THE ANTIBACTERIAL EFFECT OF NEW INTRACANAL MEDICAMENTS AGAINST ESTABLISHED MULTISPECIES BIOFILM

by

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INTRODUCTION

During the past few decades, the treatment of non-vital immature teeth has shifted from an apexification procedure to a novel approach resulting in the regeneration of pulp-like tissue and continued development of the root width and length. This has been termed regenerative endodontics. [1] The protocol for this procedure relies mainly on various disinfection techniques to render the pulp system aseptic. It includes the use of root canal irrigation solutions and intracanal medicaments to create an aseptic environment. [2, 3] In order for pulp-like tissue to be regenerated in the pulp system, the apical papillae must be lacerated with a hand file. This not only induces bleeding and subsequent clot formation, but it also supports the ingrowth of stem cells. [4, 5] These steps create a scaffold where an accumulation of undifferentiated stem cells can organize within the canal system and cause regeneration of pulp-like tissues in necrotic immature teeth. [6]

Previously used procedures for necrotic teeth with immature apices have included apexification with various obturation materials. These include calcium hydroxide or MTA which can be used to create an apical plug while also inducing hard tissue formation. [7-9] These teeth are left with undeveloped roots and thin walls, which significantly reduce their long-term prognosis. [10, 11] Although these issues have made the treatment of necrotic teeth with immature apices problematic, new regenerative endodontic procedures (REPs) have been able to address these issues. Unlike apexification procedures, teeth treated with REPs have been shown to develop their radicular dentin in length and thickness to mature form. [12-14]

Regenerative success is dependent on key factors—disinfection, stem cells, scaffolds, and growth factors. [15] The current recommended technique by the American Association of Endodontists consists of canal disinfection followed by induction of bleeding in that canal. The disinfection of the root canal system in REPs has included irrigants and medicaments used previously in endodontics as well as novel medicaments. These include sodium hypochlorite (NaOCl), calcium hydroxide, triple antibiotic paste (TAP), and double antibiotic paste (DAP).

NaOCl has been a gold standard irrigant in endodontics, due to its ability to disinfect as well as dissolve tissue. [16] However, its use in REPs is abbreviated due to its cytotoxicity to stem cells and decreased stem cell attachment. [17, 18] For this reason, recommendations suggest its use in lower concentrations of 0.1 mg/mL to 1.0 mg/mL in the first appointment only. Calcium hydroxide has been classically used as an inter-appointment medicament in endodontics. Although calcium hydroxide's alkalinity has been shown to inactivate lipopolysaccharide, other medicaments have been shown to have greater utility and effectiveness in REPs than calcium hydroxide. [19-22]

An array of antibiotic pastes has been used in regenerative endodontics including various types and concentrations of antibiotics. The most popular is triple antibiotic paste (TAP), a mixture of ciprofloxacin, metronidazole, and minocycline, and has been effective at removing microbes on radicular dentin. [2, 3, 23] Triple antibiotic paste, however, is not without its weaknesses. These include its tendency to discolor teeth, demineralize dentin, and create a cytotoxic environment for stem cells. [24-26] Double antibiotic paste (DAP) has also been used successfully for clinical asepsis and regeneration of necrotic immature teeth.[27] DAP uses ciprofloxacin and metronidazole,

but unlike TAP does not include minocycline. Although this allows DAP to prevent staining, it can still be cytotoxic to stem cells at high concentrations. [28]

There have been several studies in which DAP was used as the antibiotic medicament for regenerative therapy. One study used a mixture of ciprofloxacin and metronidazole as a medicament in a case of an immature tooth with a necrotic pulp. They placed DAP into the canal system and monitored the tooth over several months. Their 30-month follow-up radiograph revealed closure of the apex and thickening of dentinal walls. [27] Nevins and Cymerman also used a mixture of ciprofloxacin and metronidazole in four clinical regenerative cases, in which they found significant reduction in symptoms and size of periapical radiolucencies. They also concluded that omitting minocycline from the antibiotic paste did not adversely affect tissue repair. [29]

Further studies on DAP have compared it to TAP and calcium hydroxide against common endodontic pathogens such as *E. faecalis* and *P. gingivalis*. They not only found that DAP disinfected the canal system equally as TAP, but also that DAP did not discolor the teeth as TAP typically does. [30] It has also been found that 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, and 10 mg/mL dilutions of DAP have an antibacterial effect against *E. faecalis*, but the 0.125 mg/mL dilution of DAP had the additional effect of being the least cytotoxic to dental pulp stem cells. [31, 32]

Previous studies by Sabrah et al. have explored the various qualities of DAP in relation to a particular bacterial species, commonly *E. faecalis*; yet this is unlikely to resemble the actual microbiota found in a clinical case. [31] Tzanetakis et al., performed a recent study using pyrosequencing to evaluate the bacterial composition of primary and recurrent endodontic infections. [33] The bacterial phyla found in primary endodontic

infections, from most abundant to least, are Bacteroidetes, Firmicutes, Actinobacteria, Synergistetes, Fusobacteria, Proteobacteria, Spirochaetes, Tenericutes, and include 43 individually identified genera. To the best of our knowledge, no study has yet been performed on the efficacy of DAP against multispecies biofilm. Subsequently in this study, we plan to evaluate the antibacterial effect of DAP against two multispecies biofilms. Mature and immature permanent teeth with necrotic pulps have been found to have similar profiles of microbiota. [34] Understanding how DAP works against a biofilm similar to that found in an immature tooth with a necrotic pulp would allow clinicians and researchers to better understand how DAP affects microbial biofilms, and could help lead to more effective endodontic regeneration protocols.

OBJECTIVES

Specific Aims

- To investigate the antibacterial effect of various concentrations of DAP on radicular dentin infected with clinically isolated multispecies biofilms from immature and mature teeth.

Hypotheses

- Null: All tested concentrations of DAP will not have an antibacterial effect against established multispecies biofilm regardless of the source of biofilm.
- Alternative: All tested concentrations of DAP will have significant antibacterial effects against both clinically isolated biofilms.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Dentistry has an intricate history dating back to several ancient cultures. The treatment of oral disease has classically been managed via the removal and replacement of teeth. It wasn't until the 18th century when the profession's paradigm shifted to the treatment of oral disease and maintenance of compromised teeth. Pierre Fouchard emerged as a pioneer in this regard, and is considered the father of modern dentistry. [35] In his 1728 book *The Surgeon Dentist,* Fouchard describes many procedures that foreshadow many modern concepts of dentistry and endodontics. One description illustrated a process of creating a hole in the pulpal space in an effort to drain any potential infection housed by the hard tissue of the tooth. This could be done on a short-term basis or for months at a time. After proper drainage occurred, the access was "sealed" with lead foil as a type of elementary obturation material. [35, 36] Fouchard continues to describe dental procedures that include the removal of the dental pulp with a rudimentary instrument, as well the application cloves to extensive carious lesions. [35, 37] These procedures reflect, in a very basic way, the modern procedures of pulp extirpation and pulp capping, respectively.

Other pioneers of dentistry in the 18th century described further endodontic procedures. Phillip Pfaff attempted to maintain the vitality of the pulp via capping the pulp with metallic materials. [35] Codman also was able to accurately describe the clinical aim of pulp capping, as well as identify and describe the function of secondary dentin in relation to pulp exposures. [38]

Robert Woofendale was able to complete the first endodontic procedure in the US in the 1700s. He performed a procedure similar to a pulpotomy in order to diminish odontogenic pain by using a heated instrument to effectively burn and cauterize the pulp, followed by the application of cotton pellets. [39] Further advances were proposed later by Frederick Hirsch, who began to evaluate the periapex via percussive tests. The conclusion was that those teeth that exhibited odontogenic pain tended to also be painful to those same percussive tests. His treatment methodology was very similar to that of Woofendale's. It included the application of a hot instrument into the pulp, thereby cauterizing it. [37]

Beginning in the 1800s, the dental community began to realize the importance of pulp vitality and the treatment modalities to preserve it. This movement was known as the "The Vitalistic Era." [35] Charles Bew was able to attempt to define pulpal circulation. It included blood flow into the pulp through the apical foramen, which then exits through the periodontal membrane. [40] In an early text by Leonard Koecker, *Principles of Dental Surgery,* he posited that the necrosis of a dental pulp would result in necrosis of the remaining dental tissues, resulting in a foreign body. [40] With the aim to prevent the removal of dental tissues as well as foreign body reaction, Koecker and Pfaff suggested a similar pulp capping therapy. [38, 40]

