THE EFFECTS OF CONCENTRATION AND TREATMENT TIME ON THE RESIDUAL ANTIBACTERIAL PROPERTIES OF DAP IN METHYLCELLULOSE

by

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INTRODUCTION

Recently, there has been a shift in the treatment options of immature teeth with pulpal necrosis. This is due to the better understanding of the inherent potential of tissue to proliferate into the necrotic pulp space of an immature tooth which is often referred to as regenerative endodontics.[1] A protocol that has been effective and has resulted in better decontaminating pulp space uses disinfection techniques in combination with minimal or no mechanical debridement.[2] A triple antibiotic paste made of ciprofloxacin, metronidazole, and minocycline [3] is placed for a period of 1 to 4 weeks further enhances this process of creating a disinfected space and allows for tissue growth and repair. A hand file instrumented into the periapical tissue induces bleeding and clot formation, which serves as a scaffold to support the ingrowth of stem cells and the formation of pulp-like tissue that can contain PDL, cementum and bone.[4, 5] This evoked bleeding step in regenerative endodontic procedures (REP) triggers the significant accumulation of undifferentiated stem cells into the canal space where these cells might contribute to the regeneration of pulpal tissues seen after antibiotic paste disinfection of the immature tooth with pulpal necrosis.[6]

Pulpal necrosis of immature teeth is a clinical challenge in endodontics. These immature teeth with pulpal necrosis have a compromised prognosis because the thin root walls increase the risk for cervical root fracture and the short roots result in a poor crown to root ratio. [7, 8] Treatment strategies for these teeth have evolved over the last few decades. Conventional endodontic therapy can be difficult due to the challenge in disinfection and prevention of obturation material from extending beyond the immature open apex. Apexification methods utilizing calcium hydroxide (CH) or MTA to induce

hard tissue formation as an apical barrier serve to prevent overextension during obturation. [9-11] Although these developments improve the quality of obturation, the tooth maturation remains arrested.

Fortunately, this problem is being addressed with modern regenerative endodontic therapy. Teeth treated with regenerative endodontics show continued root development in both length and thickness. [12-14] Regenerative endodontics incorporates all the components necessary for success: stem cells, scaffold, and growth factors [15]. Blood from the apical area contains mesenchymal stem cells of the apical papilla, [6] the blood clot serves as the scaffold, and growth factors are derived from platelets or from the dentin itself. (i.e., VEGF, EGF, P1GF FGF2). [16, 17]

The AAE has provided the following protocol for regenerative therapy. At the first appointment, the canal is irrigated with 20 mL of 1.5% of NaOCl irrigation for 5 minutes, dried with paper points, filled with triple antibiotic paste (TAP) (0.1 mg/ml) or $Ca(OH)_2$, and temporized with Cavit and intermediate restorative material (IRM) for one week to four weeks. At the second appointment, the canal is irrigated with 20 mL of 17% EDTA, irrigated with sterile saline and dried. Bleeding is induced by extending a sterile instrument through the apex into the apical tissues, a collagen membrane is placed on the blood clot 3 mm below the CEJ; MTA is placed 3 mm to 4 mm thick, and the access is restored with glass ionomer. [18]

As with other endodontic therapies disinfection is an important and essential part of regenerative endodontics. Some materials such as NaOCl, $Ca(OH)_2$, TAP, double antibiotic paste (DAP) and EDTA are recommended for disinfection and conditioning of the root canal system.

NaOCl is a powerful germicidal irrigation solution that has been shown capable of dissolving necrotic tissue; [19] though, it is ineffective against lipopolysaccharide (LPS), [20, 21] cytotoxic to stem cells, [22] and its cytotoxicity is correlated with decreased stem cell attachment. [23] Therefore, if it is to be used it is recommended to only be used in lower concentrations (1.5%) at the first visit. It's use is completely avoided at the second visit when stem cell attachment and growth is desired. [24]

EDTA is a non-acidic (pH = 7.7) chelating agent that is used to condition the dentin after disinfection. It demineralizes dentin by removing calcium and phosphorus[25] and removes the smear layer thereby exposing dentin tubules [26] and facilitating the release of growth factors from dentin. [27-29] Furthermore, dental pulp stem cells demonstrate "intimate association" with dentin that has been pre-treated with EDTA. [16]

$Ca(OH)_2$ is an effective alkaline bactericide capable of LPS hydrolysis. [20, 30-32] However, negative effects include superficial collagen degradation, [33] decreased tooth fracture strength when used over 30 days [34] and reduced flexural strength of dentin. [35]

TAP, a combination of ciprofloxacin, metronidazole and minocycline, was formulated by Hoshino et al. and proved to be an effective topical antimicrobial on root canal dentin. [3, 36] However, there are several negative effects including minocycline-induced tooth discoloration, [37, 38] dentin demineralization, [33] potential for adverse drug reaction[39, 40] and stem cell cytotoxicity at higher concentrations. Therefore, it has been suggested to use lower concentrations. [24 41, 42]

DAP is another antibiotic mixture of equal parts of metronidazole and ciprofloxacin that has been used successfully in endodontic regeneration. [26] Due to the tooth discoloration properties of minocycline on dentin it has been removed from TAP to

form DAP. [43] While the staining characteristic has been removed, DAP, like TAP, is still cytotoxic at higher concentrations [41] and still has a potential for adverse reactions.[39, 40] Both antibiotics in DAP are effective in preventing DNA replication. Ciprofloxacin works by inhibiting DNA gyrase, and metronidazole affects the helical structure of DNA. [44, 45] Antibacterial action occurs through binding of the antibiotic or its metabolite (i.e., metronidazole) to essential enzymes in the bacterial cells and preventing their growth. [44] While its use is also recommended at lower concentrations, the ideal concentration to balance antimicrobial efficacy and stem cell cytotoxicity is still being established.

DAP has been used clinically and in *in vitro* studies. In a successful case report in 2001, by Iwaya et al. a combination of two antibiotics, including ciprofloxacin and metronidazole, was used on an immature lower right second premolar with a necrotic pulp. After initiation of the regenerative regimen DAP was placed from weeks 2-5 of treatment. The tooth was closed and followed over the next several months. At the 30 month post-operative appointment an updated radiograph revealed complete closure of the root apex and increased thickness of the canal wall [46]. Their work has since been repeated and modified. [43]

In 2015, a clinically successfully treated case series of immature teeth was reported by Nevins and Cymerman [47] in which they placed “a creamlike consistency of ciprofloxacin and metronidazole mixed in equal amounts” into the canals of teeth in four different cases. In their findings they concluded that eliminating minocycline from the intracanal antibiotics did not appear to adversely affect tissue repair in their cases. In each of the cases with DAP as part of their protocol they found a significant reduction of

symptoms and in the size of the periapical radiolucency at follow-up appointments than was noted at treatment initiation.

