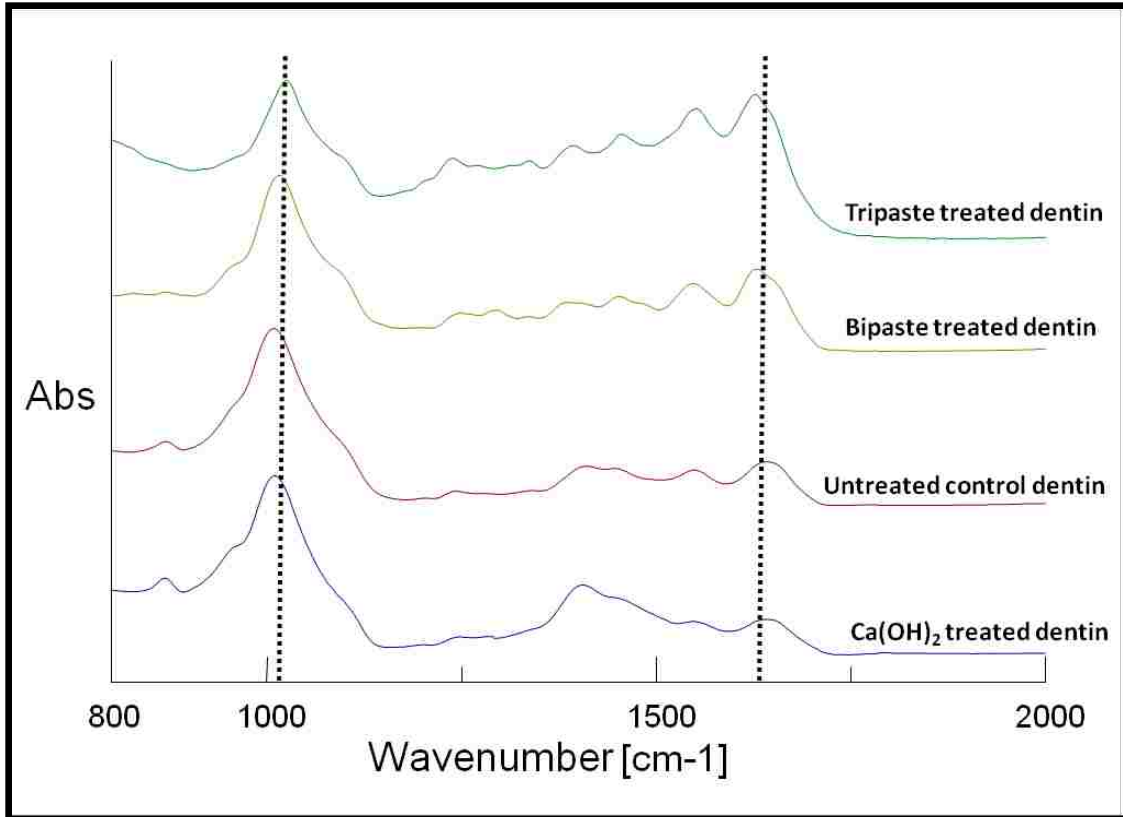


Figure 17. Representative ATR spectra of radicular dentin specimens after exposure to Ca(OH)₂, DAP, TAP, and de-ionized water (control) for four weeks.