Shortly thereafter, the *System of Dental Surgery* was published by S.S. Fitch, who described and championed his vitalistic theory. [35] He posited that the entire tooth was vital, and all tissues required a blood supply. The pulpal blood supply was vital to the crown of the tooth, and the pulpal as well as periodontal blood supply was vital to the root of the tooth. It was from this ideology that led to decoronation therapy, a procedure

in which a tooth whose pulp had been extirpated also had its crown removed. The crown was later restored on top of the root. This can be compared with the "nonvitalisic" theory. Conversely, this theory posits that the enamel and dentin, once mature, lack any circulation; therefore, they also lack the abilities of normally perfused tissues such as pain perception or healing abilities. If one were to accept this theory, extirpation of the pulp and its blood supply would not compromise the integrity of the remaining tooth structure. [40]

Shortly thereafter, several new medicaments would emerge in the field of endodontics. Those first medicaments aimed to alleviate the pain that was typically a hallmark of vital pulp extirpation at the time. One of the first practitioners to attempt this was Shearjashub Spooner, who in 1836 used arsenic trioxide to devitalize the pulp in preparation for vital pulpectomy, a technique which has roots in historical Chinese medicine. [41] This treatment became popular due to its ability to relieve pain and remained popular until the 1920s. [42] Formocresol, a fixative agent initially used by John P. Buckley, became very popular in the 1940s and is still used to some extent in modern times. [43] Others such as Jacob and Joseph Linderer advocated the use of essential or narcotic oil over pulp exposures. [44]

In the same century several new methods of root canal sealing and filling were created. This initial development came from Edward Hudson, who in 1809 began packing root canals with gold foil. [41] This same concept was echoed by Baker. He filled root canals with gold foil but also described cleaning of the root canal in conjunction with the obturation in the 1839 *American Journal of Dental Science*. This publication is known as one of the first works to expand on the central ideas of pulp extirpation, canal cleaning,

and canal filling. [35] In the middle of the 19th century another mode of root canal filling was utilizing plugs of beechwood that was impregnated with creosote. [36]

It wasn't until shortly thereafter, 1867, that the contemporary root canal filling material of choice was introduced, gutta percha, by Dr. G. A. Bowman. [36] Clarke Dubuqe was able to expand on this idea by utilizing an obturation technique including heated gutta percha.[35] At the end of the 1800s Dr. Bowman expanded on his idea of obturating with gutta percha. He began using a solution of chloroform to soften the gutta percha point in an effort to adapt it to the apical anatomy. This became known as chlorapercha. [41]

Many of the contemporary endodontic instruments have a design based on historical used endodontic armamentarium. Edwin Maynard is credited as developing the first pulpal extirpation instrument. This consisted of a twisted watch spring, resembling a modern broach. A New York dentist, Sanford Barnum, began utilizing rubber dams during gold foil applications for the purpose of isolation.[36] The rubber dam would later become a contemporary staple of endodontic therapy and is currently part of the standard of care. This is due to its ability to prevent leakage of saliva and maintain an aseptic environment during endodontic therapy.

As the endodontic community began to realize the role microorganisms play in endodontic disease, the importance of an aseptic working field would become paramount. Even as early as 1878, Dr. G. O. Rodgers commented that odontogenic disease may be the result of a microbial insult in his 1878 *Dental Cosmos* article. It would then be logical that the endodontic disease would not be remedied until that microbial insult was

eradicated. [45] This central theory was continued by Arthur Underwood, who attempted to eradicate microbes from root canal systems by employing modern antiseptics. [35]

The turn of the 20th century became a pivotal time for dentistry and endodontics, as revolutionary advances in technology were introduced. The first of these initial advances would become a hallmark of dentistry for the next half century, Novocaine. Novocaine or Procaine was developed in 1905 and allowed clinicians superior anesthetic efficacy. These drugs combined with block anesthesia developed in the 1920s resulted in a greatly increased anesthetic technique. [42, 46] Another pivotal advance in dental technology was the application of X-radiation to dental and endodontic diagnosis and procedure. Although it was first introduced in 1913, the dental x-ray unit wasn't mass marketed until 1919. This was because dental x-rays required a focused beam, which wasn't achieved until the development of the Coolidge tube. [47] Understandably, this revolutionized dentistry as not only caries but periapical radiolucencies could be visualized radiographically. This allowed clinicians at the time to begin to see the connection between pulpal and periapical disease. [48] Specifically to endodontics, radiography allowed a more intricate understanding of the root canal system via pre-operative and working radiographs. Using this new technology endodontic diagnosis and procedure became more exact, comprehensive, and efficacious. [43]

The "Focal Theory of Infection" was introduced at the beginning of the 20th century and brought the practice of endodontics into question. In part this is because the theory suggests that micro-organisms that cause odontogenic infection can disseminate into bodily tissues and cause various systemic diseases. [49] This theory was championed by British physician William Hunter of McGill University. [47, 49] He published a

lecture entitled “The Role of Sepsis and Antisepsis in Medicine,” in which he stated, “gold fillings, gold caps, gold bridges, gold crowns, fixed dentures, built in, on, and around diseased teeth, form a veritable mausoleum of gold over a mass of sepsis to which there is no parallel in the whole realm of medicine or surgery.” [50] This ideology reinforced physicians of the time to recommend removal of endodontically treated teeth, non-vital teeth, and compromised yet restorable teeth. Some physicians would even recommend prophylactic removal of all teeth in order to prevent systemic disease. [49] This relationship between oral infection and systemic disease wouldn’t be discredited until the 1930s and 1940s. These decades, known then as “the scientific era,” was a time of several dental advances, and these weakened the foundation of the focal infection ideology which began to lose ground in the medical and dental community. [47]

One redefining advance in endodontic medicaments came from Hermann, who began using basic calcium hydroxide for both vital therapy and as an interappointment medicament in the 1920s and 1930s. He believed that the previously used endodontic materials became absorbed by the periradicular tissues; and, therefore, a more biocompatible medicament should be utilized. For Hermann, calcium hydroxide represented this more biocompatible medicament, which also found to aid in dentin bridge formation. [47]

It was during this time that advances in obturation materials began progressing. A natural way to improve obturation was to utilize cement. U.G. Rickert was the first practitioner to do this by coating a gutta percha cone with sealer before inserting it into the canal. Subsequent improvements were made to this basic obturation procedure by the

advent of instruments to laterally condense the gutta percha as well as lentulo spirals to help apply medicaments and sealer into the canals. [47]

Following the advent of the first antibiotic, penicillin, dentists began utilizing this medication for odontogenic infections. It was first used as an adjunct to root canal therapy in the 1940s by Adams and Grossman. [47] Grossman was more concerned with utilizing the drug in a local rather than systemic application. He suggested applying penicillin directly into the infected canals via a non-aqueous carrier as well as paper points that were impregnated with the drug. [41] This was an important event in endodontics as it allowed practitioners to envision treating infected root canals chemo-therapeutically. Previous thinking posited that the physical debridement of the root canal space rendered the system aseptic. However, neither suspended antibiotics nor other medicaments can completely render a canal system aseptic. It was this thought process by dentists such as Adams and Grossman that gave rise to the idea of chemo-mechanical root canal preparation, which would later become a hallmark of endodontics. [51]

The formation of the American Association of Endodontics in 1943 spearheaded the movement to organize endodontics nationally. Almost a decade later, that same governing body was able to form the American Board of Endodontics in 1956. [52] Despite the hard work of the American Association of Endodontists, its governing members, and the endodontic community, endodontics wasn't recognized as a specialty by the American Dental Association until 1963. The vote at the annual session that year would forever change endodontics; and just two short years later the first diplomats were certified. [47]

THEORY OF ENDODONTICS

One of the most influential studies conducted in the field of endodontics was that of Kakehashi, Stanley, and Fitzgerald in 1965. Their study became the foundation upon which endodontics built its basis of endodontic disease pathogenesis. The experiment showed that pulps of germ-free rats, when exposed and left open remained vital. Despite trauma from food impaction and exposure to the oral cavity, the pulp did not necrose and periapical tissues remained healthy. Rats under identical conditions that were not germ free showed pulp necrosis and periapical pathosis. [53] The main conclusion that the endodontic community was able to garner from this study was that bacteria are responsible for pulpal and periapical disease. This deduction would drive endodontic therapy for the next half century.