Another recent study was conducted to investigate the effect of various dilutions of DAP on the survival of human dental pulp stem cells (DPSCs) and to determine their antibacterial effect against established *E. faecalis* biofilm. [48] DAP dilutions (0.125, 0.25, 0.5, 1, and 10 mg/mL) were tested against *E. faecalis* established biofilm and DPSC. Established bacterial biofilm was exposed to antibiotic dilutions for 3 days. The biofilms were then collected, spiral plated, and the numbers of bacterial colony forming units (CFU/ml) were determined. Results showed that all tested dilutions had an antibacterial effect against *E. faecalis*. However, 0.125 mg/ml of DAP showed a significant antibacterial effect with no cytotoxic effects on dental pulp stem cells (DPSCs). [48] However, in this study the biofilm was grown for only 3 days. Sabrah et al. [49] also found a significant residual antibacterial effect of dentin treated for two weeks with 1 and 1000 mg/mL of DAP up to 14 and 30 days, respectively. However, the DAP dilutions used in that study were in liquid form which cannot be easily applied into root canals clinically. Previous studies [49, 50] did not explore the effect of application time of DAP on the residual antibacterial effect. There is a relatively wide range of antibiotic application times according to the AAE (1-4 weeks).[24] To the best of our knowledge no previous study has explored the residual antibacterial effect of low dilutions of DAP in a system that can be applied clinically with a vehicle such as methylcellulose and no studies have explored the effect of the application time of DAP dilutions on the residual antibacterial properties. The gel like consistency of methylcellulose may be able to contain DAP in the

canal in direct contact with the dentin for a longer period of time and this may be clinically relevant.

CLINICAL SIGNIFICANCE

The ability of DAP in a methylcellulose vehicle to prevent recolonization of a bacterial species after it has been rinsed out of a canal is unknown. Re-infection after inadequate disinfection of the canal space may lead to failure of REP. Optimizing the variables such as concentration and treatment duration that maximizes the residual antibacterial effect of DAP could lead to future increased success in regenerative endodontics. Understanding how these variables effect the residual antibacterial properties of DAP will help to identify the ideal concentration and treatment duration of this intracanal medicament for more improved and predictable outcomes regarding regenerative endodontic therapy.

OBJECTIVE

The aims of this *in-vitro* study were to investigate how concentration and time of treatment affect the residual antibacterial properties of DAP in preventing *E. faecalis* biofilm formation on human dentin.

HYPOTHESES

Null: There is no significant difference in residual antibacterial effect among the tested concentrations of DAP.

Alternative: There is a significant difference in residual antibacterial effect between the different concentrations of DAP.

Null: There is no significant difference in residual antibacterial effect among the different treatment times.

Alternative: There is a significant difference in residual antibacterial effect between the different treatment times.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

While the first book about dentistry in the English language was written in 1867 by Charles Allen, the history of dental pain and pathology can be traced back several millennia when tooth aches were believed by many to be caused by the infamous “tooth worm.” The tooth worm would bore holes in teeth causing dental pain. [51] This idea first appeared in a Sumerian text around 5,000 BC. Other references to tooth worms can also be found in other countries as well many years before the belief became accepted into Western Europe around the 800 AD.

Treatment of dental pain cause by tooth worms varied depending on the severity of the symptoms. Often, practitioners would try to ‘smoke’ the worm out by heating a mixture of beeswax and henbane seed on a piece of iron and funneling the fumes into the cavity. Afterwards, the hole was filled with powered henbane seed and gum mastic. This may have provided people temporary relief given that henbane is a mild narcotic. Many times, though, this tooth had to be removed altogether in what was certainly an extremely painful procedure in a period before anesthetics. [52]

The tooth worm theory came under attack in the 18th century when Pierre Fauchard, known today as the father of modern dentistry, theorized that tooth decay was linked to sugar consumption and not little creatures burrowing inside the tooth. In the 1890s, W.D. Miller took this idea a step further, and discovered through a series of experiments that bacteria living inside the mouth produced acids that dissolved tooth enamel when in the presence of fermentable carbohydrates. [53]

Many other advancements in medicine and dentistry over the years have been instrumental in making the specialty of endodontics what it is today. Back in the 1700s, endodontic treatment consisted of incomplete treatment of the pulp with various mechanical techniques, the use of a variety of chemicals and medicaments to treat the canals; however, obturation was limited to the pulp chamber. Fauchard's 1728 book entitled Surgical Dentist described endodontic procedures such as mechanical debridement, the use of medicaments, and obturation of the pulp chamber with lead foil. [54] In 1766, Robert Woofendale provided the first recorded description of an endodontic procedure in this country using heat cauterization of the pulp and a filling made of cotton. To relieve pain and for treatment of the pulp he recommended the use of oil of cinnamon, cloves, turpentine, opium, and camphor. [55]

During the early 1800s, pulp and periradicular physiology became better understood; pulpal anesthesia was introduced, and new instruments for debridement were designed. The concept of pulp vitality and the ability to retain a non-vital tooth were introduced. [56] In 1839 Baker is credited with writing the first complete account of root canal therapy including pulpal debridement, cleaning, and obturation of the canals with gold foil. [54] At this time, the etiology of a toothache was speculated to be nerve exposure, fungus of the nerve (pulp polyp), pus, or periodontitis.

In the late 1800s improvements in instrumentation, disinfection, obturation materials, surgical endodontics, diagnostic tests, and prognostic factors were discovered and advanced the field of endodontics. One of the most important materials to surface was discovered during this period. In 1835, the first attempts were made to control moisture in the oral cavity during dental treatment. Dr. Sanford Christie Barnum was the

first dentist to use a thin piece of rubber to isolate teeth in 1869. Barnum's method was soon adopted as a solution for the problem of moisture control. And to this day the rubber dam is still the most important tool with which to extend warranty with respect to antisepsis and moisture control as well as to protect patients from aspiration of endodontic instruments or toxic materials. [54]

In the late 1890's and early 1900's advancements in dentistry continued: local anesthesia was applied and canal length and size determination were developed as procedures. In 1905 Einhorn introduced procaine (Novocaine) for local anesthesia. [57] In 1908 Dr. Rhein first described radiographic working length determination using a wire in the canal. [58, 59] In the 1920s, the focal infection theory was introduced, which claimed that a focus of infection would cause other diseases throughout the body. [60] It was proposed that bacteria present in infected organs could spread via the bloodstream to a new location and establish an infection. [60, 61] Many people understood that this could also come from infection inside a tooth. In 1910 William Hunter's presentation entitled "The Role of Sepsis and Antisepsis in Medicine," drove this theory. As a result, endodontic therapy was disparaged and extraction was endorsed for all non-vital or previously endodontically treated teeth. Surprisingly, there were even some practitioners who recommended extraction of all teeth for prevention of endodontic infection. [60, 62] Thankfully, in 1930 this theory began to lose its popularity and more conservative approaches rebounded. In 1937 Burket reported 200 arthritis cases that had not resolved with removal of infection foci and concluded that the relation between the foci and arthritis was not causative but rather associative. [63] This refuted the focal infection era.