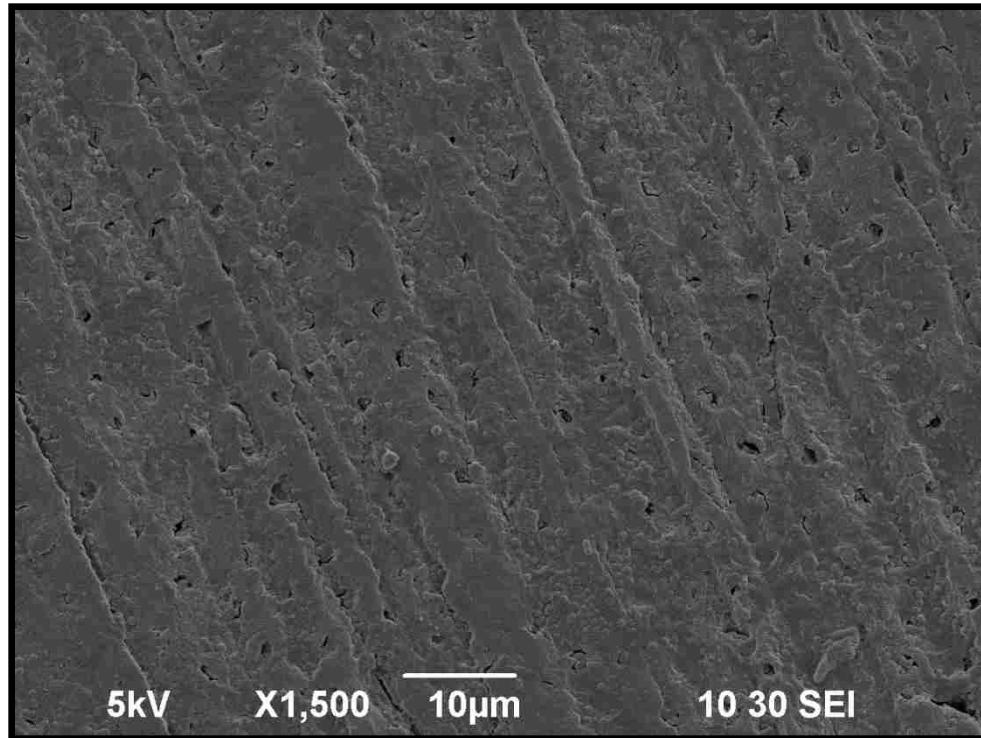


Figure 18. Representative SEM image of radicular dentin from the leveled canal surface area after four-week exposure to de-ionized water (control).

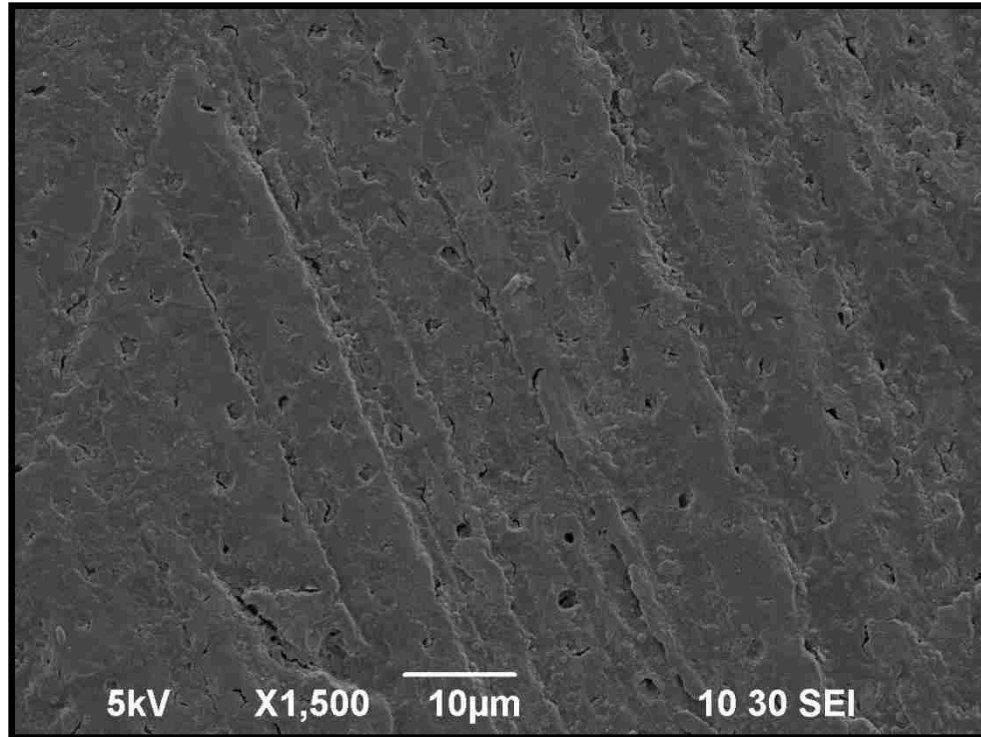


Figure 19. Representative SEM image of radicular dentin from the leveled canal surface area after four-week exposure to Ca(OH)_2 .

