THE ANTIBACTERIAL EFFECT OF A RADIOPAQUE DOUBLE ANTIBIOTIC PASTE AGAINST BOTH AN ESTABLISHED MULTISPECIES AND A SINGLE *ENTEROCOCCUS FAECALIS* BIOFILM

by

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INTRODUCTION

Endodontic therapy of immature teeth with necrotic pulps has proven to be challenging. If a tooth is undergoing the early stages of tooth development and is traumatized, bacteria may be introduced into the root canal system leading to pulpal inflammation, pulpal necrosis and impaired tooth development.[1] This disruption of tooth development can halt the continued formation of the root leaving thin dentinal walls and an open apical foramen.[2] This becomes a challenging clinical situation regarding instrumentation, disinfection and obturation due to a lack of an apical barrier.[3]

Treatment modalities such as apexification have been used to treat immature teeth with a necrotic pulp. The aim of this procedure is to address incomplete apical closure by forming a calcified barrier across the apex of the immature root.[4,5] This barrier becomes a platform for obturation with gutta percha and decreases the likelihood of overextension of obturation materials.[3,6,7] In the past the medicament of choice for apexification procedures was long term use of calcium hydroxide. It has been suggested that calcium hydroxide leaves reduced organic support in the dentin structure reducing the flexural and fracture strength of the tooth.[2,8] Furthermore, these teeth can become a liability long term by being more prone to cervical root fracture and hindering their ability to be adequate abutments due to questionable crown to root ratio's.[2,9]

The field of endodontics has tried to address the possible shortcomings of long-term calcium hydroxide and its effects on dentin through the introduction of bioceramic products such as Mineral Trioxide Aggregate (MTA). MTA contains calcium silicates

which have been shown to promote cell differentiation, to have osteoconductive effects and excellent biocompatibility.[10] Orthograde placement of MTA as an apical plug appears promising and has demonstrated high success rates.[11]

Even with clinical success, the apexification procedure does not increase thickness or length of the arrested immature root with pulpal necrosis.[12] This ultimately increases the long-term risk of fracture of the stunted root.[8,9] Therefore, a treatment that could promote continued growth and development of the immature root with a necrotic pulp is needed.

Throughout the past few decades the field of endodontics has put great emphasis on the treatment of immature teeth with infected necrotic pulps through regenerative endodontic procedures (REP). The ultimate goals of regenerative endodontic therapy are 1) obtain resolution of the apical pathosis as well as restore functionality of the tissues associated within and around the tooth, 2) regenerate a pulp-dentin complex that enables continued root development for the immature tooth and 3) a positive response to electrical pulp tests.[13-14]

The regenerative process has been studied for decades. Nygarrd-Otsby observed during a revascularization study that inducing bleeding into the root canal system from the apical tissues leads to a blood coagulum within the canal that enhances the reparative process. Continued research has suggested that in order to regenerate pulp-like tissues the concepts of tissue engineering needed to be employed.[15] The three requirements needed in this process are a source of undifferentiated mesenchymal stem cells, a scaffold that will support and promote cell growth and differentiation, and growth factor signaling molecules capable of stimulating and directing cellular differentiation.[16-18] It has been

shown that evoked-bleeding from the manipulation of the periapical tissue will release stem cells into the canal system. The blood clot produces a scaffold that is biocompatible, nontoxic with potential physical and mechanical strength that promotes cell adhesion and migration.[14] Continued proliferation and differentiation are facilitated by the release of growth factors such as VEGF, EGF, and P1GF that are imbedded within dentin.[19] This release of growth factors from dentin can be facilitated by irrigating with ethylenediaminetetraacetic acid (EDTA)[20,21]. The American Association of Endodontists' (AAE) current guidelines emphasize the need for disinfection prior to initiating a blood clot into the canal system to enhance the regenerative results.[22]

This protocol leans heavily on various disinfection techniques such as different irrigation solutions and intracanal medicaments to create an aseptic environment foundational for regeneration of pulp-like tissues and continuation of root width and length.[22,23] *E. faecalis* has historically been a difficult bacterial species to disinfect in endodontic infections due to its many virulence factors and its ability to survive in the root canal system during long nutritional deprivation.[24] It has been shown that *E. faecalis* can survive in the root canal system as a single organism rather than a polymicrobial community[25] and is capable of forming its own biofilm in the presence of intracanal medicaments.[26] The most common disinfection agents recommended during REPs are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), calcium hydroxide ($Ca(OH)_2$), triple antibiotic paste (TAP), and double antibiotic paste (DAP).[27]

NaOCl has been used in endodontics for its adequate disinfection and tissue dissolving properties.[28] However, NaOCl has been shown to be cytotoxic to stem cells in higher concentrations and is therefore used in lower concentrations (1.5%) during REP.[29]

EDTA is also a common irrigant and can facilitate the release of important growth factors from the dentin by a chelating effect that can be utilized by stem cells to grow and differentiate. It executes this process by removing the inorganic contents of the dentinal smear layer. EDTA can reverse the harmful effects of NaOCl on stem cells.[20,21] EDTA-treated dentin can facilitate newly introduced stem cells to differentiate into odontoblast-like cells that have the ability to extend their processes into the dentin tubules.[19]

To further assist in the disinfection process of REPs three main intra-canal and inter-appointment medicaments have been studied and used. Traditionally calcium hydroxide ($Ca(OH)_2$) has been used as an intracanal medicament due to its ability to effectively eradicate bacteria. It was shown that $Ca(OH)_2$ efficiently eliminated bacteria from the canal following a 7-day inter-appointment dressing.[30,31] Its alkaline nature can cause LPS hydrolysis of the bacterial cell.[32] However, $Ca(OH)_2$ can negatively impact dentin structure by degradation of collagen as well as decrease flexural and fracture resistance.[8,30,33,36] Recently, research has focused on different intracanal antimicrobial pastes such as triple antibiotic paste (TAP) and double antibiotic paste (DAP).

TAP is a novel mixture of antibiotics ciprofloxacin, metronidazole and minocycline. Its use as an intracranial medicament has proven successful and predictable and has become a treatment modality in REP.[23,27] Although predictable, TAP has also been shown to demineralize dentin, cause stem cell cytotoxicity when used in higher concentrations, as well as discolor tooth structure due to its inclusion of minocycline. In an attempt to reduce the negative effects of TAP, DAP has been introduced.

Recent research has investigated the antibacterial efficacy of DAP containing metronidazole and ciprofloxacin against different bacteria.[38-41] DAP has been shown to

have an antibacterial effect against a biofilm obtained from bacterial isolates from human mature and immature teeth in concentrations of 1-5 mg of DAP. A concentration as low as 1.0 mg/mLand 5 mg/mL of DAP had significant direct antibacterial effects against a three-week-old bacterial biofilm.[42,43] Studies recently conducted have shown DAP to have residual antibacterial effect on treated dentin possibly enhancing regenerative endodontic result.[42-44] It has been shown the elimination of minocycline from the antibiotic paste did not adversely affect tissue repair nor its antibacterial activity.[45] DAP has addressed a major drawback of TAP in that it does not discolor the treated tooth.[46]

The application of DAP and other medicaments within the canal system must be in association with microorganisms to have predictable antibacterial effects.[47,48] Therefore, intracanal medicaments should have properties making them radiopaque to allow the endodontist to verify intraradicular placement of DAP and visualize its position in the canal on the radiograph. This would minimize under or over application of antibiotic preparations when used on immature teeth with open apices.

Current radiopacifiers used in dental products are barium, zirconium and bismuth, which are all insoluble salts of heavy metals. Both zirconium oxide and barium sulfate rendered DAP radiopaque after being added to the paste.[49] A recent pilot study at IUSD demonstrated significant antibacterial effects of 1.0-mg/mL radiopaque DAP, created with the addition of 30-percent barium sulfate, against a three-week-old bacterial biofilm. The radiopacifier zirconium oxide was found to have possible additional antibacterial properties by itself.[50] This added antibacterial effect may be beneficial for regenerative endodontic procedures because an effective antibacterial environment is needed to achieve a good regenerative endodontic result.[42,44] When 30-percent zirconium oxide is

added to DAP, it has a radiodensity similar to commercially available Ultracal, a commercially available calcium hydroxide medicament (Figure 22). Therefore, it is our aim to investigate the direct antibacterial properties of radiopaque DAP using zirconium oxide opacifier against a multispecies biofilm from a bacterial isolate from an infected immature tooth with a necrotic pulp and *E. faecalis* single species biofilm.

OBJECTIVE

The purpose of this project is to evaluate the antibacterial efficacy of 1.0-mg/mL and 10-mg/mL radiopaque double antibiotic paste on radicular dentin infected with a multispecies biofilm from an infected immature tooth with a necrotic pulp as well as a single species *E. faecalis* biofilm.