In the mid-1900s recognition of specialty of endodontists was becoming more prominent. The American Association of Endodontists (AAE) and the American Board of Endodontics (ABE) were formed and advanced this field of dentistry. The specialty of endodontics gained recognition by the ADA in 1963 and as of 2015 the AAE had 8,015 members.

There have been and will continue to be more and more improvements in the field of endodontics as we move forward learning from the past and working in the present to make a better future for this endodontic specialty. One of the most exciting areas of endodontics has been the emergence of regenerative endodontics over the past two to three decades which is where my study focuses.

THEORY OF ENDODONTICS

Endodontic pathology can be initialized by introducing bacteria into the pulpal tissue by restoration, caries or trauma. This can cause and increase in inflammation which can ultimately lead to pulpal necrosis. This process can continue and further lead to periapical disease. The study that brought to light the crucial role of microorganisms in the pathogenesis of pulpal and periapical pathology was demonstrated in the 1965 classic study by Kakehashi, Stanley, and Fitzgerald. [64] This article reported that traumatized dental pulp tissue in germ-free rats was able to survive even after being subjected to food impaction under sterile conditions. However, in the exposed dental pulp tissue of conventional rats, which were not germ free, there was inflammation, necrosis, and abscess formation. This study showed that the presence or absence of microbial flora was the major determinant in healing. Moller further demonstrated that infected pulp tissue, and not necrotic tissue alone, caused periapical inflammation histologically in monkeys.[65]

Therefore, the objective of endodontic therapy is to restore the tooth to form and function by reducing the microbial load and byproducts in order to prevent and treat pulpal and periapical pathology. Along with this, to restore the tooth in a manner that prevents reinfection and promotes tooth function long-term. [66-68] Principles describing successful endodontic therapy and have been separated into three phases: chemomechanical preparation, microbial control, and obturation. [69, 70] However, it should be emphasized that microbial control must be considered throughout all treatment phases. These phases are described more commonly today as instrumentation, irrigation, and obturation. Successful endodontics is directly related to reduction of bacteria in the root canal system through chemomechanical cleaning and the ability to seal the tooth to prevent recontamination. [64, 66, 69, 71] Chemomechanical preparation of the tooth is achieved by first debriding the pulpal area with mechanical filing of the root canal system to allow for chemical irrigants to reach the entire pulpal length of the tooth. The standard of care requires treatment to be completed under rubber dam isolation to help further aid in asepsis. [71] Once chemomechanical debridement is complete three dimensional obturation to the cementodentinal junction or 0.5 to 1 mm from the radiographic apex is required. [66] After successfully cleaning and obturating the root canal system a final coronal restoration seal is required to prevent bacterial leakage to recontaminate the canal space.[71] Successful completion of these factors contributes to favorable outcomes in root canal therapy.

IMMATURE TEETH WITH PULPAL NECROSIS

Despite the 97% success rates achieved with conventional endodontic therapy, [72] immature teeth with pulpal necrosis do not have a good long term prognosis. It has been

documented that immature teeth that develop pulpal necrosis have a compromised prognosis and this was attributed to the thin and short roots increasing the risk for cervical root fracture and result in a poor crown-to-root ratio. [7, 8] Also, because there is greater risk for overextension of canal filling material through the open apex, obturating these teeth becomes very difficult. [43] Over the past few decades treatment options for these teeth with these conditions have evolved.

Apexification

An apical barrier technique was first suggested and is now referred to as apexification. [9] This technique used $Ca(OH)_2$ to induce the formation of a calcified barrier across the apex; this technique proved successful which led to widespread acceptance. [11] $Ca(OH)_2$ has a high pH and is responsible for microbial protein denaturation and causes a low-grade irritation that induces a mineralized apical barrier. The prolonged waiting period for the apical barrier to form $Ca(OH)_2$ apexification required good patient compliance.

Mineral Trioxide Aggregate or MTA was introduced in 1995. [73] Similar to $Ca(OH)_2$, MTA possesses a high pH and induces apical hard tissue formation, but it does so with increased consistency. [74] MTA is hydrophilic and able to form a better apical seal in the presence of moisture which before was not possible. [75] MTA also serves as an apical stop, so that obturation is possible at the same visit with patient compliance less of a concern. As a result, MTA apexification has reported high success rates in the short-term. In 2007 a study reported 81% success at 1 year [76] and in 2008 another reported 93.5% success at 1.5 years. [77] In summary, apexification techniques have improved the prognosis for immature teeth with pulpal necrosis. However, the long-term prognosis

may still be compromised due to the thin walls and short roots which lend themselves to early fracture. Fortunately, the emergence of regenerative endodontics has provided potentially better outcomes for these teeth.

Regenerative Endodontics

Regenerative endodontics utilizes tissue engineering principles that help the development of damaged or missing tissues of the immature tooth. The three requirements for tissue engineering are stem cells, scaffolds, and growth factors. [78] REP have been defined as biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex. [79]

History of Regenerative Endodontics

Early work in this field are attributed to Nygaard-Ostby in 1971 where he documented the first attempted dental regenerative cases. Mature vital or necrotic teeth were mechanically debrided, chemically disinfected, and obturated. [80, 81] In necrotic teeth a 4% formaldehyde solution was used for disinfection. Although growth of fibrous connective was observed in previously vital canals, there was no growth observed in the previously necrotic teeth. [80] Later in 1974 Myers treated infected mature and immature teeth in monkeys. The teeth were disinfected with full strength (5.25%) NaOCl; the apical constrictions of the mature teeth were enlarged, and bleeding was induced into the canals. After 24 weeks tissue growth had occurred in many of the teeth; however, it was usually accompanied by periapical inflammation and root resorption, perhaps indicating inadequate disinfection or coronal seal. In these cases it was noticed that immature teeth responded better than the mature teeth; they demonstrated continued root growth and the

largest amount of connected tissue in-growth. [82] In 1976 Nevins treated pulpless immature teeth in monkeys with biomechanical debridement followed by collagen-calcium phosphate gel for 12 weeks. Histologic evaluation revealed "revitalization" of the canal with various forms of soft and hard connective tissue including "cementum, bone, and reparative dentin." [83]