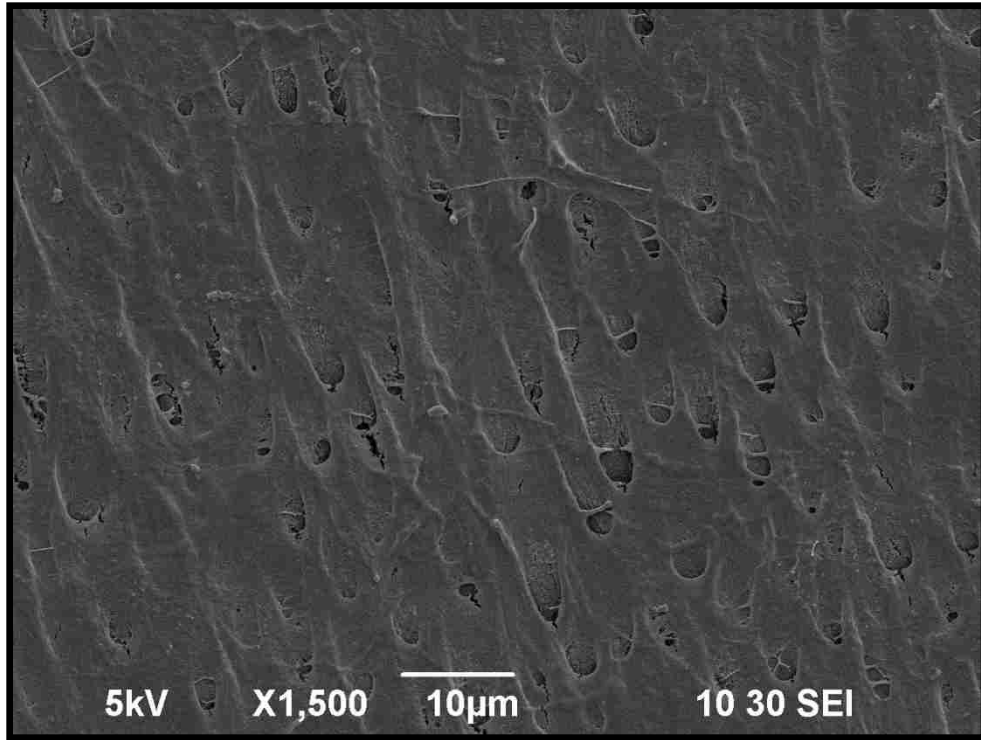


Figure 20. Representative SEM image of radicular dentin from the leveled canal surface area after four-week exposure to DAP.