Using those deductions from Kakehashi, et al., the primary goal for endodontic therapy became the eradication of microorganisms, their toxic byproducts, and the media upon which they live from the root canals system. [54, 55] This is achieved not only through cleaning and shaping of the root canal system, but also the application of chemical antimicrobial medicaments. [54, 55] Endodontic pathogens found within the canal space of an infected tooth cause periapical and periradicular inflammation by emerging from the apical foramen, lateral canals, and accessory canals. [56] When endodontic therapy is unable to eradicate those endodontic pathogens from the root canal system, the result is apical periodontitis, inflammation of the periapical tissues. [57] Endodontic success is therefore directly correlated to the reduction of pathogenic microorganisms. [53, 58]

In 1955 Stewart emphasized three major phases of endodontic treatment: chemomechanical preparation, microbial eradication, and obturation of the root canal. [59] Both Stewart and Grossman identified the process of chemomechanical preparation as the most integral step of endodontic therapy because it involves the eradication of microbes from the root canal system. Grossman also identified the following 13 principles of effective root canal therapy:

1. Aseptic technique.
2. Instruments should remain within the root canal.
3. Instruments should never be forced apically.
4. Canal space must be enlarged from its original size.
5. Root canal system should be continuously irrigated with an antiseptic
6. Solutions should remain within the canal space.
7. Fistulas do not require special treatment.
8. A negative culture should be obtained before obturation of the root canal.
9. A hermetic seal of the root canal system should be obtained.
10. Obturation material should not be irrigating to the periapical tissues.
11. If an acute alveolar abscess is present, proper drainage must be established.
12. Injections into infectious areas should be avoided.
13. Apical surgery may be required to promote healing of the pulpless tooth.

Schilder, a pioneer in the endodontic community, expanded upon the idea of root canal therapy objectives. He posited in 1967 that successful endodontic therapy requires the removal of necrotic tissue and the contents responsible for the inflammation of the

periapical tissues. He not only believed in the chemomechanical cleaning and shaping of the root canal system, but also proper obturation of that system. Schilder's obturation technique involved a "three-dimensional" obturation in which increments of heated gutta percha were sequentially placed and condensed. This allowed a homogenous obturation of gutta percha from the cementoenamel junction to the cementodentinal junction. [60] Ford echoed the efficacy of Schilder's three-dimensional obturation technique, and expanded on the basic principal via three concepts. Firstly, it allows less space for bacterial colonization. Second, a three-dimensional obturation prevents apical contamination. Third, it prevents apical migration of bacteria along the periphery of the canal. Ford not only stressed the importance of an adequate obturation but also the necessity of an aseptic technique. Following the conclusions of Kakehashi et al., preventing recontamination of the root canal space with a proper aseptic technique became paramount. This includes the use of rubber dams, adequate coronal restoration, and appropriate recall of endodontically treated teeth. [61] Adequate debridement of the canal to reduce bacterial load, three dimensional obturation to seal the root canal system, adequate coronal restoration to prevent coronal leakage, and appropriate maintenance are all important factors for successful endodontic therapy.

APEXOGENESIS

Apexogensis is a procedure performed on vital immature teeth. Typically these teeth have a pulpitis secondary to either caries or trauma. The primary goal of apexogenesis is to preserve the vital tissue found within the dental pulp with the hope that primary radicular dentin will continue to form. [62] The clinical procedure consists of a shallow or full pulpotomy to remove inflamed pulp tissue, followed by a pulp dressing

and restoration. [63] The amount of tissue removal depends upon the gross size of the pulp exposure as well as the time between the initial pulpal insult and treatment. Historically, calcium hydroxide has been the medicament of choice when considering apexogenesis as pulp therapy. It renders the tissue aseptic, is biocompatible, and stimulates tissue formation. However, some disadvantages include incomplete dentin formation as well as pulpal inflammation due to its basic pH. The advent of MTA allowed more predictable results compared with calcium hydroxide when using an apexogenesis protocol. Rather than forming an incomplete and irregular reparative dentin layer, MTA forms a complete reparative dentin layer and does not induce pulpal inflammation. [64] However, MTA is more expensive than calcium hydroxide, can take up to 24 hours to set, and can discolor the dentition as well. [65] Additional materials have recently come to market such as Biodentine and other MTA derivatives, which are used in a similar manner to MTA in apexogenesis. Once the inflamed pulp tissue is removed, hemorrhage is controlled, and asepsis is achieved; MTA is placed in direct contact with the vital pulp tissue followed by a coronal restoration is placed to insure an adequate seal. The patient is recalled in order to monitor for symptoms and radiographic root development. Apexogenesis remains the treatment of choice for immature teeth with vital pulps needing endodontic therapy. Alternative treatment modalities for immature teeth with necrotic pulps include apexification and regenerative endodontic procedures.

APEXIFICATION

Apexification is a treatment modality first introduced in the 1960s in order to treat immature teeth with necrotic pulps. This is due to the difficulties found in obturating an immature tooth with an open apex. Apexification involved treating the tooth with long

term calcium hydroxide in order to create a calcified barrier at the apex of the tooth, which acts as a matrix against which one can condense their obturation materials. [9] The clinical procedure consists of accessing the tooth under rubber dam isolation followed by radiographic working length determination. Disinfection is achieved through mostly irrigation, with minimal mechanical instrumentation due to the existing large canal and thin friable dentinal walls. Calcium hydroxide is applied to the canal in intervals of 3 months until a radiographic dentinal barrier is formed. Once this barrier has formed, the canal is obturated either with MTA or gutta percha. Total treatment time typically lasts from 9 to 24 months. [62]

Although the apexification preferably results in the apical closure of the root canal system, that closure is typically a cementoid material with small remnant communications with the periapex. Furthermore, apexification does not result in the addition root length or thickness [9, 66]. As aforementioned, this treatment modality stretches over long treatment periods, requiring disciplined patient compliance, which can become an issue, especially with the younger patient demographic typically requiring this therapy. Long-term calcium hydroxide treatment is not without its own risks and complications. It has been found to weaken the mineralized dental hard tissue, which increases root fracture risk. [67-70]

An alternative to apexification is the artificial apical barrier technique. This technique differs from apexification primarily by the mode of canal obturation. Once the tooth is asymptomatic following calcium hydroxide application, the canal is dried and packed with an apical plug that is 4-5mm in thickness. [71] By omitting the sequential calcium hydroxide applications, this treatment modality severely reduces treatment time.

This allows the tooth to be permanently restored much sooner allowing more immediate increases is fracture and leakage resistance. Furthermore, only one to two applications of calcium hydroxide allows the root dentin to retain more of its inherent strength. Success rates for this procedure have been reported from 85 percent to 93.5 percent. [72, 73] Despite its published success, this technique, like apexification, does not allow for increased dentin root thickness or length, and this inherent weakness continues to leave the tooth susceptible to fracture in the future.

HISTORY OF REGENERATIVE PROCEDURES

In 1961 Nygaard-Østby conducted clinical tests involving necrotic teeth obturated at least partially by patients' endogenous blood clot. In his study, seventeen patients underwent root canal therapy and foraminal enlargement, followed by a medicated dressing. Bleeding was stimulated in those same teeth followed by obturation with kloroperka immediately adjacent to the blood clot. Subsequently the teeth were extracted ranging from 17 days to 3.5 years, and were examined microscopically. Although he found some failures probably due to leakage, most teeth showed resolution of inflammation and, in some cases, radiographic evidence of apical closure. [74] Unknown to many at the time, this was a revolutionary study in endodontic regeneration, because it was the first to show patients' endogenous biologic tissue obturating a previously instrumented canal space. However this tissue was not pulp-like, included undesirable cells, and lacked desirable cells such as odontoblasts. [75]

Shortly later in 1966, investigators attempted to treat vital immature teeth by instrumenting short of the apical tissue. This was followed by application of a polyantibiotic mix intra-radicularly, and subsequent obturation. All reported cases

showed resolution of signs and symptoms of disease and continued root development. [76] This case report hallmarked the first attempt to disinfect the root canal system of immature teeth with antibiotics. [75]

In recent decades, case reports of continued root development following root canal therapy in immature teeth continue to emerge in published media. Commonly, these reports utilize a combination of antibiotic pastes in order to render a root canal system aseptic; and have, therefore, regenerated interest in utilizing antibiotics for that application. Iwaya published the first contemporary regenerative endodontic procedure, using a double antibiotic paste (DAP) composed of ciprofloxacin and metronidazole to disinfect an immature necrotic tooth. [27] Iwaya's protocol included chemical disinfection via 5.0-percent sodium hypochlorite and 3.0-percent hydrogen peroxide, with an application of DAP as an interappointment medicament. This treatment regimen spanned 6 visits and resulted in continued root development and positive vitality testing after 30 months. Following this reports, Banchs and Trope published a case report using triple antibiotic paste (TAP), composed of ciprofloxacin, minocycline, and metronidazole. This report outlined the treatment of a mandibular premolar using 5.25-percent sodium hypochlorite without instrumentation and TAP application for 28 days [2]. After the antibiotic paste was removed, intracanal bleeding was stimulated, and a restoration placed coronal to the blood clot. Banchs and Trope were able to achieve resolution of periapical inflammation, continued root development, as well as positive vitality tests. Hallmark features in these early case reports were immature apices, young age of the patient, minimal instrumentation, sodium hypochlorite irrigation, calcium hydroxide or TAP interappointment medication, and the formation of a blood clot as a

scaffold. [1] The publication of these early case reports formed the bedrock upon which current regenerative endodontic procedures' methodology is based.