Iwaya reported treatment of an immature necrotic tooth with periapical involvement in 2001. [46] In this case there was a concern that mechanical instrumentation would remove potentially remaining apical vital tissue that might aid in revascularization. So the canal was disinfected non-mechanically with 5% NaOCl and 3% H_2O_2 followed by a combination of metronidazole and ciprofloxacin. After disinfection, a layer of $Ca(OH)_2$ was placed against the apical tissue and the access was sealed with glass-ionomer cement followed by a composite resin. At the 30-month follow-up, radiographic exam revealed continued root growth and apical closure. While early work by Nygaard-Ostby brought this idea to the endodontic world, a case report in 2004 by Banchs and renewed excitement surrounding this treatment option. Their findings provided a specific protocol for revascularization of immature necrotic teeth. [84] This protocol was based on the healing observed in the avulsed immature tooth that is replanted. It was suggested that if the same environment could be created for the necrotic immature tooth, revascularization should occur. First, the canal was disinfected non-mechanically with 5.25% NaOCl, Peridex, a mixture of ciprofloxacin, metronidazole, and minocycline for 26 days, and 5.25% NaOCl again. After disinfection, apical tissue was irritated with an explorer to induce bleeding into the canal to the level of the CEJ and left to clot for 15 minutes. Finally, an MTA seal was placed. After 2 years, the patient was asymptomatic,

root growth was observed, and the tooth responded positively to the cold test. Consequently, this documented protocol was repeated by others and led to many successful case reports with formation of vital tissue in the canal. [85-88]

There have been a variety of findings regarding the type of tissue that is formed following REP. In 2010 Wang et al. treated immature dog teeth and found three types of new tissue: intracanal cementum (IC), intracanal bone (IB), and other connective tissue.[89] The IC was located on the dentin walls and appeared similar to cellular cementum. The IB was located in the lumen and contained bone-like tissue. Surrounding the IC and IB, a connective tissue similar to periodontal ligament was also present. In 2011 Yamauchi et al. treated immature dog teeth and found two types of new tissue: dentin-associated mineralized tissue and bony islands. The dentin-like tissue was located near the dentinal wall, was devoid of vasculature, and was less cellular. Whereas the bony islands were located in the canal lumen; they were vascular, contained many cells, and were similar to bone marrow. [90] In 2013 Martin et al. performed REP *in vivo* on a mandibular molar and identified healing with mineralized tissue and fibrous connective tissue, but histology reports concluded a lack of odontoblast-like cells lining the hard tissue present [91]. Many cases reported healing without re-innervation as determined by no response to EPT or cold testing. Based on these findings, one would conclude that such cases are healing by repair rather than regeneration. With that said, there have also been other cases reported in the literature with findings more suggestive of regeneration. Several cases reported the presence of innervation as verified by EPT or cold testing. [37, 84, 92, 93] In 2012 Shimizu et al. identified loose pulp-like connective tissue and cells that resembled odontoblasts [94] and Torabinejad and Faras also described pulp-like vital connective tissue. [95]

Indications and Outcomes for REP

REP so far have been largely reserved for adolescents with necrotic immature teeth with open apices. This is based on findings in trauma research that suggest apical diameters >1mm are more likely to undergo revascularization. [96] Assessment of outcomes for REP in immature necrotic teeth depends on the definition of success. The AAE described three goals, in order of importance, for measuring the success of REP: [18]

1) Elimination of symptoms and periradicular healing

2) Continued root growth

3) Positive response to vitality testing

However, the level of evidence for the outcome of REP is low because current research is limited to case reports and case series.

Disinfection for REP

We understand from Kakehashi et al. that healing occurs only in germ-free rats. [64] In 2007 Thibodeau et al. performed REP on immature necrotic dog teeth and confirmed histologically that vital tissue would only form in teeth that were first disinfected. [97] The most common disinfection strategy combines irrigation with NaOCl followed by an intracanal medicament such as $Ca(OH)_2$ or antibiotic pastes including TAP. [88]

Although NaOCl solution is an powerful antimicrobial, it has several disadvantages in the context of REP. [19] First, NaOCl has a concentration-dependent cytotoxicity effect on stem cells. [98, 99] Secondly, this cytotoxicity has been correlated with decreased stem cell attachment. [23] Lastly, NaOCl has also been shown to reduce the modulus of elasticity and flexural strength of dentine at 3% and 5% concentrations.

Therefore, it is recommended to use a lower 1.5% concentration during the disinfection phase and to avoid use during the induction of bleeding and stem cell phase. [18]

$Ca(OH)_2$ is an effective alkaline bactericide capable of LPS hydrolysis. [20, 30-32] $Ca(OH)_2$ has also been found to be conducive to SCAP survival and even significantly increase the proliferation of SCAP at a concentration of 1 mg/mL. [41, 100] However, $Ca(OH)_2$ also has disadvantages in the context of REP. Andreasen et al. found that a four-week application decreased tooth fracture strength.[34] $Ca(OH)_2$ has also been found to be less effective compared to triple antibiotic paste against *E. faecalis* and *P. gingivalis* biofilm. [50]

Triple antibiotic paste (TAP) contains a combination of ciprofloxacin, metronidazole and minocycline and was first formulated by Hoshino et al. [3] Metronidazole is broad spectrum, bactericidal, and is effective against obligate anaerobes present in necrotic pulp. TAP, as previously mentioned, was found to be significantly more effective than $Ca(OH)_2$ against *E. faecalis* and *P. gingivalis* biofilm. [50] TAP has also been shown to be effective against cultivatable bacteria of endodontic lesions *in vitro* at 0.3mg/mL. [101] However, TAP has several disadvantages in the context of REP including discoloration, demineralization, and cytotoxicity.

Minocycline, one component of TAP, has been shown to cause discoloration in several case reports. [37, 38] Minocycline binds calcium ions via chelation, forms an insoluble complex, and remains incorporated in the tooth matrix. [102] The chelating effect combined with the extremely low pH (2.9) also causes demineralization. [33] Adverse drug reactions have been observed from topical application of these antibiotics outside of dentistry. [39, 40, 103] Stem cell cytotoxicity is also a concern with TAP. The initial

concentration of TAP that was first used was 1000 mg/mL. However, in 2012, Ruparel et al. found *in vitro* that direct exposure of 1 mg/mL TAP to SCAP caused the death of 50% of the cells (LC_{50}). [41] Therefore, to minimize stem cell cytotoxicity, a reduced concentration of 0.1 mg/mL is now recommended. [18] Additional disinfection with 2% chlorhexidine shown to cause cytotoxicity to SCAP has been removed from the AAE recommended protocol.[18, 98]

Dentin Conditioning for REP

EDTA is thought to improve the environment for regeneration by several mechanisms. EDTA is a non-acidic (pH = 7.7) chelating agent that demineralizes dentin by removing calcium and phosphorus ions. [25, 104] Irrigation with 17% EDTA is capable of removing the smear layer thereby exposing dentin tubules. [26, 105] Exposure of dentin tubules has been shown to facilitate the release of growth factors from dentin. [27-29] EDTA has also been shown to increase dentin surface roughness, which may be associated with increased adherence by stem cells to dentin. [106] Dental pulp stem cells have also been shown to demonstrate "intimate association" with dentin that has been pre-treated with EDTA. [16] Finally, EDTA has been shown to partially reverse the cytotoxic effects of NaOCl thus promoting increased survival of SCAP. [99]

Considerations for Regenerative Procedures updated by the AAE 4/2015 are: [18]

Case Selection:

- Tooth with necrotic pulp and an immature apex.
- Pulp space not needed for post/core, final restoration.
- Compliant patient/parent.