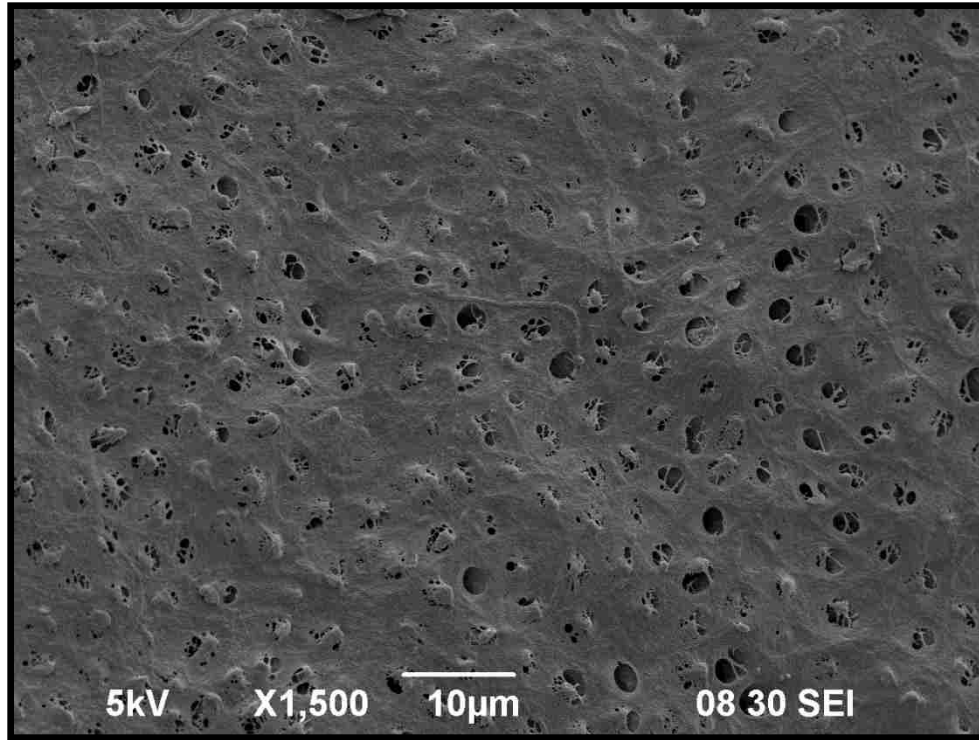


Figure 21. Representative SEM image of radicular dentin from the leveled canal surface area after four-week exposure to TAP.

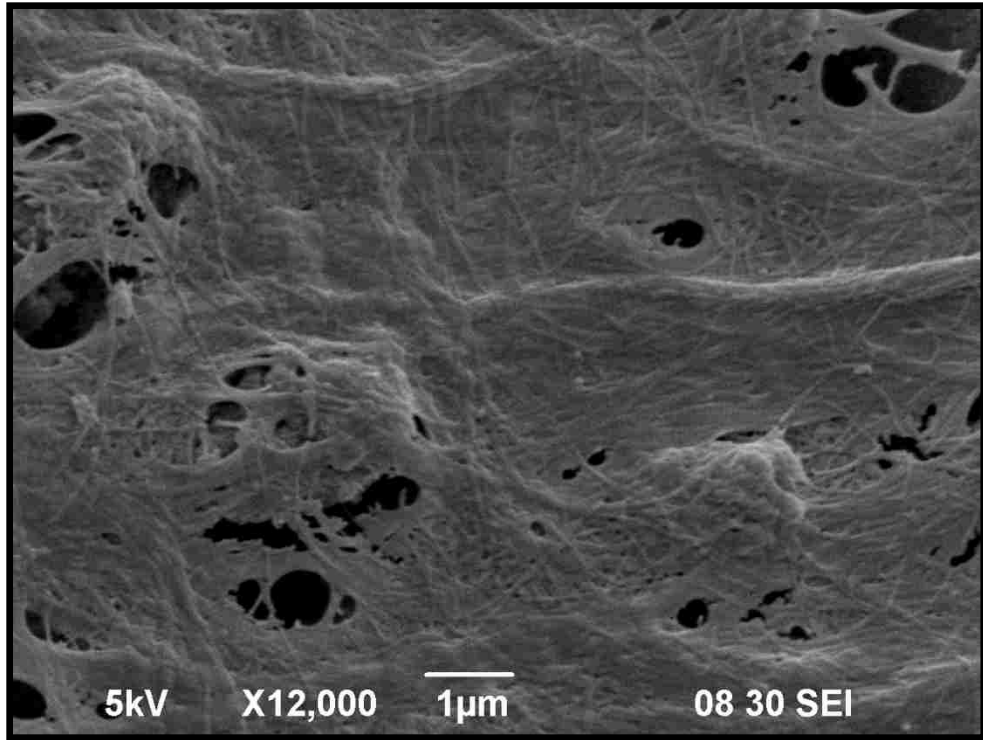


Figure 22. Representative SEM image of radicular dentin from the leveled canal surface area after four-week exposure to TAP (12,000 magnification).