There are various goals of regenerative endodontic procedures—to heal apical pathology, allow continued root thickness and length, and regain pulpal vitality and function. [77] Regenerated pulp tissue should ideally have the following properties: vascularity, innervation, similar cell density and architecture, and the formation of new odontoblasts. [78] However, the body's ability to regenerate this tissue in the face of active microbial insult is impossible. Therefore, pulpal regeneration requires that the canal must first be disinfected in order to provide an environment conducive to host tissue growth. [79] Following canal disinfection three keystone requirements for regeneration must be satisfied—stem cells, a scaffold, and growth factors. [80]

DISINFECTION

Apical periodontitis is a direct result of a microbial infection of the canal system of a tooth with pulpal necrosis. The spectrum of this microbial environment is one of both anaerobic gram-negative and gram-positive bacteria.The predominant gram-negative bacteria are *Fusobacterium, Dialister, Porphyromonas, Prevotella, Tannerella, Treponema, Campylobacter* and *Veillonella;* and predominant gram-positive bacteria are *Parvimonas, Fillifactor, Pseudoramibacter, Olsenella, Actinomyces, Peptostreptococcus, Streptococcus, Propionibacterium* and *Eubacterium*. [81] Furthermore, Nagata, et. al. found the *Actinomyces naeslundii* was the predominant species in primary endodontic infection of immature permanent teeth. [34] The mode by which these microorganisms live within and adhere to the tooth structure is a biofilm, a complex structure composed of several species of microorganisms and their byproducts. [82]

As with many dental infections, the composition of the microbial profile changes with regard to the location of the biofilm as well as the stage of the infection. These factors affect nutrient availability, host defense mechanisms, and oxygen saturation; which in turn affects the microflora composition. [83] Initially, the infection involves facultative bacteria due to the presence of oxygen. Shorty thereafter, oxygen becomes limited due to limited blood flow and the microbial profile shifts to predominantly anaerobic bacteria.

Historically, the goal of endodontic therapy with regard to an immature tooth with pulpal necrosis is the resolution of apical inflammation. This inflammation is reduced by means of eliminating the microbial infection via chemical disinfection with irrigants and medicaments. The most common interappointment medicament used in clinical case reports is TAP, composed of ciprofloxacin, metronidazole, and minocycline. [75] In 1996 this combination was shown to be effective against bacteria found in infected root canals *in-vitro* and *in-vivo.* [3, 23]

However, the minocycline component of TAP severely stains the dental hard tissue. [26] This becomes problematic especially in anterior teeth in the esthetic zone, in which immature teeth are typically traumatized. In an effort to reduce or eliminate this adverse effect of minocycline, recent studies suggested substituting the minocycline with another antibiotic, such as amoxicillin, cefaclor, or clindamycin. [75, 84] Clindamycin has been found to be effective against various endodontic pathogens. [85, 86] For that reason, a modified triple antibiotic paste (MTAP) composed of metronidazole, ciprofloxacin and clindamycin was suggested. It was successfully used as an intracanal medicament to disinfect immature teeth with pulpal necrosis during endodontic regeneration procedures. [84]

Routinely, practitioners mix these antibiotic powders in a suspension of saline chair side until a paste-like consistency is achieved, and this tends to result in an antibiotic concentration of about 1 g/mL. [28] However, this concentration has been found to have deleterious effects on both human stem cells of the apical papilla [28, 87] and human dental pulp cells. [88] In an effort to reduce the cytotoxic effects of the antibiotic pastes, concentrations ranging from 0.1 mg/mL to 2mg/mL have been suggested. [28, 87, 88] In addition to the effects found on endogenous cells, high concentrations of antibiotic pastes were found to reduce mechanical mechanical [89] and chemical properties [90] of radicular dentin *in vitro*.

Canal disinfection is achieved not only by interappointment medicaments but also irrigation solutions. In tandem with mechanical instrumentation, sodium hypochlorite has a greater effect than saline alone on intracanal microorganisms. [91] Sodium hypochlorite has been found to kill gram-negative anaerobic rods typically found in apical periodontitis within fifteen seconds. [92] Despite this, current irrigant solutions have difficulty killing *E. faecalis;* Estrela et al. concluded that NaOCl and CHX are not effective in completely eliminating *E. faecalis* within the complex anatomy of the root canal system. Despite *E. faecalis'* tenacity, sodium hypochlorite remains an important endodontic irrigation solution due to its ability to kill microbes and dissolve organic tissue.

Sodium hypochlorite's efficaciousness is affected by several inherent characteristics including concentration and temperature. Sodium hypochlorite's antimicrobial and tissue dissolution capabilities are increased at both higher concentrations and higher temperatures. [93, 94] More specifically, the bactericidal rate

for sodium hypochlorite is doubled for every increase of 5°C in temperature. [95] Commonly used concentrations of sodium hypochlorite such as 5.0-percent to 6.0-percent solutions have been found to be both safe and efficacious for root canal therapy. [96] However, when considering irrigation solutions used in regenerative endodontic procedures, different considerations must be measured. One must first consider the natural anatomy of the immature tooth. Its foremost difference to mature teeth is the open blunderbuss apex. This puts the immature tooth at greater risk of periapical irrigant extrusion. Furthermore, care must be taken to preserve the viability of any remaining stem cells of the apical papilla. Essner showed that higher concentrations of sodium hypochlorite resulted in greater cytotoxicity to stem cells. [17] Additionally, sodium hypochlorite reduces the differentiation potential of stem cells of the apical papilla by affecting the radicular dentin. [97] The American Association of Endodontics has considered the published data regarding sodium hypochlorite and recommends a concentration of 1.5 percent when using for regenerative endodontic applications. [98]

Ethylenediaminetetraacetic acid (EDTA) is a chemical compound used to chelate ionic compounds; in dentistry it is used to remove the smear layer in cavity preps and instrumented root canals. [99] Dentin shavings during cavity preparations and endodontic instrumentation become matted and pushed into the dentinal tubules—the accumulation of this creates a smear layer. This smear layer has been found to inhibit the seal of obturation materials in endodontic therapy; and its removal by EDTA has been shown to improve the seal. [100] EDTA requires a one-minute intra-radicular rinse to remove the smear layer. [62]

Rather than smear layer removal, EDTA is utilized in regenerative endodontics to allow release of growth factors and cell survival. Trevino performed endodontic studies to determine the cytotoxicity of various irrigants. The least cytotoxicity was found in those teeth irrigated only with 17-percent EDTA. The group irrigated with 6.0-percent sodium hypochlorite, 17-percent EDTA, 6.0-percent sodium hypochlorite had slightly more cytotoxicity. Two-percent chlorhexidine was found to be the most cytotoxic, with no remaining sustainable cells. [101] Accordingly most regenerative endodontic procedure protocols omit the use of chlorhexidine in order to preserve the viability of apical stem cells. EDTA also allows adherence of newly mineralized tissue to canal dentin, and releases growth factors from radicular dentin involved in SCAP differentiation. [77, 102]

STEM CELLS

Stem cells are those found within organisms that possess the inherent ability to differentiate into different cell phenotypes. Those that are multipotent are able to differentiate into specific and limited cell lines; those that are pluripotent, like embryonic stem cells, are able to differentiate into any cell line. Stem cells are further divided into sub-categories based on their sources, autologous, allogeneic, and xenogeneic. Autologous stem cells are derived from the organism in which those cells are to be utilized. Allogeneic stem cells are derived from the same species of organism in which the cells are to be used. Conversely, xenogeneic stem cells are those derived from another species altogether.