- Patients not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2).

Informed Consent

- Two (or more) appointments.
- Use of antimicrobial(s).
- Possible adverse effects: staining of crown/root, lack of response to treatment, pain/infection.
- Alternatives: MTA apexification, no treatment, extraction (when deemed nonsalvageable).
- Permission to enter information into AAE database (optional).

First Appointment

- Local anesthesia, dental dam isolation and access.
- Copious, gentle irrigation with 20 ml NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g., needle with closed end and side-vents, or EndoVac™). Lower concentrations of NaOCl are advised [1.5% NaOCl (20 mL/canal, 5 min) and then irrigated with saline or EDTA (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues.
- Dry canals with paper points.
- Place $Ca(OH)_2$ or low concentration of triple antibiotic paste. If the triple antibiotic paste is used: 1) consider sealing pulp chamber with a dentin bonding agent [to minimize risk of staining] and 2) mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 0.1 mg/mL.

- Deliver into canal system via syringe
- If triple antibiotic is used, ensure that it remains below CEJ (minimize crown staining).
- Seal with 3-4 mm of a temporary restorative material such as Cavit™, IRM™, glass ionomer or another temporary material. Dismiss patient for 1-4 weeks.

Second Appointment (1-4 weeks after 1st visit)

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
- Anesthesia with 3% mepivacaine without vasoconstrictor, dental dam isolation.
- Copious, gentle irrigation with 20 ml of 17% EDTA.
- Dry with paper points.
- Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (induce by rotating a pre-curved K-file at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the cemento–enamel junction). An alternative to creating a blood clot is the use of platelet-rich plasma (PRP), platelet rich fibrin (PRF) or autologous fibrin matrix (AFM).
- Stop bleeding at a level that allows for 3-4 mm of restorative material.
- Place a resorbable matrix such as CollaPlug™, CollaCote™, CollaTape™ or other material over the blood clot if necessary and white MTA as capping material.

- A 3–4 mm layer of glass ionomer (e.g., Fuji IILC™, GC America, Alsip, IL) is flowed gently over the capping material and light-cured for 40 s. MTA has been associated with discoloration. Alternatives to MTA should be considered in teeth where there is an esthetic concern.
    - Anterior and Premolar teeth - Consider use of CollaTape/CollaPlug and restoring with 3 mm of RMGI followed by bonding a filled composite to the beveled enamel margin.
    - Molar teeth or teeth with PFM crown - Consider use of CollaTape/CollaPlug and restoring with 3 mm of MTA, followed by RMGI or alloy.

Follow-up

- Clinical and Radiographic exam
    - No pain, soft tissue swelling or sinus tract (often observed between first and second appointments).
    - Resolution of apical radiolucency (often observed 6-12 months after treatment)
    - Increased width of root walls (this is generally observed before apparent increase in root length and often occurs 12-24 months after treatment).
    - Increased root length.
    - Positive pulp vitality test response
- The degree of success of regenerative endodontic procedures is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:

- Primary goal: The elimination of symptoms and the evidence of bony healing.
- Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential)
- Tertiary goal: Positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue)

MATERIALS AND METHODS

Dentin sample preparation

An overview of experimental design can be found in the flowchart (Figure 1). Sound human permanent teeth (n=120) were collected after obtaining Institutional Review Board approval (IRB, 1408897632). The teeth were kept in 0.1% thymol solution at 4°C and used within 6 months. The crowns were removed using a water-cooled diamond saw (Figure 2) and the roots were used to obtain 120 standardized dentin specimens with the dimensions of $4 \times 4 \times 1$ mm^3 (Figures 3-5). Dentin specimens were placed on cylinders with wax in preparation for smoothing and polishing (Figure 6). A Rotoforce 4 polishing unit (Struers, Cleveland, OH) (Figure 7) was used to polish the pulpal side of each dentin specimen with abrasive papers (1200–4000 grit; Struers) (Figure 8) under running water. Dentin slabs were then sonicated with 1.5% NaOCl (Value Bleach; Kroger, Cincinnati, OH) and 17% EDTA (VISTA, Racine, WI) for 4 minutes. Each sample was then wrapped with a cotton pellet saturated with sterile water, placed in Whirl-pak bags (Sigma-Aldrich, St Louis, MO), sterilized with ethylene oxide gas, and kept at 4°C until used. (Figure 9-10)

Preparation of Medicaments Used in the Study

A total of 5 antimicrobial preparations were investigated in the current study including clinically used concentrations of DAP (500 mg/mL), three low concentrations of DAP (1, 5 and 50 mg/mL) and $Ca(OH)_2$ (UltraCal XS, Ultradent, South Jordan, UT). The clinically used DAP was prepared by mixing 500 mg of equal portions of metronidazole and ciprofloxacin (Champs Pharmacy, San Antonio, TX) with 1 mL of

sterile water.[107] The low concentrations of DAP (1, 5, and 50 mg/mL) were loaded into a methylcellulose system as described in previous studies [108, 109] to create a clinically applicable antibiotic medicament that can be injected into a root canal system. [110] In summary, 2500, 250 and 50 mg of DAP were dissolved independently in 50 mL of sterile water. Then, 4 gm of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St. Louis, MO) was slowly added to each DAP solution over 120 minutes under maximum stirring to obtain pastes with 1, 5, and 50 mg/mL of DAP. A DAP free placebo paste were also prepared and used as a control group. No untreated positive control group was used in the current study as our earlier pilot study has shown no difference between the untreated positive control samples and dentin samples treated with the placebo paste. A recent study has also found no difference between infected dentin treated with aqueous methylcellulose based paste and that treated with normal saline. [109]

Treatment of dentin samples

In order to be able to precisely quantify the residual indirect antibacterial effects of medicaments, sterilized rather than infected dentin samples were pretreated with various medicaments in the current study as described in a recent report. [111] Sterilized dentin slabs were placed individually in separate wells of sterile 96 well microtiter plates (Fisherbrand, Fischer Scientific) with the pulpal side (treatment side) facing upward (Figure 13). Samples were then randomly divided into 5 treatment groups and 1 control group (n=20 per group). The pulpal side of each dentin slab received 200 µL of one of the treatment pastes (1, 5, 50 and 500 mg/mL of DAP or $Ca(OH)_2$) or the control placebo paste. Treatment and rinsing of dentin samples were completed under a ventilated hood (Figure 11-12). All treated samples were then incubated for 1 or 4 weeks (n=10 per group at each time point) at 4°C and 100% humidity (Figure 13). The two treatment times were

selected based on the clinical endodontic regeneration procedure recommended by AAE.[18] After the assigned treatment time, the treatment paste was rinsed off from each sample using 5 mL of sterile saline followed by 5 minutes of irrigation with 5 mL of 17% EDTA. Dentin slabs were then immersed in 200 µl of sterile phosphate buffered saline (PBS) and incubated at 37°C for 3 weeks before growing the bacterial biofilm.