NULL HYPOTHESES

The null hypotheses are as follows:

- The 1.0-mg/mL and 10-mg/mL concentrations of radiopaque DAP will have no significant antibacterial effect on the formation of multispecies biofilms from bacterial isolates from an infected immature tooth with necrotic pulp.
- The 1.0-mg/mL and 10-mg/mL concentrations of radiopaque DAP will have no significant antibacterial effect on the formation of a single species *E. faecalis* biofilm.

ALTERNATIVE HYPOTHESES

The alternative hypotheses are as follows:

- The 1.0-mg/mL and 10-mg/mL concentrations of radiopaque DAP will demonstrate a significant antibacterial effect on the formation of multispecies biofilms from bacterial isolates from an infected immature tooth with necrotic pulp.
- The 1.0-mg/mL and 10-mg/mL concentrations of radiopaque DAP will demonstrate a significant antibacterial effect on the formation of a single species *E. faecalis* biofilm.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Dentistry has a deep enriched history of treating dental pain that amass several different ancient cultures and span multiple early civilizations dating back to 5,000 BC. Early accounts charged a "tooth worm" as the offending culprit causing tooth caries and oral periodontitis. The gnawing worm was held responsible for many evils, mainly for toothaches.[51] As the decades progressed, the theory of a gnawing worm causing toothaches began to mature as new discoveries by Anton Von Leeuwenhoek were made introducing the theory that microorganisms cause dental caries and lead to dental pain.

The father of modern dentistry, Pierre Fouchard added to Leeuwenhoek's pioneering work and additionally denounced the tooth worm theory. In *The Surgeon Dentist*, written in 1728, Fouchard links tooth decay to sugar consumption. His studies further influenced Willoughby Dayton Miller in the mid-1800s who discovered that dental caries are caused by oral bacteria who produce acid as a bioproduct following the consumption of fermentable sugars.[52] Fouchard's foundational work was a beginning effort in many modern day dental and endodontic clinical concepts, as if shifted the focus of removing and replacing diseased teeth to maintaining dentition through the treatment of oral disease. Fouchard's book described a process of draining a potential tooth infection by creating a hole in the pulpal space creating a pathway through which the infection could drain. Following a period short term period of days to weeks or even a long term period of months the pathway of drainage in the pulpal space was closed and sealed with lead foil. This rudimentary idea of treating an infection was the first

procedure of its kind to attempt to obturate root canals.[53,54] He continued his novel approaches in foundational endodontics as he detailed pulp extirpation and treatment of extensive caries by the use of small pins, cinnamon, and clove.

Later advancements in endodontics came in the late 18th century. Phillip Pfaff attempted to invent a pulp capping technique by placing metallic materials such as small pieces of lead or gold cut in sizes that could specifically cover the exposed vital pulp then carefully placed as a capping material. W.W. Codman described the aim in pulp capping procedures was to create a dentin bridge. He accurately identified and described the function of this dentin bridge as secondary dentin in relation to pulp exposures.[55]

The first endodontic procedure was successfully completed in the 18th century by Robert Woofendale. He described the procedure of treating odontogenic pain of a disease pulp by heating an instrument and placing on the pulp as a form of cauterization, followed by the application of cotton pellets.[56] He continued to promote products such as turpentine, opium, camphor, cinnamon, and clove in attempts to diminish and relieve odontogenic pain.[55] Further advancements came by Frederick Hirsch who was one of the first to evaluate the periapical tissues by using a percussion test. He concluded that people who responded positive to percussion testing on a given tooth tended to have odontogenic pain associated with the same tooth. His treatment mirrored Woofendale's in that he similarly advocated the use of hot instruments to cauterize pulpal tissue in an attempt to alleviate pain.[57]

As the 19th century began to surface the dental community started to understand the magnitude of maintaining pulp vitality and attempted to create appropriate and predicable treatment options. This was the beginning of ushering in "The Vitalistic

Era."[54] Pulpal circulation started to be defined by Charles Bew as the flow of blood entering in through the pulp through the apical foramen and finding its exist into the periodontal membrane.[58] In *Principles of Dental Surgery,* Leonard Koecker in 1826 hypothesized that the tooth following pulp removal would become a foreign body. He stated this was due to the further necrosis of remaining pulp tissue once pulp tissue was removed. Koecker and Pfaff suggested a similar pulp capping therapy with the purpose to prevent removal of dental tissues as well as foreign body reactions.[55,56]

Later in 1829, S.S. Fitch described in *System of Dental Surgery* his vitalistic theory that the entire tooth, in comparison to bone, was vital and all tissue of the tooth required a blood supply. He simply stated that the crown of the tooth received its blood supply from pulpal blood flow and the roots of the tooth received its blood supply from pulpal and periodontal blood flow. This hypothesis lead to the development of the decoronation procedure. This procedure was described as removing the crown of the tooth following pulpal extirpation while leaving the root of the tooth in the socket to be later restored. This theory was conversely argued by a "nonvitalistic" theory stating that enamel and dentin, once mature, lack circulation. Due to the lack of circulation these aspects of the tooth could not act like normal tissues with blood supplies and therefore could not sense pain and were incapable of healing themselves. This theory claimed and built on the idea that the integrity of the remaining tooth structure would be unaffected by the removal of pulpal tissue and blood supply.[58]

In decades to come the field of endodontics saw the emergence of several new medicaments aimed at treating pain associated with pulp extirpation which seemed to be unavoidable at the time. Shearjashub Spooner in 1836 dedicated his time as one of the

first practitioners to attempt to use arsenic trioxide to devitalize the pulp before a pulpectomy.[59] This ancient technique used in Chinese medicine became popular and carried itself into the 1900s due to its success in relieving pain during pulpectomies. John P Buckley in the 1940s pioneered the use of formocresol as a pulp fixation modality which as continued into some modern day use and practice.[60] The use of narcotic oils promoted by other practitioners such as Jacob and Joseph Linderer were further used to attempt to alleviate pulpal pain.[61]

New practices for filling and sealing the root canal space were introduced in the 19th century. Edward Hudson, a pioneer and champion in this era, began packing root canals in 1809 with cold foil.[59] Baker utilized, replicated and slightly altered this same concept. He published in the *American Journal of Dental Science* his attempts at cleaning the canal system prior to obturation with gold foil. He has been the first to be credited with fundamental ideas of pulp removal, cleaning, and filing of the canal space.[54] Later in the 1850s, beechwood saturated in creosote would be used as an obturation material.[53]

It was around this time in 1867 that Dr. G. A. Bowman introduced the contemporary root canal filling material of choice, gutta percha.[53] This idea was modified and expanded in a process of using heated gutta percha introduced by Clarke Dubuque.[54] Dr. Bowman's technique of using gutta percha was used for the rest of the 19th century. He later elaborated on his original idea and developed a modality to adapt the gutta percha to various apical anatomy. He introduced the use of chloroform to soften the gutta percha, later known as chlorapercha, in order to give gutta percha the ability to mold and adapt to nonuniform root canal anatomy.[59]

The 19th century was also a time in which other endodontic armamentarium such as files and the rubber dam were invented and put to clinical use. Edwin Maynard created a pulpal extirpation instrument, resembling the modern-day broach, by winding and twisting a spring from his watch. The rubber dam was invented in 1862 in Monticello, NY, by Sanford Barnum for the isolation of the endodontic environment during preparation for placement of gold foil restorations.[53] The rubber dam would eventually become the standard of care in the treatment of nonsurgical endodontic disease by preventing leakage of saliva and providing an aseptic environment.

As time progressed the role of microorganisms began to come into focus in the treatment of pulpal and periapical disease. Establishing the proper aseptic environment and working field became priorities in the clinical practice of endodontics. Dr. G. O. Rodgers 1878 article published in *Dental Cosmos* stated that a microbial insult may be the cause of odontogenic disease. Therefore, the educated clinician could deduce that eradication of the microbial insult would be necessary to successful treatment of endodontic pathology because the cause of the disease process had been removed.[62] Arthur Underwood in 1882 continued to expand this theory by suggesting that pulpal suppuration could be treated by the use of antiseptics by elimination of microbes.[54]

Dentistry and the field of endodontics took revolutionary steps forward at the turn of the 20th century as advancement in new technology came to the forefront. These mainly consisted of the hallmark introduction of dental radiography and local anesthetics. The origination of Novocaine advanced the field of dentistry, especially by changing the treatment of patients with symptomatic pulpal disease. Novocaine was developed in 1905 and replaced cocaine as the anesthetic of choice. In the 1920s block anesthesia was

developed, and when combined with Novocaine, the result was a local anesthetic with more efficacy.[63,64]

Almost equal in importance was the development of dental radiology. At the time, dental radiographs required a focus beam. Therefore, even though it was developed in 1913, the dental radiograph unit was not marketed until 1919, when the Coolidge tube was developed to focus and centralize the x-ray beam.[62] This was a benchmark in endodontics because the radiograph provided visualization of not only caries but periapical pathology. It allowed a connection to be made between pulpal and periapical disease.[65] By implementing this technology, clinicians could more exactly and efficiently diagnosis and perform endodontic therapy. Root length determination during cleaning and obturation and the understanding of root canal anatomy became more exact and thorough.[60]