During regenerative endodontic procedures, local autologous stem cells are used to regenerate pulp like tissue rather than stem cells, which migrate from other areas of the

body. [6] These local stem cells are multipotent. They have the capacity to differentiate into various cells found within the dental pulp but cannot differentiate into *any* cell type. [103] These stem cells found within the dentino-aveolar complex are classified as dental pulp stem cells (DPSCs), [103] stem cells from human exfoliated deciduous teeth (SHEDs), [104] periodontal ligament stem cells (PDLSCs), [105] dental follicle progenitor stem cells (DFPCs), [106] and stem cells from apical papilla (SCAPs), [107, 108]. Dental pulp stem cells are found near the cell rich zone, adjacent to the odontoblastic layer. [62] These cells have the capacity to differentiate into mature odontoblasts following exposure to growth factors released from radicular dentin. For this reason regenerative research has focused on this potential. [109] Stem cells of the apical papilla, or SCAP, have also shown promise with regenerative endodontic procedures partially due to their ability to produce dentin. [108, 110]

SCAFFOLD

The purpose of a scaffold in regenerative endodontic procedures is multifactorial. It allows an environment for cell growth, stem cell differentiation, as well as the formation of new vasculature. [77] The idea of a scaffold in regenerative endodontics first emerged as a collagen gel scaffold in 1976 by Nevins. [111] Shortly after, the blood clot from a lacerated apical papilla was found to have functions of a regenerative scaffold. [112] This was a comparative *in-vivo* study comparing the revascularization potential of a blood clot, a collagen scaffold, and the combination of the two. The conclusions of the study were that a blood clot alone was found to be more efficacious in pulpal regeneration than a collagen scaffold only. Therefore, the author concluded that an

ideal scaffold provides not only a physical latticework for cell growth, but also endogenous growth factors.

Hutmacher [113] identified the following six properties of an ideal scaffold for tissue regeneration:

1. Porous structure for tissue and vascular integration
2. Biodegradable at a rate of tissue formation
3. Allow cellular attachment for differentiation and proliferation
4. The mechanical properties of the site being implanted must be adequate
5. Does not elicit any adverse reactions
6. Easily formed into different sizes and shapes

The main scaffold that has been used clinically for regenerative endodontic procedures is the blood clot from a lacerated apical papilla. [77] However, several researchers have studied scaffold alternatives, including the use of platelet-rich plasma for the addition of endogenous growth factors. [4, 101, 114]

GROWTH FACTORS

Growth factor is a broad term for a group of endogenous molecules that serve to promote growth, healing, and maturation. In the dento-alveolar complex, these growth factors serve as an important signaling molecule in the pulp tissue regeneration cascade. Evidence suggests they are entombed within the dentinal matrix following normal physiologic dentinogenesis. [115] Several important growth factors in pulp regeneration include: transforming growth factor beta (TGF-*B*), bone morphogenic protein (BMP), and vascular endothelial growth factor (VEGF). TGF-*B* serves to induce odontoblast differentiation, pulp tissue mineralization, wound healing, and anti-inflammatory

signaling. BMP promotes odontoblast differentiation. VEGF regulates angiogenesis by acting as a chemotactic agent for cells critical to vascular growth. Though we know these cell signaling pathways are an essential component to pulpal regeneration, further research needs to be attempted in order to understand how they can be utilized clinically to promote pulpal regeneration.

CLINICAL DECISION-MAKING

Several factors must be considered when making clinical decisions regarding the management of an immature permanent tooth with pulpal necrosis—these include outcome data, patient desires, patient compliance, and dentinal thickness, to name a few. Studies show favorable success rates for both calcium hydroxide and MTA apexification at 74 percent, 100 percent and 90 percent, respectively. [72, 116, 117] A recent retrospective study by Jeeruphan, et al compared outcomes of 22 calcium hydroxide apexification cases, 19 MTA apexification cases, and 20 revascularization cases. [12] It found survival rates of 100 percent for teeth treated with pulpal regeneration, 95 percent for teeth treated with MTA apexification, and 77.2 percent for teeth treated with calcium hydroxide apexification. It is important to note that even if regeneration of pulp-like tissue did not occur, retention of the immature tooth was considered a success. Furthermore, the revascularization group averaged a significantly greater increase in both root length and thickness compared to the other two groups. This thickening of the dentinal walls decreases the risk of root and crown fracture in these compromised teeth. Additionally, long-term use of calcium hydroxide has been connected to increased risk of fracture. [67, 69] In fact, Jeeruphan noted root fractures in 23 percent of the teeth treated with calcium hydroxide apexification. [12]

Although regenerative endodontic procedures has demonstrated great interest in the dental community and shown great promise, the quality of published evidence regarding the subject needs to be strengthened. Many clinical publications are case reports, and there are no published randomized clinical trials or meta-analyses on the subject. Case reports may initially stimulate interest in the area; but they do not have the capacity to analyze data, from which one can make deductions regarding treatment protocol. [75] Additionally, case reports skew perceived success rates because journals publish only successful cases. Although there is poor and at times conflicting evidence regarding regenerative endodontic procedures, the potential benefit and seeming lack of adverse effects continues to drive clinicians to utilize it as a treatment modality.
The AAE echoed this sentiment by compiling and analyzing the available data regarding regenerative endodontic procedures. This resulted in their most recent document of recommendations and guidelines for regenerative endodontic procedures. [118] These as well as other publications help to guide clinicians as they continue to provide regenerative procedures to their patients.

MATERIALS AND METHODS

COLLECTION OF CLINICAL ISOLATES

This study was approved by the local institutional review board (IRB # 1510640949). Two subjects without any systemic disease who had not received antibiotics in the last 6 months were selected for the study and signed the informed consent and assent forms before collection of the bacterial isolates from their infected root canals. One of the subjects had an immature tooth with an infected root canal and a periapical lesion that was indicated for endodontic regeneration treatment. The other subject had a mature tooth with an infected root canal and a periapical lesion that was indicated for conventional root canal therapy. The clinical isolates were collected as described in a previous protocol. [119] The selected tooth was isolated with a rubber dam. The operative field was then cleansed with 3.0-percent hydrogen peroxide solution and disinfected with 6.0-percent NaOCl. The coronal root canal access was then performed using a sterile round bur. After that, the pulp chamber was disinfected using a swab soaked in 6.0-percent NaOCl and the residual NaOCl was inactivated with sterile 5.0-percent sodium thiosulfate. The bacterial isolate was collected from the infected root canal using a sterile #15 file with the handle cut off. The file was introduced 1 mm short of the apical foramen and a filing motion was performed for 30 seconds before taken out. After that, 3 sterile paper points were inserted into the root canal at the same working length and left inside for 1 minute in order to absorb the tissue fluid. Both the file and paper points were then placed into 5 mL of brain heart infusion broth supplemented with 5 g/L yeast extract and 5.0 percent (v/v) of vitamin k and hemin (BHI-YE), vortexed to

elute the bacteria, incubated anaerobically at 37˚C for 48 h and frozen at -80˚C until used. A visual summary of this and the remainder of the methodology are found in Figure 1.

DENTIN SAMPLE PREPARATION

Intact permanent human teeth were collected, stored in 0.1-percent solution, and used within 6 months. Inclusion criteria include: caries-free, complete root formation, and at least 4-mm microdot diameter in either buccolingual or mesiodistal direction. Exclusion criteria include: caries, restorations, hypocalcification, hypoplasia, cracks, incompletely formed roots, dentinogenesis or amelogenesis imperfect, and dental fluorosis. These findings will be identified visually. Standardized radicular dentin slabs (n = 112) were obtained from the collected teeth as described in previous studies. [120, 121] Briefly, 4×4×2 mm^3 slabs were obtained from each root using a low speed diamond saw under continuous water irrigation. The diagram found in Figure 2 summarizes this process. Both saws used to cut the dentin specimens are shown in Figures 3 and 4. Figures 5 and 6 show the product after sectioning and mounting on the polishing jig. Both sides of each slab were polished using a Roto Pol 31 polishing unit (Struers, Cleveland, OH). Figure 7 shows the polishing unit; Figure 8 shows polishing discs of increasing grit; Figure 9 shows a polished 4x4mm dentin specimen. The samples were sonicated using 1.5-percent NaOCl, distilled water and 17-percent EDTA for 4 minutes to remove the smear layer. Samples were independently sterilized with ethylene oxide, stored at 4ºC and used within 4 weeks. Figure 10 shows a sterilized and packaged dentin specimen.