Bacterial Strain and Media

E. faecalis (American Type Culture Collection #29212; Manassas, VA) was grown initially on anaerobic blood agar plates (Bio-Merieux, Durham, NC). A sterile broth of brain heart infusion (BHI) supplemented with 5 g of yeast extract/L (BHI-YE) was inoculated with colonies of *E. faecalis* and incubated at 37°C anaerobically for 24 hours. (Figure 14)

Biofilm Growth on Treated Dentin Samples

After 3 weeks of immersion in sterile PBS, dentin slabs were transferred independently into wells of sterile 96-well microtiter plates with the treated surface facing upward. Then, 190 µL of fresh BHI-YE and 10 µL of an overnight *E. faecalis* culture (10^6 CFU/mL) were added to each well. The slabs were incubated anaerobically for 3 weeks at 37°C and the culture media was replenished 2 times a week. After incubation, each dentin specimen was utilized for biofilm disruption assays. A negative untreated control group was also used in the current study to confirm the absence of any bacterial contamination from outside sources within the experimental setting of this study. Briefly, untreated sterilized dentin slabs (n=3) were individually placed in 200 µL of bacteria-free BHI-YE media and incubated for 3 weeks under the same anaerobic conditions described earlier with regular replacement of BHI-YE. After 3 weeks, biofilm

disruption assays were performed to confirm the lack of any bacterial biofilm in the uninfected dentin slabs.

Biofilm Disruption Assay

The biofilm disruption assays were conducted as described in recent studies.[109, 111] Briefly, the dentin specimens were placed into sterile test tubes containing 2 mL of sterile saline. To detach the bacterial biofilm, samples were sonicated for 20 seconds and vortexed for 30 seconds (Figure 15). The ability of this protocol to maintain the viability of the sonicated bacteria as well as to detach the biofilm from the surface of the dentin slabs and from dentin tubules was confirmed in a pilot study. The obtained biofilms were then diluted in sterile saline, spirally plated on blood agar plates, and incubated anaerobically for 24 hours (Figures 16-18). An automated colony counter (Synbiosis, Inc, Frederick, MD) was used to evaluate the number of colony-forming units (CFU)/mL (Figure 19).

Statistical analyses

Some experimental groups did not exhibit any bacterial growth. Therefore, Fisher's Exact tests were used to determine the significant differences in the presence or absence of any bacterial growth. Furthermore, Wilcoxon rank sum tests were used to compare the between antibiofilm effects of various experimental groups that demonstrated bacterial growth. The significance level was set at 0.05.

RESULTS

Residual antibacterial effect

Comparisons after 1 week of treatment: (Figure 20)

The treatment groups had increasing bacterial counts in the following order: 500 mg/mL DAP, 50 mg/mL DAP, $Ca(OH)_2$, 5 mg/mL DAP, 1 mg/mL DAP and MC. Comparisons between treatment groups demonstrated significant differences ($p<0.01$) except for 1 mg/mL DAP compared with MC ($p=0.54$) and 5 mg/mL DAP compared with $Ca(OH)_2$ ($p=0.38$). No complete eradication of bacterial biofilm was observed among any 1-week dentin treatments. Furthermore, all dentin samples demonstrated bacterial growth. Figure 22 demonstrates that dentin samples pretreated with 500 mg/mL of DAP for one week developed a significantly higher residual antibacterial effect in comparison to all other 1-week treatments ($p<0.01$). A one week dentin pretreatment with 50 mg/mL of DAP induced a significantly higher residual antibiofilm effect in comparison to 1 week dentin pretreatment with placebo paste, $Ca(OH)_2$, 5 or 1 mg/mL of DAP ($p<0.01$). A one week pretreatment of dentin with 5 mg/mL of DAP or $Ca(OH)_2$ had a significant but limited residual antibacterial effect in comparison to a 1 week dentin treatment with 1 mg/mL of DAP and placebo paste ($p<0.05$). However, no significant residual antibacterial effect was observed between 1 week pretreatment of dentin with 1 mg/mL of DAP and placebo paste.

Comparisons after 4 weeks of treatment: (Figure 21)

Bacteria counts were significantly lower for 5 mg/mL DAP, 50 mg/mL DAP, and 500 mg/mL DAP than for 1 mg/mL DAP, $Ca(OH)_2$, and MC ($p=0.0001$). Additionally, 5

mg/mL DAP, 50 mg/mL DAP, and 500 mg/mL DAP were not significantly different from each other ($p=1.00$). Furthermore, 1 mg/mL DAP, MC, and $Ca(OH)_2$ were not significantly different from each other ($p=0.052$ for 1 mg/mL DAP vs $Ca(OH)_2$, $p=0.86$ for 1 mg/mL DAP vs MC, and $p=0.09$ for $Ca(OH)_2$ vs MC). Four weeks of pretreatment of dentin with 500, 50, or 5 mg/mL of DAP demonstrated significantly higher residual antibacterial effects in comparison to 4 weeks of dentin pretreatment with 1 mg/mL of DAP, $Ca(OH)_2$ or placebo paste ($p<0.001$). Furthermore, no significant differences in residual antibacterial effect were detected in dentin pretreated for 4 weeks with placebo paste, $Ca(OH)_2$, or 1 mg/mL of DAP.

Comparisons of 1 week vs 4 weeks: (Figure 22)

Bacterial counts were significantly higher at 1 week than at 4 weeks for 1 mg/mL DAP ($p=0.0134$), 5 mg/mL DAP ($p=0.0001$), 50 mg/mL DAP ($p=0.0001$), 500 mg/mL ($p=0.0001$), and MC ($p=0.0341$). Bacterial counts were significantly lower at 1 week than at 4 weeks for $Ca(OH)_2$ ($p=0.0062$). Four weeks of dentin pretreatment with 5, 50 and 500 mg/mL of DAP demonstrated significantly higher residual antibacterial effects and complete eradication of biofilm in comparison to a 1 week pretreatment of dentin with the same concentrations of DAP ($p<0.01$) (Figure 24). Four weeks of dentin pretreatment with 1 mg/mL of DAP or placebo paste had a significant but limited residual antibacterial effect in comparison to a 1 week pretreatment with the same pastes ($p<0.05$). However, dentin pretreated with $Ca(OH)_2$ for 4 weeks had significantly higher biofilm formation in comparison to those that received 1 week of pretreatment with $Ca(OH)_2$ ($p<0.01$).

FIGURES

FIGURE 1. Experimental design flow chart.

FIGURE 2. The high-speed saw with water irrigation.

FIGURE 3. Overview of specimen preparation: Each tooth was sectioned, cut to 4x4 mm, and the inner surface was flattened.