A controversial theory brought forth at the beginning of the 20th century began to criticize the foundation and treatment modalities of endodontics. This theory, the "Focal Theory of Infection," was championed by William Hunter of McGill University. A physician and a pathologist, Hunter said micro-organisms that caused odontogenic infection and disease could disseminate themselves and their toxins into the body and eventually cause various diseases.[66] He later popularized this hypothesis by publishing a lecture titled, "The Role of Sepsis and Antisepsis in Medicine." His lecture 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."[67] This way of thinking influenced dentist and physicians to recommend the extraction of a variety of diagnosed

teeth such as: non-vital teeth, endodontically treated teeth, or compromised but able to restore teeth. It had such influence that some physicians went to the extent of prophylactically removing healthy teeth in order to prevent the dissemination of microorganisms from the oral cavity systemically in an attempt to reduce the incidence of various diseases.[66] In the decades to follow, called the "the scientific era," causation of systemic disease by oral disease was discounted, and the focal theory of infection began to lose its grip on the medical and dental societies. The theory was eventually discredited in the 1930s and 1940s.[68]

In the mid-20th century Hermann began to recharacterize endodontic treatment with the implementation of a revolutionary intracanal medicament, calcium hydroxide. Hermann wanted to focus on the biocompatibility of intracanal products used in endodontics. He believed the materials spread through the tooth into the surrounding periapical tissues. Calcium hydroxide became the material of choice as it was far less caustic then other medicaments or pulp capping materials of that time. Hermann was able to demonstrate that the use of calcium hydroxide had the ability to also form a dentin bridge.[69]

Obturation materials advancements started occurring during this same time period. UG Rickert developed the first obturation cement meant to enhance the obturation process. Rickert's technique was simple and foundational in that he coated the gutta percha in sealer before carefully placing it into the canal. Lentulo spirals were invented in this period and could be used as a system to deliver the sealer more effectively in the canal. Successive obturation procedures began to be created to enhance the compaction laterally of gutta percha encased in sealer.[70]

Following the advent of the first antibiotic, penicillin, dentist began utilizing the medication to treat odontogenic infections. It was first introduced as an endodontic adjunct therapy by Adams and Grossman in the 1940s.[70] Grossman suggested and recommended antibiotic use locally rather than systemically. He started a technique that would use a paper point as well as a nonaqueous carrier for the direct application of penicillin into the canal in hopes to better disinfect and sterilize the environment.[59] This thought process spawned an interest in treating root canal infections chemotherapeutically by using a combination of chemotherapeutic agents alongside physical mechanical debridement. This combination gave rise to the idea of chemo-mechanical preparation, which would characterize endodontics as a staple treating endodontic pathology and shaping modern endodontics.[69]

In 1943 the American Association of Endodontics (AAE) was founded and formed and began to establish the movement to organize endodontics nationwide and direct the profession under one body. The AAE in 1956, formed a specialty board called the American Board of Endodontics (ABE). It was not until 1963, despite the diligent effort and hard work of the AAE, that the field of endodontics was recognized as a dental specialty by the American Dental Association (ADA).[71] Just two years later, due to this landmark vote at the annual AAE session, the first diplomats were certified and the endodontic profession never looked back.[70]

At the turn of the 21st century endodontics continued to push the boundaries of technology in radiography and endodontic materials. The field of endodontics now is highlighted by several of these advancements such as: cone beam computed tomography, dental operating microscope, nickel titanium rotary files and bioceramic endodontic

materials such as mineral trioxide aggregate (MTA).[72,73] Regenerative endodontics has recently emerged as a treatment option used for immature teeth with necrotic pulps. Nova Southeastern University held the first conference in regenerative endodontics just over a decade ago.[74] As the field of endodontics continues to evolve and adapt to current hypothesis and theories, there will still be much to gain and learn as endodontics advances into and beyond the 21st century.

THEORY OF ENDODONTICS

Today the endodontic community is well rehearsed that pulpal and periapical disease begins with a bacterial insult to pulpal and periapical tissue by caries or trauma. A foundational study that advanced this idea was done in 1965 by Kakehashi, Stanley, and Fitzgerald. They found that pulps in germ-free rats, when exposed and left open to the outside environment, remained vital and healthy despite incurring trauma from mastication. In stark contrast and comparison, they found that rats in identical conditions, but that were not germ-free, experienced pulpal necrosis and periapical pathosis.[75] The conclusion of this landmark study was that bacteria was indeed responsible for pulpal and periapical disease and became the key in the way endodontic disease is treated today.

Kakehashi's work resulted in the continued knowledge base that achieving maximal reduction in bacterial pathogens as well as their toxins is the primary goal of endodontics. Minimizing bacteria would now mold the way the field of endodontics treated pulpal and periapical pathosis.[76,77] This was mainly achieved by proper debridement of the canal system through mechanical instrumentation to clean and shape the canal walls. This cleaning and shaping would allow a convenient path for chemical disinfection through the application of irrigants and intracanal medicaments.[76,77] Bacteria

and their byproducts have the ability to exit the canal system and enter the periapical and periradicular tissue causing inflammation as an extension of pulpal disease. They can exit the system through the apex and lateral or accessory canals.[78] In the circumstance where endodontic therapy via proper chemomechanical instrumentation and irrigation do not fully eradicate the microorganisms, the result is apical periodontitis or inflammation of the periapical tissues. Therefore, the reduction of pathogenic microorganisms within the root canal system is directly related to the success of the treatment of endodontics.

Dr. G. G. Stewart in 1955 focused and stressed to the endodontic community that there are three major phases to endodontic treatment namely chemomechanical preparation, eradication of microbial insult, and obturation in order to seal the root canal system.[79] Dr. Grossman in later studies built on Stewart's emphasis and theory confirming the most crucial of theses phases was the chemomechanical process in order to strive to eliminate or decrease the microbial insult within the canal system.

Grossman described 13 effective principles that should be followed in root canal procedural therapy[80]:

1. Proper use of an 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 irritating 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.

Another exceptional pioneer in the field of endodontics was Dr. Schilder who took these visionary principles and expanded them. In 1967 Schilder emphasized not only the complete removal of necrotic tissue and other intracanal contents but stressed the importance of creating a seal through a technique of "three-dimensional" obturation. This technique involved heating increments of gutta percha and placing them into a properly chemomechanically instrumented canal system and then condensing the increments in a vertical and horizontal manner to fill the canal space. This allowed for a homogenous obturation that was dense and theoretically filling the three-dimensional irregular shape of the canal from cementoenamel junction to the cementodentinal junction.[81]

Dr. Pitt Ford continued with this technique and emphasized three concepts that supported this three-dimensional obturation technique. First, Ford described that it decreased the space for bacteria to colonize. Second, a three-dimensional fill would allow the prevention of apical contamination. Third, by filling in three dimensions it would decrease and even prevent the bacterial migration along the periphery of the canal.[82] Throughout all of this Ford was an advocate for the necessity of an aseptic technique that would obstruct bacterial contamination. He highlighted the need for rubber dam isolation, a coronal seal completed by a sufficient coronal restoration, and continued and appropriate follow-up protocol of teeth treated with endodontic therapy.[82]

Therefore the theory in successful endodontic treatment outcomes of conventional root canal therapy brought forth through the hard work of endodontic pioneers is to obtained adequate intracanal chemomechanical debridement in order to reduce and possibly eliminate the bacterial presence, provide and sufficient seal through three-dimensional obturation, prevent leakage by creating an sufficient coronal seal through an adequate coronal restoration, as well as proper long term follow-up care. By following this biologically based theory, successful outcomes can be achieved in conventional endodontic therapy.

MANAGEMENT OF IMMATURE TEETH WITH NECROTIC PULP

Conventional root canal treatment can be predictable and successful when treating tooth roots that are mature.[82] Mature teeth in contrast to immature teeth have closed, fully developed apices whereas immature teeth have developing roots that until fully developed are thin and short with open apices. Therefore, immature teeth especially those whose development is stopped by pulpal necrosis present unique challenges to conventional endodontic therapy. Careful thought and planning must be made to manage the open apices to avoid easily extruded materials such as irrigants and obturation materials.[3] Due to the incomplete formation of immature teeth with necrotic pulps their root dentinal walls are short and thin and therefore have an increased risk of root fracture and tooth loss.[1] These complicated treatment cases have proposed different strategies to for the proper management of underdeveloped root with an open apex.