PREPARATION OF INTRACANAL MEDICAMENTS

Low concentrations of DAP (1 and 5 mg/mL) loaded into methylcellulose hydrogels were prepared as described in recent publications. [120, 122] Ten mg and 50 mg of equal portions of metronidazole and ciprofloxacin (Champs Pharmacy, San Antonio, TX) were dissolved in 10 mL of sterile water to form 1and 5 mg/mL DAP solutions, respectively. Then, 0.8 g of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St. Louis, MO) was gradually incorporated into each DAP solution over 90 minutes under vigorous stirring to obtain a creamy injectable consistency of 1 and 5 mg/mL of DAP intracanal medicament. A DAP-free methylcellulose-based hydrogel and a commercial $Ca(OH)_2$ paste (UltraCal XS, Ultradent, South Jordan, UT) were also used as additional treatment groups in this study.

BACTERIAL GROWTH ON ROOT SPECIMENS

Sterile dentin slabs (n = 104) were independently placed into wells of sterile 96-well microtiter plates (FisherBrand, Fischer Scientific) with the pulpal sides oriented outward as shown in Figure 11. Half of the dentin slabs received a mixture of 190 µL of fresh BHI-YE and 10 µL of overnight culture (approximately 1×10^5 CFU/mL) of biofilm bacteria obtained from an immature tooth with pulpal necrosis (n = 52) as shown in Figure 12. The other half of the dentin slabs received a mixture of 190 µL of fresh BHI-YE and 10 µL of overnight culture (approximately 1×10^5 CFU/mL) of biofilm bacteria obtained from the mature tooth with pulpal necrosis (n = 52). All infected dentin slabs were incubated anaerobically at 37°C for 3 weeks and the growth media was replaced every 7 days. Weekly replacement of growth media was selected to limit the nutritional supply during the 3-week period of *in-vitro* biofilm formation in order to

maintain the original taxa of clinical isolates as much as possible. [123, 124] Uninfected sterile dentin slabs (negative control) were used in this experiment to confirm the absence of any external bacterial contamination though the course of the study (n = 10). The negative control dentin slabs received fresh BHI-YE (200 µL per sample) and incubated anaerobically for 3 weeks as described earlier with weekly replacement of BHI-YE.

CONFIRMATION OF POLYMICROBIAL BIOFILMS

After the 3 week incubation period, two random dentin slabs were selected from each type of biofilm and processed for scanning electron microscopy (JEOL 7800F; JEOL, Peabody, MA) as described in a previous study. [120]

DENTIN SPECIMEN TREATMENT

The remaining dentin slabs infected with each type of biofilm were transferred to new wells of sterile 96-well microtiter plates containing 50 µL of fresh BHI-YE and randomized into 5 experimental groups (n = 10 per group). Infected slabs received 100 µL of 1 mg/mL DAP, 5 mg/mL DAP, placebo paste, $Ca(OH)_2$, or no treatment positive control group (n = 10 for each type of biofilm). Figure 13 shows a beaker of DAP paste; Figure 14 shows the DAP paste placement, and Figure 15 shows calcium hydroxide placement. The uninfected dentin slabs in the negative control group received no treatment as well. All slabs were then incubated for 1 week at 37°C and 100-percent humidity in an anaerobic gas pack shown in Figure 16.

BIOFILM DISRUPTION ASSAYS

After that, samples were independently exposed to 5 mL of sterile water for 1 min under mild agitation to wash off the treatment pastes. All dentin slabs were subjected to

biofilm disruption assays as detailed in recent studies. [121, 125] Each dentin specimen was gently washed twice with sterile saline to remove the experimental paste and transferred to a new plastic test tube containing 1 ml of sterile saline. The tubes were sonicated for 20 seconds and vortexed for 30 seconds to detach biofilm cells. The detached biofilm cells were diluted 1:10 and 1:1000, shown in Figure 17, and spirally plated on blood agar plates (CDC, BioMerieux), as shown in Figure 18 and Figure 19. The plates were incubated for 48 h in 5.0-percent CO_2 at 37°C (an example of bacterial growth is shown in Figure 20) and the number of CFUs/mL determined by using an automated colony counter (Synbiosis, Inc., Frederick, MD), shown in Figure 21.

STATISTICAL ANALYSES

For the direct antibacterial effects, Wilcoxon Rank Sum tests were used to compare the effect of treatment and type of biofilm on bacterial counts. Pair-wise comparisons among the treatment combinations were performed using the Sidak method to control the overall significance level at 5-0-percent for both direct and residual antibacterial effects.

RESULTS

BIOFILM VALIDATION

The scanning electron microscopic images confirm the presence of a polymicrobial biofilm. The biofilm structure is thick and varied, with multiple species evident on the surface of the dentin. Figure 22 shows a scanning electron micrograph of the biofilm obtained from a mature tooth with pulpal necrosis. Figure 23 shows a scanning electron micrograph of the biofilm obtained from an immature tooth with pulpal necrosis. These bacteria were grown on dentin slabs for three weeks and exhibit a polymorphic heterogeneity. Numerous cocci and rod-shaped bacteria are evident in both the immature and mature biofilm samples. These microscopic images confirm the polymicrobial nature of the biofilm used to inoculate the dentin slabs.

DIRECT ANTIBACTERIAL EFFECTS OF VARIOUS TREATMENTS

The tested biofilm sample groups treated with 5 mg/ml of DAP, 1 mg/mL of DAP and calcium hydroxide exhibited a significant and statistically relevant antimicrobial effect when compared to both the control groups as well as the placebo paste group ($p < 0.008$). The mean values of the tested groups for the immature biofilm are as follows: 5.37 for the control group, 5.77 for the placebo group, 1.55 for the 1mg/mL DAP, 0.00 for the 5 mg/mL DAP, and 0.00 for the calcium hydroxide group. The mean values of the tested groups for the mature biofilm are as follows: 4.60 for the control group, 4.51 for the placebo group, 0.00 for the 1mg/mL DAP, 0.00 for the 5 mg/mL DAP, and 0.00 for the calcium hydroxide group. These data are summarized in Table 1. Additionally,

no significant differences were found between the biofilm groups and the tested groups of 5 mg/ml of DAP, 1 mg/mL of DAP and $Ca(OH)_2$. Both 5 mg/mL of DAP and $Ca(OH)_2$ caused total obliteration of the bacterial biofilms obtained from both mature and immature teeth. Although 1 mg/mL of DAP caused complete eradication of bacterial biofilm obtained from the mature tooth with pulp necrosis, the 1 mg/mL DAP was not able to completely eradicate biofilm obtained from the immature tooth with pulpal necrosis. Despite the difference between the eradication of biofilms obtained from both mature and immature teeth, the differences were not statistically significant. Bacterial biofilms obtained from the immature tooth were significantly more prevalent than that obtained from the mature tooth in untreated dentin and dentin treated with placebo paste ($p < 0.005$).

FIGURES AND TABLE

FIGURE 1. Flowchart of experimental methodology.

FIGURE 2. Roots (A) were sectioned, cut longitudinally (C), and polished flat on the pulpal side (D).

FIGURE 3. Saw used to initially section whole teeth.

Figure 4: Low Speed saw used to section teeth into 4x4mm samples.

Figure 5: Example of properly sectioned tooth.

FIGURE 6. The 4x4-mm dentin samples mounted on polishing jig.

FIGURE 7. Dentin polishing unit.