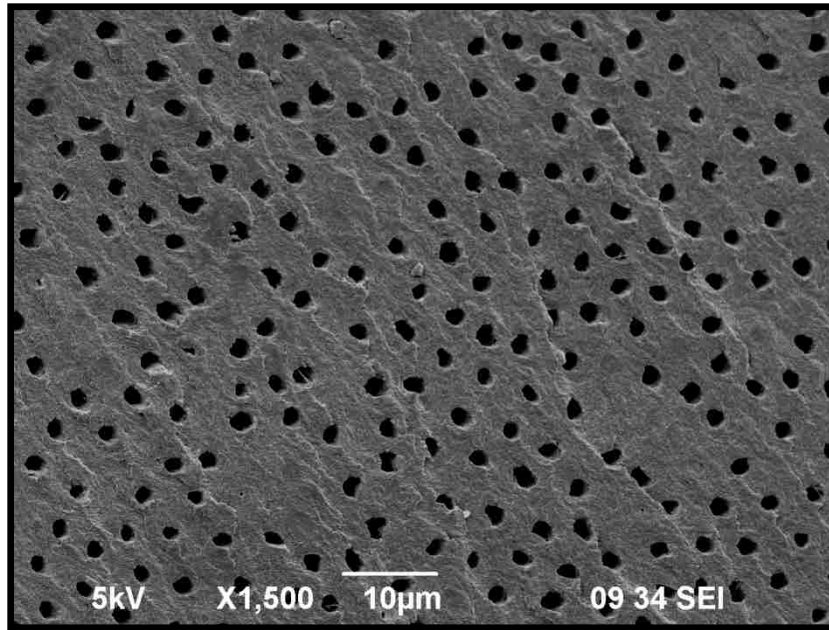


Figure 23. Representative SEM image from three-month TAP treated root canal.

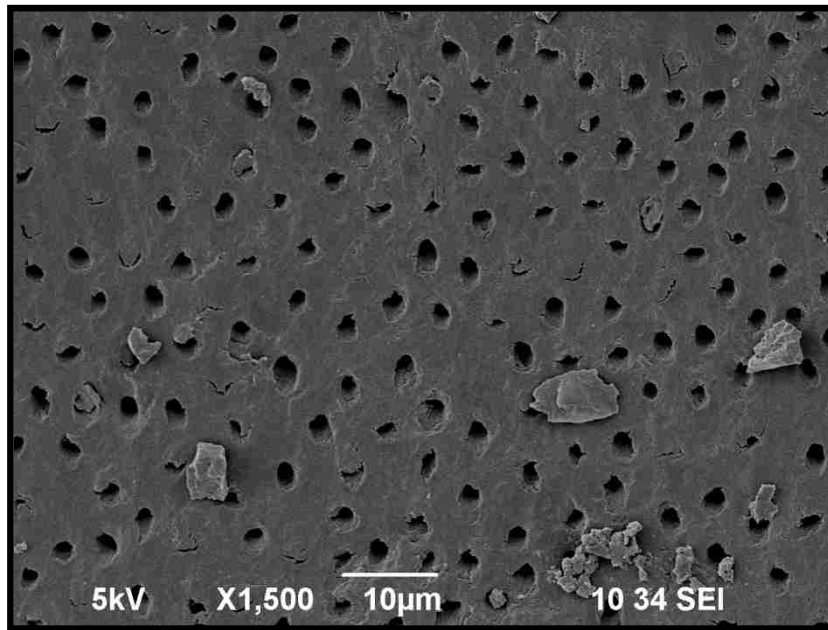


Figure 24. Representative SEM image from three-month DAP treated root canal.