APEXOGENESIS

A treatment strategy for teeth with vitals pulps and open apices is a procedure termed apexogenesis. These immature teeth with open apices have had a microbial insult either by caries or trauma in which the pulp has become inflamed. The primary objective in the procedure of apexogenesis is to preserve and maintain the vital more healthy tissue remaining within the dental pulp with the aim and hope that primary radicular dentin formation will continue to occur, therefore continuing the development of the root in length and width with the eventual closure of the apex.[84] The process consists of an aseptic environment where the clinician performs and partial or full pulpotomy in order to remove the pulp tissue that is inflamed. Once removed, a pulp dressing and restoration are placed over the healthier pulp tissue.[27,85] Treatment planning of inflamed pulp removal is dictated on the size of the pulp exposure and the amount of time surpassed from the initial exposure. Calcium hydroxide has been used historically as the traditional vital pulp dressing in an apexogenesis procedure due to its biocompatibility and ability to render the tissue aseptic and stimulate hard tissue formation. With advantages come disadvantage with calcium hydroxide vital pulp dressings such as incomplete dentin formation as well as its basic pH around 12 that can cause pulpal inflammation.[86]

The advancement of MTA began to change the climate of pulp capping materials in apexogenesis procedures.[86] It would allow for more predictable results because in comparison with calcium hydroxide, MTA stimulates the formation of a complete reparative dentin layer and does not induce inflammation of the pulp tissue. MTA unlike calcium hydroxide will discolor the dentition, can take up to 24 hours to set providing adequate moisture for setting, and is a more expensive material.[87] MTA paved the way

for eventual advancements of other bioceramic materials such as Biodentine and other derivatives of MTA, which addressed long setting times and discoloration of dentition following application.

The endodontic clinician now has options of different valuable and predicable pulp capping materials in the apexogenesis procedure that can be used to directly dress the uninflamed vital pulp. Once placed and proper setting times have been established, the cavity preparation is sealed with a final restorative material. Over time a stringent follow-up period should be advised and maintained to monitor the clinical and radiographic development of the tooth and its root.

APEXIFICATION

Another treatment option for an immature tooth with pulpal necrosis where the root development has halted leaving thin, short root and an open apex is a procedure termed apexification. First introduced in 1960, apexification is a way to address the open apex by creating an artificial barrier at the apex. When teeth have open apices, obturation of the canal becomes a challenge due to its breadth, which also poses a challenge to managing the open apex. To counter this apexification attempts the formation of an apical barrier by creating one or artificially making one. Historically long-term treatment of with calcium hydroxide has been shown to stimulate an formation of a hard tissue barrier at the apex of the immature open root apex.[5] This process involves rubber dam isolation, access and root length determination radiographically, mainly chemical disinfection through irrigants to avoid mechanical debridement of already fragile root surfaces of immature teeth, and then placement of long-term calcium hydroxide. The calcium hydroxide is normally changed and replaced at three-month intervals until

radiographically a hard tissue barrier can be seen. Follow-up periods may be lengthy and could last anywhere between 9 to 24 months for a hard tissue barrier to for at the apex. Once an apical barrier is obtained radiographically the canal can then be obturated using MTA and/or gutta percha plugged gently at the apex coronally along the space of the canal. Once obdurate, a final seal created by a coronal restoration is necessary.[84]

Apexification using long-term calcium hydroxide does have some limitations. It has been shown that the hard tissue barrier is typically a cementoid material with small remnant communications with the periapex similar to a Swiss cheese appearance.[4,5,88] One of the main draw backs of any apexification procedure is the lack of additional root length and width formation. Patient management can become problematic due to the length of time it takes to form a barrier at the apex, especially when the demographic of people with immature teeth with open apices tend to be children and adolescents. *In-vitro* studies have shown that calcium hydroxide used over long periods of time in contact with dentin can weaken the dentin possibly resulting in higher root fracture risks.[8, 89-91]

In attempts to modify and address some of the drawback of apexification with long-term use of calcium hydroxide an alternative apexification treatment option of making an artificial apical barrier was introduced. Once the canal has been treated with chemical irrigation and intracanal medicaments and rendered clean an artificial barrier is placed at the apex as an apical plug usually 4-5 mm in thickness extending coronally.[92] This treatment modality mainly addresses the length of time that is calcium hydroxide is in contact with dentin. By being able to place an artificial barrier at the apex and omitting the sequential calcium hydroxide applications, this modality significantly reduces treatment time. By so doing the tooth can be restored permanently much sooner allowing

for less chance of coronal leakage and recontamination of the root canal system. When calcium hydroxide is used as the intracanal medicament in these cases its exposure time with the dentin is less therefore reducing the chance of weakening the dentin and decreasing the root fracture risks. MTA apical barrier apexification techniques have reported outcomes ranging from 85 percent to 93.5 percent successful.[93,94] Though highly successful both apexification treatment options do not provide the root with the potential for continued development of both root length and width, therefore rendering the tooth and its root to possible future failure due to root fractures.

HISTORY OF REGENERATIVE ENDODONTICS

The work of Nygaard-Ostby in 1961 became foundational in the regenerative concepts that have altered treatment for immature teeth with open apices. In his ground breaking study, he attempted to elucidate periodical and intracranial tissue healing and repair through the patient's own endogenous blood clot. Within his study, seventeen individuals with necrotic or vital pulps underwent root canal treatment. During the procedure the canals were debrided in a reaming fashion and the apex was enlarged. For those teeth with pupil necrosis, an intracranial medicament was placed. Following medicament placement, the patient returned, and bleeding was induced from the pulpal and periapical tissues and was allowed to partially fill the canal apically. The area coronal to the bleed was obturated with kloroperka being directly placed on top of the bleed. Once the tooth was obturated with kloroperka, the coronal access was sealed. In the period following, the teeth were extracted and examined microscopically. His findings showed that most teeth had resolution of inflammation and even apical closure of the root end.[95] He did notice some failures in which he deemed came from possible coronal

leakage. He described the apical portion of the canal where the blood clot had been formed as having vascularized fibrous connective tissue that was normal in character, but unfortunately lacking pulp tissue structure and desirable pulp cells.[96] This breakthrough study, unknown to many at the time, would be foundational in regenerative endodontics in that for the first recorded time it showed evidence of a patient's endogenous biologic tissue's healing potential.

Rule and Winter continued this effort in 1966 when they studied immature teeth with open apices. In their study they treated the apical vital tissue by instrumenting short of the vital tissue and then dressing the canal with an interappointment polyantibiotic medicament. Their findings were stunning in that they observed not only a complete resolution of symptoms of disease but continued root development both in length and width.[97] This became the first case report in regenerative endodontic that showed the potential that teeth have to continue root development following disinfection of a microbial insult with polyantibiotic pastes.[96]

As the advent of regenerative endodontics began to mature and develop others would build off earlier attempts by creating different treatment protocols. Many of these reports had been using multiple combinations of antibiotics as intracanal disinfection medicaments during the procedures. Iwaya performed the first contemporary regenerative procedure in which he used a double antibiotic paste (DAP) composed of a mixture of ciprofloxacin and metronidazole to be used as an intracanal medicament to disinfect the canal system. Knowing that bacteria were the cause of the disease and a reduction or elimination would provide the best avenue for endodontic therapy success, Iwaya used a stringent disinfection protocol. His protocol included the use of 5 percent NaOCl and 3

percent hydrogen peroxide. Following the disinfection through irrigation an application of intracanal DAP medicament was used. His treatment protocol called for 6 visits, in which the tooth was monitored for continued root development. At thirty months he described a positive vital response of the treated tooth.[98] A few years later, Banchs and Trope found success in treating an immature tooth with necrotic pulp with their rendition of regenerative endodontic protocol. They modified the steps taken by Iwaya, in that they used a triple antibiotic paste (TAP) that included the addition of minocycline to the ciprofloxacin, metronidazole DAP used previously.[22] They altered the irrigation protocol by using only 5.25 percent NaOCl and was never mechanically instrumented. Following irrigation, the TAP was applied for twenty days as an intracanal medicament. The patient then returned, and the TAP was carefully removed with a gentle irrigation of saline. Once removed an intentional bleed was created from the periapical tissue filling the canal to the coronal one third of the root. Once filled a final restoration was placed to provide a coronal seal. Similar to Iwaya, Banchs and Trope showed resolution of periapical inflammation, continued development of the root in both length and width as well as a positive vitality response. Among these successful attempts at regenerative endodontic procedures, a common thread began to develop. A theme driven by patients with immature root development, open apices, minimal mechanical instrumentation, chemical irrigation with NaOCl, interappointment polyantibiotic medicament placement, followed by the intentional blood clot formation within the root was leading to favorable results in pathologic resolution, continued root development and positive vital pulp responses.[13] These early attempts and reports laid the foundational basis of the current trends in regenerative endodontic methodology.