FIGURE 4. Each half-root was cut into a 4x4x1-mm dentine sample with a double-bladed low-speed saw with water.

FIGURE 5. The low-speed double bladed saw used with water cooling.

FIGURE 6. Dentin specimens placed on cylinders with wax preparing for smoothing and polishing.

FIGURE 7. This unit was used to flatten, smooth and polish the pulpal surface of the dentin specimens.

FIGURE 8. The abrasive papers used to smooth and polish the pulpal side of the dentin specimens.

FIGURE 9. Sterile dentin specimen individually wrapped with sterile gauze saturated with sterile water, placed in Whirl-pak

FIGURE 10. Example of polished, sterilized dentin specimen removed from packaging.

FIGURE 11. Ventilated hood where dentine unpackaging and treatment with medicaments took place.

FIGURE 12. Material and used in dentin treatment inside ventilated hood

FIGURE 13. All dentin specimens were treated for 1 or 4 weeks at 37°C with 100% humidity.

FIGURE 14. A sterile broth of brain heart infusion (BHI) supplemented with 5 g of yeast extract/L (BHI-YE) was inoculated with colonies of *E. faecalis* and incubated at 37°C for 24 hours.

FIGURE 15. Dentin samples being sonicated and vortexed to detach biofilm cells.

FIGURE 16. Spiral plating of the dilutions of the detached biofilm cells from the treated dentin samples.

FIGURE 17. Incubation of spirally plated blood agar plates for 24 hours

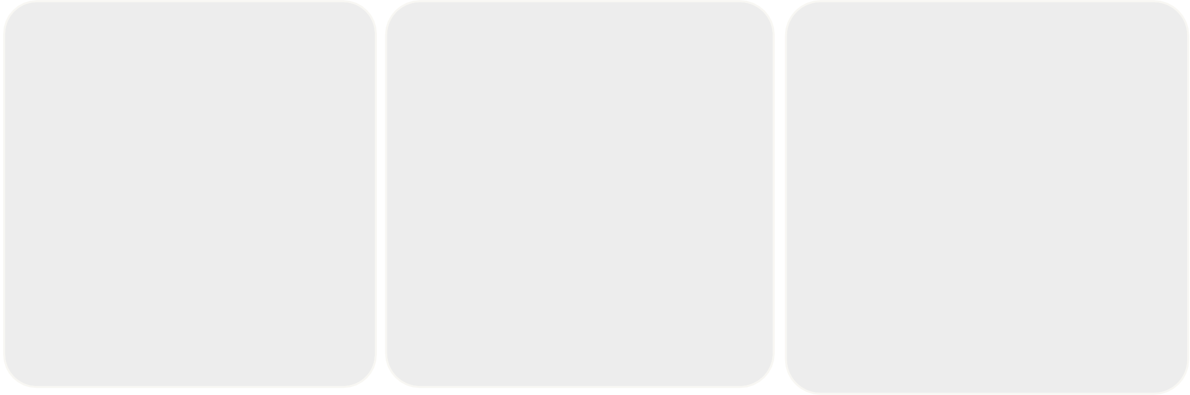

FIGURE 18. Blood agar plates incubated for 24 hours incubation after spiral plating.

FIGURE 19. Blood agar plate placed in automated colony counter after 24 hour incubation period.

FIGURE 20. The residual antibacterial effect of dentin treated for one week represented as the mean (±SD) of the log CFU/mL. Different letters represent statistical significance between different treatments.

FIGURE 21. The residual antibacterial effect of dentin treated for four weeks represented as the mean (±SD) of the log CFU/mL. Different letters represent statistical significance between different treatments.

FIGURE 22. The residual antibacterial effect of dentin treated with different concentrations of DAP represented as the mean (±SD) of the log CFU/mL. Different upper case letters represent statistical significance between different treatments within the same application time. Different lower case letters represent statistical significance between similar treatments within the two application times.

DISCUSSION

The use of DAP as an intracanal medicament was first reported in a clinical case that used contemporary principles of endodontic regeneration. [46] However, DAP has been emerging lately as the antibiotic medicament of choice in endodontic regeneration due to its significant antibacterial properties against different endodontic pathogens,[48 112] as well as its minimum tooth discoloration potential in comparison to TAP.[113]

The current study indicates that the application time of DAP plays a significant role in determining residual antibacterial properties of dentin as only 4 weeks of dentin pretreatment with 3 of the tested DAP concentrations (5, 50, and 500 mg/mL) completely prevented biofilm colonization on dentin. The antibacterial effect of antibiotics is usually achieved during the reproductive cycle of bacterial cells.[114] Therefore, a relatively long contact time between antibiotics and bacteria is required to maximize the beneficial use of antibiotics. It is also worth noting that both components of DAP, metronidazole and ciprofloxacin, are classified as concentration dependent antibiotics rather than time dependent antibiotics. However, it appears that longer contact time between DAP and dentin can increase the amount of DAP bonded/adsorbed to dentin and improve the residual antibacterial effect of dentin after DAP removal. A recent study found that complete DAP removal from root canal dentin is challenging even with activation of the irrigation solution such as with the EndoActivator system. [115] Other studies have also shown that DAP application significantly reduces the push out bond strength between the root canal and various root cements,[116, 117] which indicates the presence of a residual interaction between DAP and dentin. However, all previously mentioned studies have

used the clinically used DAP concentration (500-1000 mg/mL) to create a paste consistency. The current study indicates that 4 weeks of dentin pretreatment with 5 mg/ml of DAP, 100-200 times less than the clinically used concentration, can maintain a residual antibacterial effect for several weeks.

This study demonstrated that only 50 and 500 mg/mL of DAP treatment for 1 week were able to provide a significant and substantial residual antibacterial effect (3 $\log_{10}$ reduction in CFU/mL). These findings suggest that in the case of short term (1 week) dentin treatment with DAP, antibiotic concentration may play a significant role in the residual antibacterial properties. Our study also demonstrated that treatment for 1 week with 1 or 5 mg/mL of DAP did not provide a substantial residual antibacterial effect (only 0.05- 0.5 $\log_{10}$ reduction). On the other hand, a recent study has indicated that treatment for 3 weeks with 1 mg/mL of DAP in liquid form provided 2 weeks of residual antibacterial effect.[111] However, the previous study used liquid forms of 1 mg/mL of DAP against a 3 day old biofilm and did not provide final irrigation with EDTA after DAP removal [111]. In the current study, low concentrations of DAP were loaded into a methylcellulose vehicle system to create a clinically applicable antibiotic medicament. Furthermore, the bacterial biofilm was allowed to grow for 3 weeks after samples were immersed in PBS for 3 weeks (a total of 6 weeks after DAP removal) in order to investigate the residual antibacterial effects for longer and more challenging in vitro conditions. Additionally, final EDTA irrigation was performed after DAP removal to simulate the actual clinical scenario during endodontic regeneration.