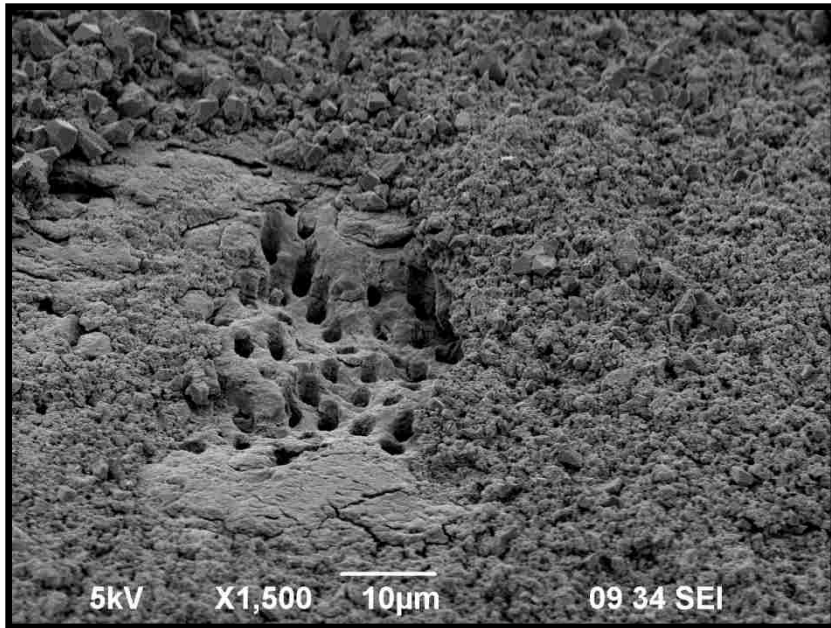


Figure 25. Representative SEM image from three-month Ca(OH)_2 treated root canal.

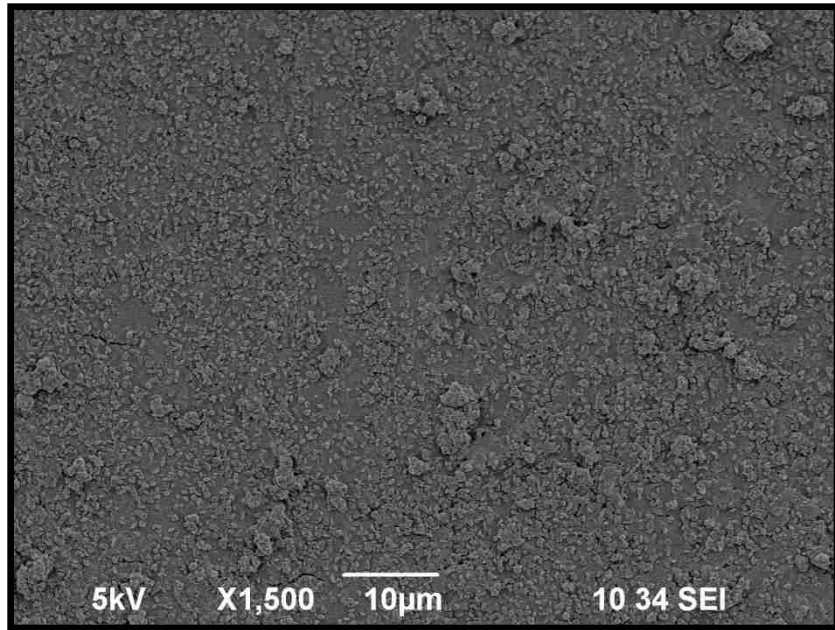


Figure 26. Representative SEM image from three-month untreated control root canal.

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Medicine, Oral Pathology, Oral Radiology and Endodontics (2009) **108**, 471-4.

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CURRICULUM VITAE

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EDUCATION

2009-2013 Doctor of Philosophy (Ph.D), Indiana University, Indianapolis, Indiana
2002-2005 M.S.D. Pediatric Dentistry, Baghdad University School of
Dentistry, Baghdad, Iraq
1995-2000 B.D.S., Mosul University School of Dentistry, Mosul, Iraq

APPOINTMENTS

Academic

2001-2002 Junior instructor, Mosul University School of Dentistry, Mosul, Iraq
2005-2009 Faculty member (lecturer), Mosul University School of Dentistry,
Mosul, Iraq
2006-2007 High diploma instructor and clinical supervisor in pediatric
dentistry, Kurdistan Ministry of Health, Sulaimani, Iraq.