The main goals of regenerative endodontic procedures are to resolve the apical pathology, continued root development in both thickness and length of the root, as well as regaining pulp vitality verified by positive pulp sensibility testing.[99] This would mean that beyond the healing of the apical pathology the regeneration of the pulp dentin complex would allow for functional cells to replace lost tooth structure. This new pulp dentin complex should have properties similar to normal pulp such as innervation, vascularity, the ability for cells to form dentin to continue root development and to counteract future microbial insults.[42] By doing so, regenerative endodontics can be a novel way of treating an immature tooth with pulpal necrosis. Therefore, by achieving a favorable environment through a process of disinfection regeneration as described by tissue engineering has the potential to take place. The three pillars of tissue engineering for endodontic regeneration are stem cells, scaffolds and growth factors. A balanced disinfection protocol sustains these pillars.[100,101]

DISINFECTION

The continued research in the field of endodontics has given us a current common understanding that the etiology of apical periodontitis it that of an intraradicular infection. This intraradicular infection contains a spectrum of both anaerobic gram-negative and gram-positive bacteria. The most prevalent and predominant gram-negative bacteria are *Fusobacterium, Dialister, Porphyromonas, Prevotella, Tannerella, Treponema, Campylobacter* and *Veillonella*; and the most prevalent and predominant gram-positive bacteria are *Parvimonas, Fillifactor, Pseudoramibacter, Olsenella, Actinomyces, Peptostreptococcus, Streptococcus, Propionibacterium* and *Eubacterium*.[102] In 2014, Nagata et al. identified *Actinomyces naeslundii* as the most prevalent species in primary

endodontic infections within immature permanent teeth.[103] This microbial environment within the canals thrives in biofilm communities both on canal walls as well as within dentinal tubules.[104]

Due to changes in of environment within the canal system such as oxygen saturation and nutrient availability, the progression of the endodontic infection as well as the microbial profile changes to adapt to the alterations in the environment.[105] This is seen in the early stages of the infectious process, when the main species that dominates the spectrum is facultative bacteria due to the higher levels of initial oxygen. As the disease process continues, it becomes more ideal for anaerobes, as the level of oxygen perfusion decreases as blood flow decreases in the environment.

The purpose and primary goal of treating immature teeth with necrotic pulps is to have resolution of signs and symptoms of pulpal and periapical inflammation. The foundational basis of chemomechanical debridement of the canal space is to reduce or eliminate the microbial infectious process. In so doing, an environment is fostered in which healing can be induced. Therefore, the disinfection process is pivotal in combating the microbial challenge. The disinfection process is mainly achieved in regenerative endodontic procedures by intracanal irrigants and a variety of medicaments. One of the most commonly used intracanal interappointment medicaments in regenerative endodontic procedures is the use of triple antibiotic paste composed of ciprofloxacin, metronidazole, and minocycline.[96] Minocycline specifically aims to disrupt protein synthesis of bacteria while ciprofloxacin and metronidazole prevent bacterial DNA synthesis. Studies have concluded *in vitro* and *in vivo* that the combination of these

specific antibiotics is extremely effective in eradicating bacteria from infected root canals.[23-106]

TAP in higher concentrations has cytotoxic effects on human dental pulp stem cells[107] as well as detrimental effects on the mechanical and chemical properties of radicular dentin.[108,109] It has been reported that concentrations ranging from 0.2 mg/mL to 2.0 mg/mL are not as toxic to stem cells and can be used with better predictability in regenerative procedures.[107] TAP contains minocycline which has been shown to cause severe staining to tooth structure.[110] With the majority of regenerative endodontic procedures taking place in immature teeth with necrotic pulps in the anterior maxilla and mandible esthetic need to be taken into consideration. The staining potential of TAP containing minocycline is detrimental to the appearance of a tooth in the esthetic zone. In an effort to reduce this adverse effect, studies started to eliminate minocycline and substitute with other antibiotics such as; amoxicillin, cefaclor, or clindamycin.[96,111] Clindamycin has an antibacterial spectrum effective against endodontic pathogens and therefore was introduced into a modified version of TAP (mTAP).[111,112] Others eliminated the third antibiotic altogether and created and mixture of two antibiotics specifically ciprofloxacin and metronidazole known as double antibiotic paste (DAP). This addresses the discoloration issues left behind by TAP medicaments containing minocycline.[113] DAP too has exhibited excellent efficacy against endodontic pathogens.[114] Similar to TAP, DAP at low concentrations was shown to be the least cytotoxic to dental pulp stem cells and has gained traction in the endodontic community as a possible intracanal medicament used during endodontic regenerative procedures.[41-46]

Another common intracanal medicament used in the disinfection stage of regenerative procedures in calcium hydroxide. Calcium hydroxide can inactive lipopolysaccharide (LPS), which is a crucial element in the inflammatory process.[32,35,112] Its mechanism of action and success is founded on its basic pH of 12.5. This pH has the ability to denature structural proteins and critical cellular enzymes. It also has the capacity to release hydroxyl ions that create free radicals. These free radicals interact with bacterial DNA inhibiting replication and other cellular functions.[48] In low concentrations of 1.0-mg/mL calcium hydroxide has been shown to promote stem cell survival of the apical papilla (SCAP).[108] Its current use in regenerative endodontic procedures is supported by the American Association of Endodontics (AAE). Though supported by the AAE, *in-vitro* studies have shown to decrease fracture resistance and microhardness of dentin when in continued long-term contact.[8] Yassen in his study showed degradation of superficial collagen in radicular dentin specimens left in contact with dentin from one to four weeks.[115] Not only can it lead to weakening to tooth structure but has been shown to be inadequate against specific and highly virulent endodontic pathogens namely, *E. faecalis* and *P. gingivalis* biofilms.[46]

The disinfection process during regenerative endodontic procedures currently not only involves the use of intracanal medicaments but also multiple irrigants. With its ability to dissolve organic tissue and eliminate microorganisms sodium hypochlorite (NaOCl) has been a staple in endodontic disinfection.[116] Studies have shown that the NaOCl has greater effect on microbials found within root canals than saline alone.[117] It has been demonstrated that gram-negative anaerobic rods in apical periodontitis when in contact with NaOCl for only 15 seconds are killed.[118] Similar to TAP and DAP intracanal

medicaments when NaOCl is used at lower concentrations, it will maintain its ability to destroy and dissolve necrotic tissue but will not have as much of a detrimental effect on important vital tissue and cells. Other characteristics that make NaOCl effective and useful in endodontic therapy is its ability to be affected by concentration and temperature.[116-119] Research demonstrates that NaOCl has the capacity to increase its tissue dissolution and antibacterial effects when temperature is increased. Interestingly and more specifically by increasing the temperature by only 5°C, the bactericidal rate is doubled.[120] NaOCl is commonly used at concentrations ranging between 5.0 percent to 6.0 percent when performing conventional root canal therapy and is considered safe at these concentrations when maintained within the canal.[121] Estrela et al. did find that even though it has the incredible capacity to kill microbes, it may be limited in its efficacy and ability to fully eradicate certain species such as *E. faecalis*.[122]

As previously discussed, when treating patients in a regenerative manner the clinician must create an environment capable and favorable for regeneration to take place. Not only do immature teeth have open apices in which extrusion of high concentrations of NaOCl could be dangerous to surround vital structures, these teeth contain viable stem cells located in the apical papilla needed for optimal results in regenerative procedures. Essner demonstrated that at high concentrations NaOCl has cytotoxic effects on stem cells.[123] The radicular dentin is also affected by NaOCl and reduces the environment of the dentin to the differentiation potential of stem cells from the apical papilla.[124] Even with these possible shortcoming, NaOCl has remained the gold standard as a disinfecting irrigant and is recommended and supported by the AAE as an irrigant at lower concentration (1.5%) in regenerative endodontic procedures.[125]

Another important irrigant in regenerative endodontic procedures is ethylenediaminetetraacetic acid (EDTA). This is a chemical compound that is used as a chelating agent to chelate ionic compounds. It has been effective in dentistry in removing the smear layer, especially in root canals following chemomechanical debridement.[126] During the instrumentation phase dentin debris, bacteria and their toxins can create this smear layer which has the potential to occlude dentin tubules, isthmuses and fins from being properly irrigated and disinfected as well as inhibiting a seal during obturation. Therefore EDTA has been shown an important agent in removing the smear layer.[127] It has been shown that with a contact time of one-minute with the canal system the smear layer is removed.[84]

EDTA also serves another purpose in regenerative endodontics. It not only functions on the smear layer but has demonstrated the ability to increase cell survival and allow growth factors to be released from treated dentin. In a study conducted by Trevino, EDTA, NaOCl and chlorhexidine cytotoxic effects were compared. It was shown that 17-percent EDTA alone has the least cytotoxic effects. When treated with 6.0-percent NaOCl, 17-percent EDTA and then again with 6.0-percent NaOCl, the results showed a slight increase in cytotoxicity. The greatest cytotoxic effects were found with 2.0-percent chlorohexidine, where there were no remaining viable cells. This study suggested that chlorohexidine should not be used in regenerative endodontic procedures due to its detrimental effects on needed viable cells.[128] Other research has shown that the use of EDTA as an intracanal irrigant releases dentinal growth factors from the dentin, which is believed to promote SCAP differentiation and survival during regenerative endodontic

procedures.[19] Work done by Yamauchi demonstrated that EDTA has the ability to increase the adherence of newly mineralized tissue to intraradicular dentin.[129]

STEM CELLS

A crucial pillar in regenerative endodontic procedures is the role of stem cells. Stem cells are specific types of cells that have an incredible and unique inherent ability and potential to differentiate into different cell lines.[130] The more embryonic the stem cell, the more potential it has to differentiate. Therefore, embryonic stem cells are considered pluripotent stem cells with the ability to develop and differentiate into nearly any type of human cell. Conversely, a multipotent stem cell has limited capabilities and can differentiate into only certain cell lines.[130] Additional subcategories can be used to define the source from which stem cells are obtained. There are three main sources in which stem cells can be obtained namely, autologous, allogeneic, and xenogeneic. Autologous stem cells originate from the same individual whereas allogeneic stem cells are derived from another individual of the same species. Furthermore, xenogeneic stem cells arise from another species completely. Regardless of their origination, stem cells from the same or different species can be utilized in the tissue engineering of the human pulp-dentin complex.