A recent study proposed that a 1 week application of 1 mg/mL of DAP loaded into an aqueous methylcellulose system was efficient in eliminating a substantial amount

of 3-week-old *E. faecalis* biofilm.[109] All things considered, it seems that 1 mg/mL can provide a direct antibiofilm effect.[109, 110] However, a longer treatment time and/or higher concentration of DAP may be required to obtain an extended indirect residual antibacterial effect. In cases of immature teeth with pulpal necrosis, the clinical manifestations and the extent of preoperative infection should always be considered before determining application time and concentration of DAP as an interappointment medicament. Furthermore, the use of low concentrations of DAP rather than the currently used higher concentrations (500-1000 mg/mL) should be advocated to minimize the adverse effects of DAP on stem cells[41, 100, 107] and dentin/root structure. [118, 119]

One of the interesting findings of this study is that treatment with $Ca(OH)_2$ did not demonstrate any notable residual antibacterial effect regardless of the treatment time. The antimicrobial properties of $Ca(OH)_2$ are mainly achieved through the release of large amounts of hydroxyl ions that raise the pH in the local environment and leads to denaturation of bacterial proteins as well as destruction of microbial cytoplasmic membranes.[120] Once $Ca(OH)_2$ is removed, the buffering capacity of dentin is expected to neutralize the acidic pH in the root canal environment and extenuate the antibacterial effect of $Ca(OH)_2$. Therefore, $Ca(OH)_2$ is not expected to have any substantial residual antibacterial effect. It is well documented that both $Ca(OH)_2$ and antibiotic pastes have direct antibiofilm effects.[50, 109, 111] However, the decision to use $Ca(OH)_2$ or DAP during endodontic regeneration should be based on the need of residual and extended antibacterial properties within the root canal system after the removal of the intracanal medicament.

The lower concentrations of DAP (1, 5, and 50 mg/mL) in this study were applied via a methylcellulose vehicle to the dentin specimens. Methylcellulose was selected as the vehicle due to the non-cytotoxic potential of the material. [49] The methylcellulose allowed for DAP in these concentrations to be applied in a thick, sticky consistency which may have allowed for more of the antibiotic to adhere to the dentin for a longer period of time. This could have played a role in allowing for an increase in the residual antibacterial property of DAP. Further investigations comparing similar concentrations of DAP with and without methylcellulose could be done to determine if methylcellulose altered the residual antibacterial properties.

The elimination of minocycline from TAP to form DAP has been advantageous in REP due to the benefit of similar antibiotic properties without the staining component. In the current study we also noted no discoloration of the dentin samples after they had been treated with the various concentrations of DAP. In a previous study by Sabrah et al., DAP was compared to TAP and $Ca(OH)_2$ against newly formed biofilms of common endodontic pathogens (*E. faecalis* and *Porphorymonas gingivalis*).[50] The study found that DAP performed equally as well as TAP and better than $Ca(OH)_2$. The authors report that “DAP can be considered an effective and comparable antibacterial substitute to TAP without the discoloration associated with the latter medicament”[50] This is an important benefit clinically as many immature teeth with pulpal necrosis are often associate with maxillary anterior teeth and as such are in the more esthetic areas of the mouth.

Among the limitations of this *in vitro* study is the limitation of the use of only a single species (*E. faecalis*) biofilm rather than a multi-species biofilm. A multispecies biofilm would be more representative of the actual clinical situation. However, recent

studies found that both 3 week old *E. faecalis* biofilm and a 3 week old multispecies biofilm retrieved from human dental plaque were more resistant to disinfectants used in endodontics.[121, 122] Further studies should aim to investigate the residual antibacterial effects of DAP against multi-species biofilm as well as clinically isolated biofilms.

SUMMARY AND CONCLUSIONS

Our null hypothesis which stated that DAP exerts similar residual antibacterial effects regardless of the concentration used or application time was rejected. Treatment with 1 mg/mL of DAP or the clinically used $Ca(OH)_2$ did not provide substantial residual antibacterial effects regardless of the application time. However, four weeks of dentin treatment with 5, 50 or 500 mg/mL of DAP provided significantly higher residual antibacterial effects and complete prevention of *E. faecalis* biofilms in comparison to a 1 week treatment with the same concentrations.

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ABSTRACT

THE EFFECT OF CONCENTRATION AND TREATMENT TIME ON THE RESIDUAL ANTIBACTERIAL PROPERTIES OF DAP IN METHYLCELLULOSE

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Introduction: Regenerative endodontic procedures are used to treat immature teeth with pulpal necrosis in order to control infection, enable continued root development and enhance formation of a pulp like tissue in the canal. Canal disinfection is an integral part the regenerative endodontic process. Double antibiotic paste (DAP; i.e., equal parts of ciprofloxacin and metronidazole) has been successfully used for canal disinfection in regenerative endodontics. A comparison of the residual antibacterial effect of dentin treated with various dilutions of DAP pastes on biofilm formation has not yet been investigated thoroughly.

Objectives: The aims of this in-vitro study were to investigate how concentration and time of treatment affect the residual antibacterial properties of DAP in preventing *E. faecalis* biofilm formation on human dentin.

Materials and Methods: Extracted human teeth were used to obtain 4x4mm radicular dentin specimens. Each specimen was pretreated for 1 or 4 weeks with the

clinically used concentration of DAP (500 mg/mL), low concentrations of DAP (1, 5 or 50 mg/mL) loaded into a methylcellulose system, calcium hydroxide ($Ca(OH)_2$), or placebo paste. After treatment, samples were rinsed and placed in sterile phosphate buffered saline (PBS) for three weeks. Samples were then inoculated with cultured *E. faecalis* and incubated in anaerobic conditions for three weeks to allow mature biofilm formation. The dentin samples were rinsed and biofilms detached. The detached biofilm cells were then diluted and spirally plated for enumeration on blood agar plates. The plates were then incubated for 24 h and the number of CFUs/mL was determined using an automated colony counter. Data was analyzed using Fisher's Exact and Wilcoxon rank sum tests were used for statistical comparisons (α=0.05).

Results: Dentin pretreatment for 4 weeks with 5, 50 or 500 mg/mL of DAP demonstrated significantly higher residual antibacterial effects and complete eradication of *E. faecalis* biofilms in comparison to a 1 week pretreatment with similar concentrations. However, dentin pretreated with 1 mg/mL of DAP or $Ca(OH)_2$ did not provide a substantial residual antibacterial effect regardless of the application time.

Conclusion: Dentin treated with 500, 50, or 5 mg/mL of DAP for 4 weeks was able to completely prevent the colonization of bacterial biofilm. Four-week treatment of dentin with DAP offers superior residual antibacterial effect in comparison to a one-week treatment. Intracanal application of DAP for 4 weeks during endodontic regeneration may offer an extended residual antibacterial effect.

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