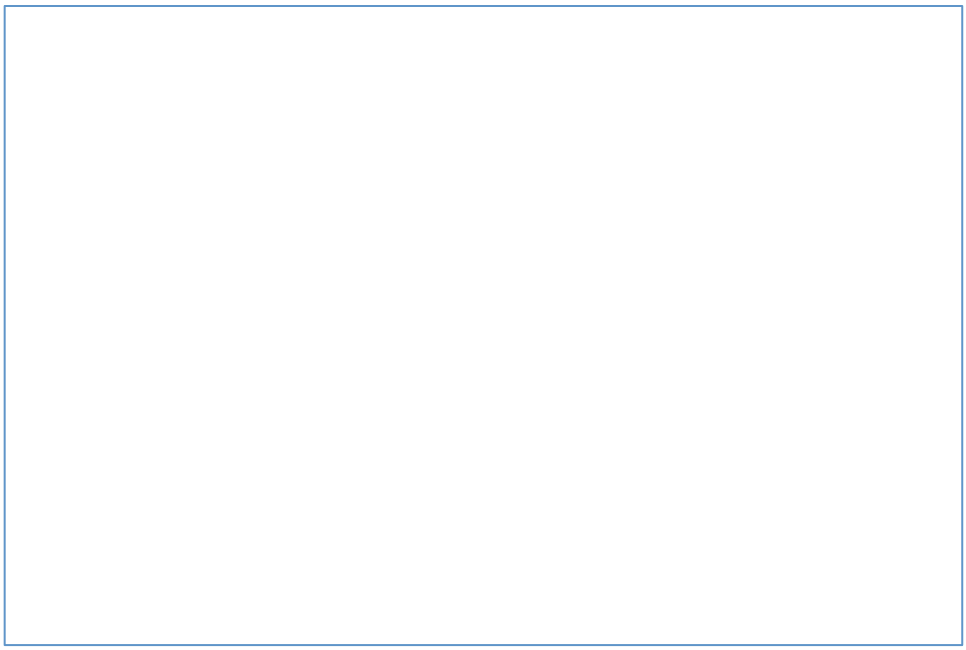

FIGURE 8. Polishing discs.

FIGURE 9. Example of polished 4x4-mm dentin sample.

FIGURE 10. Example of sterilized 4x4-mm dentin sample.

FIGURE 11. Dentin specimens placed into sterile 96-well plate.

FIGURE 12. Dentin specimens with BHI-YE broth.

FIGURE 13. Example of 5mg/mL DAP treatment paste.

FIGURE 14. DAP paste placement.

FIGURE 15. Calcium hydroxide placement.

FIGURE 16. Specimen placement into anaerobic gas pack.

FIGURE 17. Dilution tubes for enumeration of biofilm bacteria.

FIGURE 18. Aspiration of bacterial sample into spiral plater.

FIGURE 19. Spiral plating of biofilm bacteria onto blood agar plates.

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FIGURE 20. Example of bacterial growth from mature biofilm treated with methylcellulose, 1:10 dilution.

FIGURE 21. Digital colony counter.

FIGURE 22. Scanning electron microscopic images of 3-week old bacterial biofilm formed by bacteria obtained from an infected root canal of a mature tooth with necrotic pulp.

FIGURE 23. Scanning electron microscopic images of 3-week old bacterial biofilm formed by bacteria obtained from an infected root canal of an immature tooth with necrotic pulp.

TABLE I

The direct antibiofilm effects of the different antimicrobials against bacterial biofilms from mature or immature teeth with pulpal necrosis represented as the mean (SE) of the log CFU/mL

Different upper case letters indicate significant differences between biofilms from mature and immature teeth within the same type of treatment. Different lower case letters indicate significant differences between the different types of treatment within each type of biofilm.

DISCUSSION

The premature removal of immature permanent teeth presents a crucial problem for young dental patients, namely the loss of dentition as well as the loss of alveolar bone adjacent to the tooth. Therefore, the maintenance of immature teeth remains an important treatment goal for dentists. Regenerative endodontic procedures remain a novel approach to help maintain immature teeth with pulpal necrosis. These procedures aim for regeneration of pulp-like tissue and continued development of the root width and length. The nature of immature teeth – large canals, thin root walls, and blunderbuss apical foramen – severely limits mechanical instrumentation in regenerative endodontic procedures. Despite this difficulty, disinfection remains a paramount component of the regenerative protocol, without which pulpal regeneration cannot occur. Therefore, in these cases chemical disinfection plays a crucial role in reducing the microbial load within the necrotic canal space prior to regeneration.

Regenerative success itself is dependent on several key factors—disinfection, stem cells, scaffolds, and growth factors. [15] Of these, disinfection remains the foundation upon which remaining factors are built. The current recommended technique by the American Association of Endodontists consists of canal disinfection followed by induction of bleeding in that canal. Several antibiotic pastes have been used in regenerative endodontics including various types and concentrations of antibiotics. Although TAP paste historically has been most popular, DAP paste does not include the staining compound minocycline.

In the literature, there are many *in-vitro* studies focusing on lower concentrations of antibiotic mixtures. These studies test low concentrations and whether they are able to achieve adequate canal disinfection. [120, 126, 127] The challenge with antibiotic paste concentrations is obtaining the proper balance of canal disinfection, residual antimicrobial efficacy, and cytotoxicity to stem cells. Generally, higher concentrations of antibiotic paste result in a greater residual and direct antimicrobial effect, as well as a greater cytotoxic effect to the stem cells of the apical papillae.

This study showed that 1 mg/mol, 5 mg/mL, and calcium hydroxide provided a significant direct antimicrobial effect against both bacterial biofilms used in this study (from an immature tooth with pulpal necrosis and another from a mature tooth with pulpal necrosis). Indeed, the study showed tested groups with complete obliteration of microbial activity other than the 1 mg/mL DAP against the biofilm from the immature tooth with pulpal necrosis. To date, the American Association of Endodontists (AAE) recommends the use of $Ca(OH)_2$, 1 mg/mL of DAP, or 1 mg/mL of TAP as intracanal medicaments during endodontic regeneration. [118] As mentioned previously, there are issues with all of these medicaments: $Ca(OH)_2$ lacks residual antimicrobial activity; TAP tends to stain teeth due to the inclusion of minocycline, and DAP maintains some cytotoxic potential, among others. Indeed, preservation of stem cell viability is of great importance to regenerative endodontics, without which increases in root length and thickness would not be feasible. With these concerns in mind, the AAE's medicament recommendation is a result from the evidence that 1 mg/mL of DAP and/or TAP did not cause significant cytotoxic effects against stem cells from apical papillae and dental pulp stem cells. [122, 128, 129] This current study demonstrated that a low concentration of 1

mg/mL DAP was effective in killing bacteria from a three-week old biofilm cultured from two infected root canals, one from an mature tooth with pulpal necrosis, and the other from an immature tooth with pulpal necrosis. This finding generally agrees with previous studies' findings, which posited that 1mg/mL of DAP yielded a significant reduction in a three week old bacterial biofilm of *Enterococcus faecalis*. [120] Despite those findings, this study found that 1mg/mL of DAP was unable to completely eradicate a three week old biofilm obtained from an immature tooth with pulpal necrosis. Despite the differences in antimicrobial efficacy between biofilm sources, the differences were not statistically relevant. However, anecdotally this could indicate that the biofilm found in immature teeth may be more robust than the previously tested *E. faecalis* biofilms; and 1mg/mL DAP may prove to be an insufficient concentration to eradicate biofilms found in immature teeth with pulpal necrosis.

Indeed, the results found in this study regarding the increased resistance of biofilms found in immature teeth echo the findings of previous studies concerning the antimicrobial efficacy of NaOCl. Those studies found that infected root canals of immature teeth were more resistant to root canal therapy than mature teeth regardless of the NaOCl concentration used to disinfect the root canals. [130] However, the results of this *in vivo* study are likely due to the inadequacy of mechanical debridement of immature teeth rather than the higher virulence of the biofilm. One way to translate the findings of our study is to posit that the bacterial pathogens and polymicrobial biofilms found in the infected root canal of an immature tooth are more robust and resistant than those found in mature teeth. Several studies have been performed to catalogue the various microbial flora found in both immature and mature teeth with pulpal necrosis.