The regenerative endodontic theory is to utilize stem cells in the local dento-alveolar complex to regenerate the pulp-dentin structures. These localized stem cells are multipotent and therefore have the ability to differentiate into various cells.[17] The classification of some of these stem cells is as followed: dental pulp stem cells (DSPCs)[131] stem cells from human exfoliated deciduous teeth (SHEDs),[132] periodontal ligament stem cells (PDLSCs),[133] dental follicle progenitor stem cells (DFPCs),[134] and

stem cells form the apical papilla (SCAPs).[135,136] Following disinfection from the canal and intentionally causing a bleed from the periapical region stem cells, like DPSCs and SCAPs, with the help of growth factors and scaffolds have demonstrated the capability to differentiate into cells of the pulp-dentin complex. Such cells like odontoblasts then have the ability to produce dentin, which would add width and length to a halted, underdeveloped immature root, and therefore add strength and increase its long-term prognosis.[136,137] Stem cells in the local dento-alveolar region are not created equally. For instance, SCAPs compared with DPSCs have demonstrated superior proliferation rates and therefore a greater potential for regeneration.[135-137] The field of endodontics currently is focusing on stem cells function, signaling factors, as well as attachment and differentiation potentials in order to better create proper protocols for more predictable regenerative endodontic procedural success.[14]

SCAFFOLD

Another important pillar to the tissue engineering concept in regenerative endodontic procedures is the use of a scaffold. A scaffold helps to provide a physical structure and environment into which stem cells and differentiate and proliferate as well as provide a network for angiogenesis.[99] Nevins in 1976 first introduced the idea of using a collagen gel as a scaffold.[138] Later Thibodeau demonstrated that the blood clot intentionally obtained from the periapical region presented as an autologous scaffold that stem cells could utilize to differentiate and proliferate. In his study Thibodeau compared three different types of scaffolds in a dog model namely, a scaffold created by a blood clot, a collagen scaffold, and lastly a combination of scaffolds from blood clot and collagen. He established that his success rate would increase with the use of a blood clot

compared to using a solitary collagen scaffold on its own. His final conclusion was the ideal scaffold needed to have properties that would provide a physical network or latticework for cell growth as well as providing the stem cells with needed growth factors to direct their differentiation and proliferation.[139]

Hutmacher in 2001 contributed to the regenerative endodontic theory of scaffolds by identifying six properties needed by a scaffold to function properly in regenerative procedures.[140] The properties are:

1. Structure which is porous for tissue and vascular integration.
2. Biodegradable at the same rate of tissue formation.
3. Allows cells attachment for differentiation and proliferation.
4. Adequate mechanical properties in the implanted site.
5. No adverse reaction.
6. Formed into many sizes and shapes easily.

Even though the blood clot has been utilized with more frequency this may shift as the process of regenerative endodontics evolves. For example platelet-rich plasma and platelet-rich fibrin are both being studied for their possible ability to enhance the scaffold function by providing a highly concentrated network of autologous growth factors.[18,128,141]

GROWTH FACTORS

Another element in the regenerative endodontic theory is the integral role of growth factors. Growth factors cover a wide spectrum of function and use but for the purpose of endodontic regenerative theory they are a group of endogenous molecules that play a role in multiple aspects such as; maturation, healing, repair, as well as promoting

tissue growth. These microscopic molecules are critical for the success of regenerative procedures. Growth factors are signaling molecules that facilitate the directing of stem cell attachment, differentiation and proliferation. Consequently, many of these growth factors can be found embedded in the dentin matrix. If these signaling molecules are exposed to the right environment, they have the capacity to release and help orchestrate the function of stem cells in regenerating the pulp-dentin complex.[142] As discussed previously the use of EDTA during regenerative endodontic procedures has demonstrated to facilitate the release of growth factors such as transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF) and bone morphogenic protein (BMP) when in contact with dentin.[21] Each of these growth factors has a direct role in the process of regeneration. TGF-β can act on stem cells to differentiate into odontoblasts as well as aiding in the signaling of pulp tissue mineralization, wound healing and anti-inflammatory effects. VEGF has shown to be an important growth factor in the process of forming new vasculature while BMP has been identified in facilitating the odontoblast differentiation of stem cells.[142]

More recent studies in the field of endodontics have revealed that other factors can aid in stem cell differentiation. It has been shown that the use of exogenous dexamethasone or simvastatin on human DPSCs enhances their ability to differentiate into functioning odontoblasts and odontoblast-like cells, respectively.[143,144] Both these pharmacologic interventions could enhance the regenerative potential of the pulp-dentin complex.

CLINICAL INDICATIONS AND DECISION-MAKING

When presented with a case of an immature tooth with pulpal necrosis, the clinician must identify the proper mode of treatment for that particular tooth in that particular individual. Treatment options such as apexification or regenerative procedures have been previously discussed. Several factors in the decision-making process should include but are not limited to the following during case selection; clinical outcomes, indications for the stage of tooth development, patient compliance and expectations of treatment. The regenerative endodontic procedure has been shown to be a safe treatment option for an immature tooth in which the root development has halted leaving the tooth with large open apices as well as short and thin radicular dentin structure. If the regenerative procedure is unsuccessful the clinician has the ability to then move forward with an apexification procedure. The AAE has stated the following criteria to clinically and radiographically assess when determining success of a regenerative endodontic procedure. They are stated as follows in the AAE 2016 Clinical Considerations for Regenerative Procedure[145]:

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

The outcomes studies of regenerative endodontic procedures compare these procedures with those of apexification. Jeeruphan et al. found that regenerative endodontic procedures compared with apexification procedures using either calcium

hydroxide or MTA resulted in greater survival rates as well as better increase in root width and length with regenerative endodontic therapy with reported survival rates of 100 percent, 77.2 percent and 95 percent, respectively.[12]

One main drawback is that clinicians are basing their current treatment on the best available current evidence which is of lower quality. Much of the research on regenerative clinical case outcomes are from case reports and case series. The lack of a standardized protocol both in clinical and radiographic assessments makes collecting and analyzing data difficult, and therefore, solid conclusions on treatment recommendations cannot be deduced.[96] Regardless of the level of evidence, these case reports and case series may be the endodontic community's best current available evidence. This current level of evidence though considered low on the hierarchy of evidence still provides the clinician with an alternative treatment option for treating an immature tooth with necrotic pulp with potential additive benefits that apexification procedures inherently do not provide.[146] The AAE currently supports regenerative endodontic procedures and has provided recommendations, considered guidelines, based on best available evidence in treating such cases.[145] These and other publications have the potential to guide the clinician and provide them with necessary tools to predictably and safely treat immature teeth with pulpal necrosis.

MATERIALS AND METHODS

SELECTION OF HUMAN TEETH

Intact extracted human adult teeth were obtained for utilization in the study and were collected and stored in 0.1-percent thymol solution at 4^{o} C. For teeth to be included in the study certain criteria were met by visual examination. Inclusion criteria consisted of complete formation of roots, teeth without caries on crown and roots, at least 4-mm mid-root diameter buccolingually or mesiodistally. Exclusion criteria consisted of teeth with restorations, caries, hypocalcifications, hypoplasia, incomplete root formation, fractures or cracks.

HUMAN DENTIN SPECIMEN PREPARATION

After visual examination and criteria were met, the teeth selected were subsequently prepared into polished radicular dentin samples (Figure 2, Figure 3). The tooth samples were rinsed with sterile saline for 10 seconds following removal from 0.1-percent thymol solution. Using a high-speed diamond blade saw combined with cool water irrigation, each tooth crown was cut from the root, and the roots were further sectioned longitudinally. The root halves were further prepared to create dentin samples (Figure 3, Figure 4). Each half of the root was mounted with wax onto an acrylic plate (Figure 5). Following mounting, a low-speed diamond blade saw combined with cool water irrigation was used to prepare each root into 4x4 mm dentin slabs (Figure 6). These rough dentin samples were then polished using Struers Rotopol 31 polishing unit (Struers, Cleveland, OH). Each sample was secured for polishing on a 38 mm cylindrical mounting block with pulpal side facing up (Figure 7, Figure 8). The pulpal side of the

dentin sample was then polished using sequential Sic abrasive papers at 500, 1200, 2400 and 4000 grits. If surface defects were visually examined following polishing the dentin specimen was excluded and another prepared. The final polished dentin samples dimensions were 4x4 mm with a dentin thickness of 1-1.5 mm (Figure 9). Each dentin sample had its smear layer removed as described in the literature,[147] by rinsing with 8 mL 1.5-percent NaOCl and 17-percent EDTA for 4 minutes. To prevent dehydration prior to use in the study each dentin sample were rinsed in sterile water. Each dentin sample was placed into Whirl-Pak bags (Sigma-Aldrich, St. Louis, MO, USA) separately with moist cotton gauze and sterilized with ethylene oxide gas (Figure 10). The packaged samples were then stored at 4ºC until needed in the study.