Other

2005-2009 Dentist (private practice), Mosul, Iraq

HONORS AND AWARDS

2012 Sam H. Jones Community Service Award, IUPUI
2012 Educational Enhancement Research Grant, IUPUI
2011 Delta Dental Award for Innovation in Oral Care Research
2011 Sam H. Jones Community Service Award IUPUI
2011 Educational Enhancement Travel Grant, IUPUI
2010 Sam H. Jones Community Service Award, IUPUI
2010 Educational Enhancement Travel Grant, IUPUI
2010 Graduate Student Travel Award, IUPUI

PROFESSIONAL MEMBERSHIPS AND SERVICE

2000-present Iraqi Dental Association
2001-present Nineveh Dental Association
2005-present Mosul Association of University Lecturers.
2007-present Oral and Dental Care Iraqi Association (ODCIA)
2009-present Indiana Association of Dental research
2010-present American Association of Dental research
2010-present American Academy of Pediatric Dentistry
2011-present International Association of Dental research

Journal Reviewer

2010-present Indian Journal of Dental Research
2011-present International Journal of Pediatric Dentistry

2012-present Journal of Spectroscopy

TEACHING

Indiana University

2011-2012 T601, Critical Thinking (PBL Tutor, 6h/w)

2010-2011 G910, Seminar: Preventive Dentistry (Guest lecturer, 2h)

Kurdistan Ministry of Health

2006-2007 Advanced Clinical Pediatric Dentistry (clinical supervision)

2006-2007 Advanced Pediatric Dentistry (Course Director and Lecturer)

Mosul University

2005-2009 Clinical Pediatric Dentistry (clinical supervision)

2005-2009 Pediatric Dentistry (Course Director and Lecturer)

PEER-REVIEWED PUBLICATIONS

Yassen GH, Chin J, Younus MS, Eckert G. Knowledge and attitude of dental trauma among mothers in Iraq. *European Archives of Paediatric Dentistry* (In press).

Yassen GH, Vail MM, Chu TG, Platt JA. The effect of medicaments used in endodontic regeneration on root fracture and microhardness of radicular dentin. *International Endodontic Journal* (In press).

Bottino MC, Yassen GH, Platt JA, Labban N, Jack Windsor L, Spolnik KJ, Bressiani HA. A novel three-dimensional scaffold for regenerative endodontics: materials and biological characterizations. *Journal of Tissue Engineering and Regenerative Medicine* (In press).

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Yassen GH. Evaluation of mandibular infiltration versus mandibular block anaesthesia in treating primary canines in children. International Journal of Paediatric Dentistry. 2010; 20(1):43-9.

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AL-Dahan ZA, Al-Jobory GH. Dental erosion among 11–12 years old children in Mosul city. Al-Rafidain Dental Journal. 2006;6 (Sp Iss):58S-63S.

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Sabrah A, Yassen G, Gregory RL. Antibacterial Activity of Intracanal Medicaments Used in Pulp Regeneration. (International Association of Dental Researches, 2013, Accepted).

Yassen G, Eckert G, Platt J. Pulp Regeneration Medicaments Effect on Microindentation of Root Canal Dentin. (Academy of Dental Materials, 2012).

Bottino MC, Kamp T, Waeiss RA, Yassen G, Platt JA, Arthur R. Drug Containing Periodontal Membranes - Effects on Non-Periodontitis Related Bacteria. (International Association of Dental Researches, 2012)

Yassen G, Ehrlich Y, Spolnik KJ, Patel N, Bressiani AA, Platt JA, Bottino MC. Processing/Characterization of an Electrospun Composite Nano/Microfibrous Scaffold for Endodontic Regeneration. (American Association of Dental Researches, 2012).

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Yassen G, Chin J, Eckert G. Traumatic Injuries of Permanent Teeth Among 6-12 year-old Iraqi Children: A 4 Year Retrospective Study. IUPUI Civic Engagement Showcase & Symposium 2012, abstract.

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Yassen G, Lippert F, Eckert G, Eder J, Ferreira Zandona A. Dose-Response Effect of Strontium and Fluoride on Caries Lesion Remineralization. Indiana University School of Dentistry Nineteenth Annual Research Day Program 2011; 19:14, abstract.

Yassen G, Chin J, Mohammedsharif A, Alsoufy S, Othman S, Eckert G. Induced Root Apexification in Young Permanent Incisors Using Calcium Hydroxide. Indiana University School of Dentistry Eighteenth Annual Research Day Program 2010; 18:46, abstract.

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