One study conducted recently identifies two species found most often in immature teeth with pulpal necrosis – *Actinomyces naeslundii* and *Porphyromonas endodontalis.* [34] It is possible that the prevalence of these species in infected pulps of immature teeth contribute to the resistance of these bacterial biofilms when compared to those of mature teeth, and could be less sensitivity to DAP than other species. In contrast, the *Fusobacterium* and *Prevotella* species were found to be two of the most prevalent bacteria in infected mature teeth with pulpal necrosis. [131]

Another important aspect of this study was the inclusion of methylcellulose as a delivery vehicle in the DAP paste formulations. Historically, clinicians would hand mix powders of various antibiotics, typically metronidazole, ciprofloxacin, and minocycline, until they obtained the desired consistency. The main drawback to this technique is the unknown concentrations of the antibiotic mixtures, which often times would be much greater than the lower concentrations we now use today. As aforementioned, these high concentrations are problematic when considering the preservation of stem cells of the apical papillae. Our purpose for the inclusion of methylcellulose in our antibiotic preparation is to create a paste-like consistency while still maintaining low concentrations of the antibiotics. Using this method we rely on the methylcellulose rather than the antibiotic powders themselves to achieve the proper body required to adequately and evenly coat the internal canal walls of an immature tooth with pulpal necrosis. The data in this study show that the methylcellulose gel did not have any antimicrobial capacity, and it did not diminish the antimicrobial efficacy of the DAP mixtures. Despite lack of antimicrobial complications, we must consider the ramifications of the methylcellulose gel on the viability of the stem cells of the apical papillae. One study conducted recently

concluded that 1 mg/mL of TAP loaded into methylcellulose hydrogel did not have negative effects on the attachment and proliferation of dental pulp stem cells. [122] Although these results are promising considering the lack of detrimental interaction between methylcellulose and stem cells of the apical papillae, additional studies are needed to explore the effects of dentin pretreated with these DAP/methylcellulose mixtures on the proliferation and differentiation of various types of dental stem cells.

This study confirms that $Ca(OH)_2$ is an efficacious antimicrobial agent against biofilms obtained from both immature and mature teeth with pulpal necrosis. However, one must not only consider the direct antimicrobial activity but also the residual antimicrobial activity of $Ca(OH)_2$. Although $Ca(OH)_2$ has an adequate direct antibacterial effect, one study concluded its residual antimicrobial effect on dentin is limited [121]. Ongoing studies continue to be conducted in order to determine the residual antimicrobial capacity $Ca(OH)_2$ in relation to antibiotic pastes.

In this study, we used a medicament application time of one week. This application time falls within the recommended time of 1 to 4 weeks, as suggested by the AAE clinical considerations for a regenerative procedure. Although a treatment time of 1 week is in the lower end of the suggested range, the duration of medicament placement must be considered alongside the clinical findings. Medicament placement can only be discontinued after the eradication of any signs and symptoms of a persistent infectious process. Although a treatment time of 1 week has been found to eliminate bacteria and is easily reproducible in the laboratory, other studies have suggested more specific and longer treatment times.[122] Jenks et al. found that a longer treatment time of 4 weeks with DAP concentrations of 5 mg/mL, 50 mg/mL, and 500 mg/mL exhibited a

significantly greater residual antibacterial effect against an *E. faecalis* biofilm when compared to a 1-week treatment time. Further studies may investigate the direct antibacterial effect of DAP concentrations against a multispecies biofilm with a greater treatment time of 4 weeks.

Most clinically used antibiotic pastes are created by mixing antibiotic powders with saline, and can be at concentrations of 1g/mL or more. At high concentrations, clinicians must consider the deleterious effects on the chemical, physical, and mechanical properties of dentin. In reference to the chemical properties, Yassen et al. published a paper in which spectrophotometric data suggests four weeks of treatment with high concentrations of DAP and TAP significantly decrease the inorganic component of dentin. Conversely, four weeks of treatment with calcium hydroxide degrades superficial collagen. [89] Additionally, Yassen et al. measured the weight percentages of elements in dentin after four weeks of medicament treatment via energy-dispersive x-ray analysis. They found that 50 mg/mL TAP had significantly lower amounts of calcium and phosphorus when compared to 1 mg/mL DAP and calcium hydroxide, which again suggests that higher concentrations of paste cause greater demineralization effect. [132]

In reference to the physical properties of dentin, the same paper by Yassen et al., shows a significantly higher contact angle and lower wettability of dentin treated with calcium hydroxide, 1mg/mL TAP, and 50mg/mL TAP. It also shows a significantly higher roughness value from a four-week treatment with 50 mg/mL TAP compared to all other treatment groups. [132] Using an optical and contact profilometer, Nerness et al. obtained data showing a significantly greater surface loss value and surface roughness value with 1 g/mL TAP when compared with 1mg/mL TAP. [133] Furthermore, Yassen

et al. performed another study using a point indentation apparatus to measure the hardness of dentin after a four week treatment with high concentrations of TAP and DAP. They found that the TAP and DAP paste significantly decreased the hardness compared to the calcium hydroxide and control group. [134]

In reference to the mechanical properties of dentin, Yassen et al. performed fracture resistance testing on dentin samples treated with high concentrations of TAP, DAP, and calcium hydroxide. They found that after 3 months of treatment, all treatment groups had significantly lower fracture strength when compared to the control group. The calcium hydroxide group had the lowest fracture resistance of all tested groups. [135] These findings must be taken into account when considering which medicament to use during regenerative endodontic procedures and especially at which concentration.

There are several limitations of this current study, one of which includes the methodology in obtaining the clinical isolates. These clinical isolates were obtained from single cases of mature or immature teeth with pulpal necrosis. For this reason, it is difficult to make conclusions in regard to differences between mature and immature teeth with pulpal necrosis. The heterogeneity observed in this study between the 2 types of biofilms might be related to the natural differences in microbial flora between the two infected root canals rather than the apical maturity. Past studies aiming to codify various species of microbes in infected root canals have found wide ranges in species prevalence from tooth to tooth. [136] Further studies are warranted to confirm the findings of the current study, and may do so by collecting more clinical bacterial isolates from mature and immature teeth with pulpal necrosis. Furthermore, additional studies comparing

treatment times of 1 week versus 4 weeks is warranted. It would also be beneficial to test more specific concentrations between 1mg/mL

SUMMARY AND CONCLUSIONS

In summary, the purpose of this study was to determine the most effective concentration of double antibiotic paste when being used to eradicate a clinically obtained multispecies biofilm. This goal was sought to further our understanding of appropriate treatment protocols when performing regenerative endodontic procedures. The results found in this study show that 1mg/mL of DAP, 5mg/mL of DAP, and $Ca(OH)_2$ displayed clinically significant antimicrobial effects against the biofilms used in this study obtained from a mature tooth with pulpal necrosis and from an immature tooth with pulpal necrosis. Thus, our null hypothesis, that all tested concentrations of DAP will not have an antibacterial effect against the multispecies biofilm regardless of the source of biofilm, has been rejected. Although we observed total bacterial eradication from all other testing groups and biofilm combinations, 1mg/mL DAP was unable to completely eradicate the biofilm obtained from the immature tooth. Therefore, it is plausible that a concentration higher than 1mg/mL DAP is needed to render the infected root canal space of an immature tooth aseptic.

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ABSTRACT

THE ANTIBACTERIAL EFFECT OF NEW INTRACANAL MEDICAMENTS AGAINST ESTABLISHED MULTISPECIES BIOFILM

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We investigated the antibacterial effect of low concentrations of double antibiotic paste (DAP) loaded into a methylcellulose system against bacterial biofilms obtained from mature and immature teeth with necrotic pulps. Standardized radicular dentin specimens were randomly divided into six experimental groups (n = 20). Group 1: 5mg/mL DAP treatment. Group 2: 1mg/mL DAP treatment. Group 3: Calcium hydroxide ($Ca(OH)_2$) treatment. Group 4: Methylcellulose. Group 5: No treatment. Group 6: No bacteria or treatment. Clinical bacterial isolates were obtained from mature and immature teeth with necrotic pulps indicated for endodontic regeneration or routine endodontic treatment, respectively. Specimens in each group were inoculated with either bacterial isolates (n = 10) and incubated anaerobically for 3 weeks. Specimens were then treated for one week with the assigned group treatment. Treatments were rinsed with sterile saline and biofilms were detached and spiral plated using biofilm disruption

assays. Wilcoxon Rank Sum tests followed by pair-wise comparisons were used for statistical analyses. Treatment of infected dentin with 1 mg/ml of DAP, 5 mg/mL of DAP, and $Ca(OH)_2$ demonstrated significant and substantial antibiofilm effects in comparison to untreated control groups or groups treated with placebo paste.

Furthermore, 1 mg/mL of DAP caused complete eradication of biofilm obtained from mature tooth with necrotic pulp. However, the same concentration was not able to completely eradicate biofilm obtained from the immature tooth with necrotic pulp. Low concentrations of DAP (1-5 mg/mL) loaded into a biocompatible methylcellulose system demonstrated significant antibacterial effects against biofilm obtained from both mature and immature teeth with necrotic pulps.

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Professional Organizations

American Association of Endodontists
American Dental Association
Indiana Dental Association