ANTIBIOTIC PASTE PREPARATION

The concentrations of DAP utilized in this study were prepared using protocols from previously published studies.[36,40] A modification to the published protocols was accomplished by an addition of a radiopacifier, zirconium oxide. In summary, equal proportions of metronidazole and ciprofloxacin antibiotic powders (25 and 250 mg) were dissolved in 25 mL of sterile water to obtain DAP in concentrations of 1.0 mg/mL and 10 mg/mL respectively. An addition of 7.5 g of zirconium oxide power (5.0-µm powder, Sigma-Aldrich, ST. Louis, MO) was mixed into the different concentrations to achieve a 30-percent (w/v) of radiopaque DAP paste. In order to achieve a final paste-like consistency, 1.75 g of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St. Louis, MO) was sequentially added. Coupled with the above mentioned radiopaque DAP, a preparation of 1.0-mg/mL DAP without a radiopacifier and three separate antibiotic free pastes were created to be used as controls for the study. One paste was a combination

of methyl cellulose and sterile water, while the other two were a mixture of methyl cellulose, sterile water, and zirconium oxide powder or barium sulfate respectively. Finally, to create pastes with no air bubbles, the 1.0 mg/mL and 10 mg/mL radiopaque DAP were centrifuged at 7000 rpm for 15 minutes.

BACTERIAL STRAINS AND MEDIA

After IRB approval (IRB# 1510640949) a bacterial sample already collected from an immature tooth with a necrotic pulp was utilized in the study. The bacterial isolate obtained followed the protocol set forth by a previous study completed by Sassone.[148] In summary, the tooth selected for endodontic regenerative treatment was isolated using a rubber dam. Using 3.0-percent hydrogen peroxide, both the tooth and rubber dam were cleaned and then further disinfected with 6.0-percent sodium hypochlorite. Following initial cleaning and disinfection, the pulp chamber was coronally accessed using a sterile round bur with high-speed dental handpiece. Upon completion of access, the pulp chamber, using 6.0-percent sodium hypochlorite was disinfected. In order to inactivate the sodium hypochlorite solution, 5.0-percent sodium thiosulfate solution was used. After removal of the handle, a #15 endodontic hand file was used to collect bacterial samples. It was placed into the root canal system apically within 1 mm of the apical foramen. The file was then moved in an up and down motion within the canal for 30 seconds and removed. Three separate sterile paper points were placed 1 mm from apical foramen for 1 minute each, in order to wick and collect the tissue fluid in the canal. The hand file and paper points were placed in 2 mL of BHI-YE growth medium, and vortexed to elute the microbes for further growth. The microorganisms were then grown anaerobically for 48 hours at 37^{o}C. Once the growth was complete the bacterial isolates were then frozen at -

80° C with 10-percent sterile glycerol until further use in the study. Furthermore, a standard strain of *E. faecalis* (ATCC 29212) was used and prepared in BHI for 48 hours at 37°C at 5.0-percent CO_2 to form a stock culture. The cell density of the stock culture will be set to 3.2 x 10^7 CFU per milliliter. It was further frozen similar to the multispecies immature culture until used in the study.

EXPERIMENTAL GROUPS

112 total sterilized radicular dentin specimens were used for the study. These dentin specimens were divided equally into 8 experimental groups each group containing 16 dentin specimens. Within each experimental group 8 dentin samples were infected with a multispecies isolate obtained from an immature tooth with necrotic pulp. The other 8 samples were infected with a single species *E. faecalis* isolate. Groups 4-7 were used as control groups, specifically 4 and 5 as placebo groups and 7 as a sterile control group.

The groups were as follows:

Group 1 – 1.0-mg/mL radiopaque DAP

Group 2 – 10-mg/mL radiopaque DAP

Group 3 – 1.0-mg/mL DAP

Group 4 – Placebo (Methyl cellulose and sterile water)

Group 5 – Placebo (Methyl cellulose, sterile water, zirconium oxide)

Group 6 – Bacteria with no treatment

Group 7 – No bacteria and no treatment

HUMAN DENTIN SPECIMENS: BACTERIAL GROWTH

The sterilized dentin specimens were placed into sterile 96 well plates with the polished pulpal surface facing upward (Figure 11). Groups 1-7 had 16 wells each. For each well in Groups 1-6 190 µl of sterilized BHI-YE growth media supplemented with vitamin K and hemin was placed (Figure 12). The wells for Groups 1-6 then had 10 µl of the previously obtained clinical immature tooth isolated biofilm added to eight wells. The following eight wells had 10 µl of the previously obtained single species *E. faecalis* isolate added. The wells in Group 7 had 200 µl of BHI-YE growth media added with no bacteria added. Once each well in Group 1-7 had obtained the total volume of 200 µl the dentin specimens were incubated. An incubation period of 3 weeks at 37^{o}C and 100-percent humidity was followed with the growth media being replaced once every week. The presence of biofilm on the dentin samples were assessed visually. After biofilm had been established one dentin sample from Groups 1-7 were randomly chosen for SEM processing and visualization to confirm the presence and/or absence of biofilm formation (Figure 20, Figure 21).

HUMAN DENTIN SPECIMENS: TREATMENT

Once the presence and/or absence of biofilm has been confirmed, the dentin samples were treated by the experimental groups 1-7 as indicated. Prior to dentin specimen treatment new sterile 96 well plates were prepared for use with a fresh 50 µl of growth medium. The dentin specimens were placed into the new 96 well plates and treated (Figure 13). The specimens in groups 1 and 2 were treated with 100 µl of 1.0 mg/mL and 10 mg/mL of radiopaque DAP, respectively. One hundred microliters (100 µl) of 1.0-mg/mL DAP were placed for treatment in group 3. One hundred microliters

(100 µl) of placebo paste (methyl cellulose and sterile water) were placed to treat dentin specimens in group 5. Group 6 specimens were treated with placebo paste (methyl cellulose, sterile water and zirconium oxide). Dentin specimens inoculated in Group 6 had no treatment completed. The sterile control group, group 7, had no bacterial biofilm introduced or treatment rendered. Group 1 through Group 7 were incubated anaerobically at 37ºC for one week of total treatment time in 100-percent humidity.

BIOFILM DISRUPTION ASSAYS

Following the 1-week total treatment time, each dentin specimen was individually washed with sterile saline in a gentle manner to remove bacteria that had not attached to formed biofilm (Figure 14). After washing each dentin specimen twice with sterile saline, they were transferred to a sterile plastic test tube each containing 200 µl of sterile saline. In order for the biofilm on each dentin specimen to be spiral plated and quantified each tube was sonicated and vortexed for 20 and 30 seconds, respectively, and dilutions of 1:100 and 1:1000 were made (Figure 15). Blood agar plates (CDC, BioMerieux) were used for spiral plating (Figure 16). The bacterial plates were incubated at 37ºC and 100-percent humidity for 24 hours (Figure 17). Following the 24-hour incubation period, an automated colony counter (Synbiosis, Inc., Frederick, MD) determined the number of CFUs/ml in each dilution (Figure 18, Figure 19).

STATISTICAL ANALYSIS

Separate statistical analyses were performed for the multispecies and single species biofilms. The effect of treatment group on bacteria counts was made using one-way ANOVA. Pair-wise comparisons between the groups were made using the Sidak

method to control the overall significance level at 5 percent. The distribution of the data was examined and a transformation of the data (e.g. log10) or nonparametric tests were used if necessary. A 5.0-percent significance level was used.

SAMPLE SIZE

Based on previous studies the coefficient of variation was estimated to be 1.0. With a sample size of 8 samples per group for each type of biofilm, the study had 80-percent power to detect a 3.51x difference between groups.

RESULTS

BIOFILM VALIDATION

The nature of the both the polymicrobial immature biofilm and the single species *E. faecalis* biofilm were confirmed using a scanning electron microscope (Figure 20, 21). The immature biofilm structure is thick and varied, containing multiple different cellular morphologies of microorganisms evident on the surface of the dentin specimen. Numerous cocci as well as rod-shaped bacteria are evident on the dentin surface of the immature biofilm. In contrast the *E. faecalis* biofilm was likewise thick but contained only evidence of coccal-shaped microorganisms. Numerous cocci, mainly in clusters, with possible chains created are noticed on the surface of the dentin specimens. Both the immature and *E. faecalis* biofilms were grown and matured on dentin specimens for a 3-week period of time. These microscopic scanning electron microscopic images confirm the presence and nature of both biofilms inoculated on the dentin specimens.

DIRECT ANTIBACTERIAL EFFECTS OF TREATMENTS

The mean of the $\log_{10}$ CFU/mL values of the tested groups for the immature biofilm were as follows: 0.00 for the sterile group, 7.00 for the untreated group, 6.82 for the placebo group containing zirconium oxide combined with methylcellulose (RoMC), 7.06 for the placebo group containing methylcellulose (MC) only, 0.00 for the 1.0-mg/mL DAP, 0.00 for the 1.0-mg/mL radiopaque DAP (RoDAP), and 0.00 for the 10-mg/mL RoDAP. The mean of the $\log_{10}$ CFU/mL values for the *E. faecalis* biofilm were as follows: 0.00 for the sterile group, 7.08 for the untreated group, 6.96 for the placebo

group containing RoMC, 7.05 for the placebo group containing MC, 0.66 for the 1.0-mg/mL DAP, 0.00 for the 1.0-mg/mL RoDAP, and 0.00 for the 10-mg/mL RoDAP. No bacteria were observed in either sterile group of the immature and *E. faecalis* biofilms. Only one (1) colony was counted in the 1.0-mg/mL DAP group for the *E. faecalis* biofilm. These data are summarized in Table II.

POLYMICROBIAL IMMATURE AND *E. FAECALIS* BIOFILM BATERIAL COUNTS

When comparing the polymicrobial immature and *E. faecalis* species biofilms, no significant differences were found between biofilms in the following groups: no treatment group, placebo group containing MC only, 10-mg/mL RoDAP group, 1.0-mg/mL RoDAP group, 1.0- mg/mL DAP group, and the sterile control group. A significant difference was found between the polymicrobial and single species biofilms in the placebo group containing RoMC ($p = 0.01$) showing that in these specific placebo groups, the *E. faecalis* group demonstrated significantly increased biofilm growth compared to the immature group. These findings are summarized in Table III.

COMPARING THE EFFECT OF TREATMENTS

The 10-mg/mL RoDAP, 1.0-mg/mL RoDAP, 1.0-mg/mL DAP, and sterile control groups had significantly lower colony counts than the placebo group containing MC, the placebo group containing RoMC, and the no-treatment group for both polymicrobial and single species biofilms ($p \leq 0.01$).

The placebo group containing RoMC had significantly lower immature biofilm colony counts than the MC ($p = 0.02$) and no treatment ($p = 0.03$) group, but RoMC did

not have significantly different *E. faecalis* biofilm colony counts than the MC ($p = 0.09$) and the no-treatment groups ($p = 0.08$).

The MC and the no treatment groups were not significantly different from each other for either biofilm ($p = 1.00$ for *E. faecalis* biofilm, $p = 0.31$ for immature biofilm). 10-mg/ml RoDAP, 1.0-mg/ml RoDAP, 1.0-mg/ml DAP, and the sterile control group were not significantly different from each other for either polymicrobial or single species biofilms ($p = 1.00$). Figure 23 summarizes these findings.

TABLES AND FIGURES

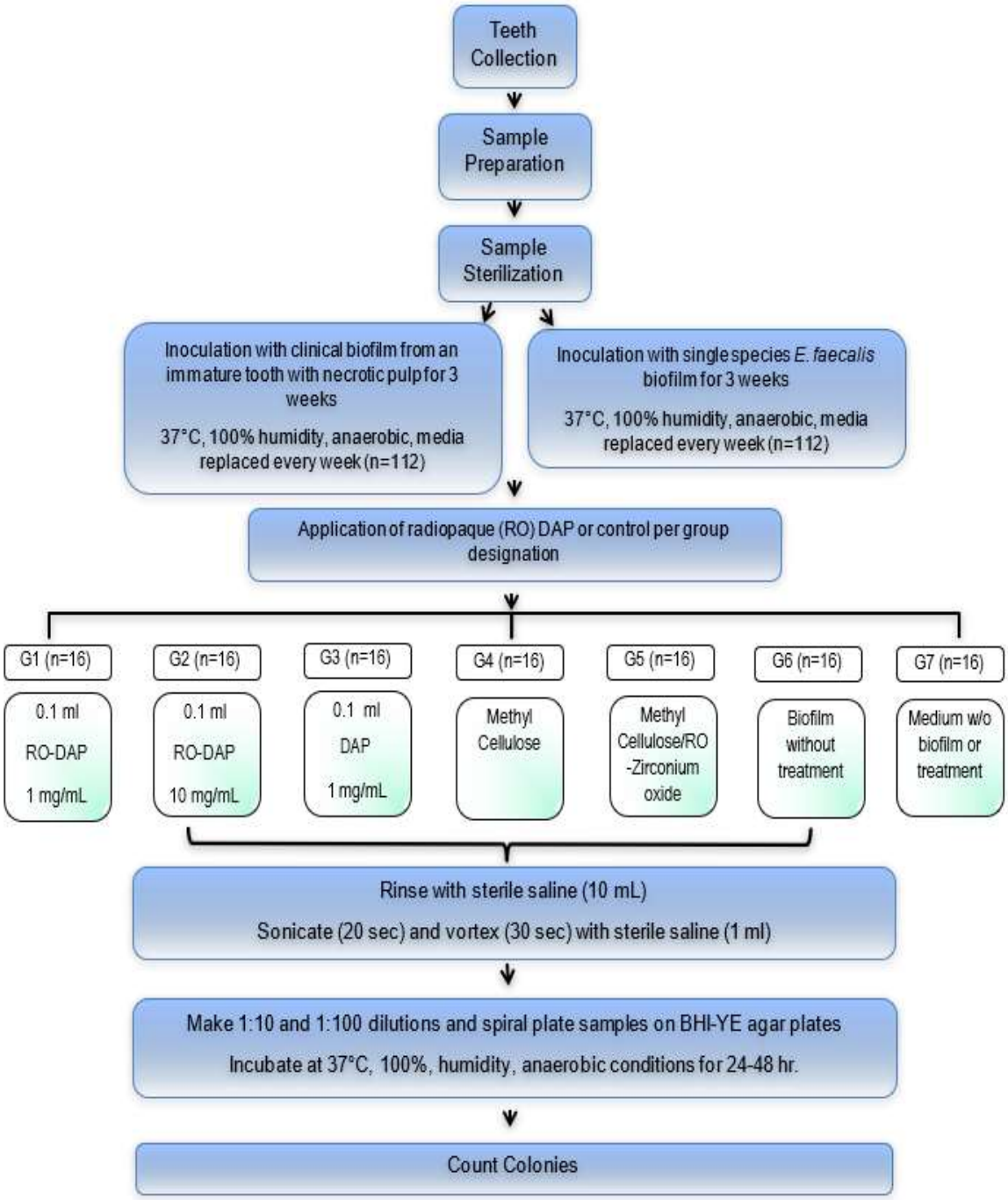

FIGURE 1. Flowchart of experimental methodology.

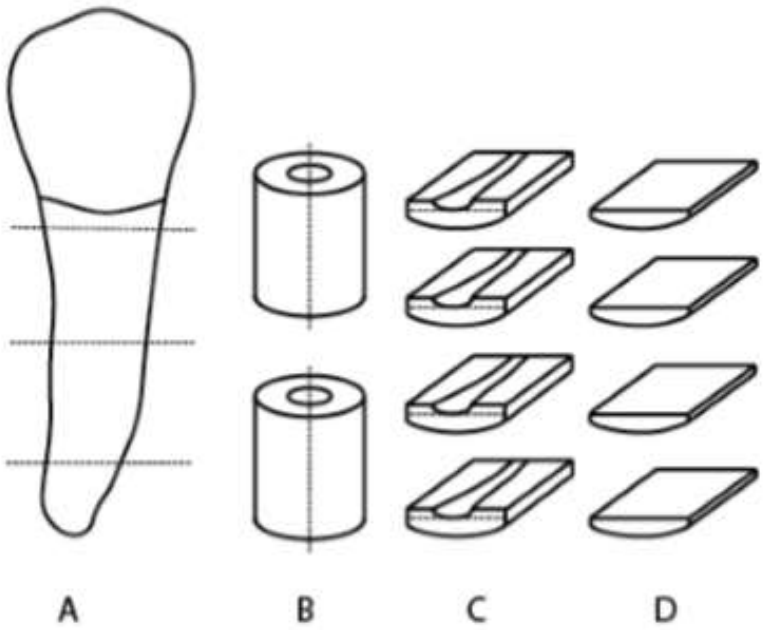

FIGURE 2. Roots (A) were sectioned, cut longitudinally (c), and polished flat on the pulpal side (D).

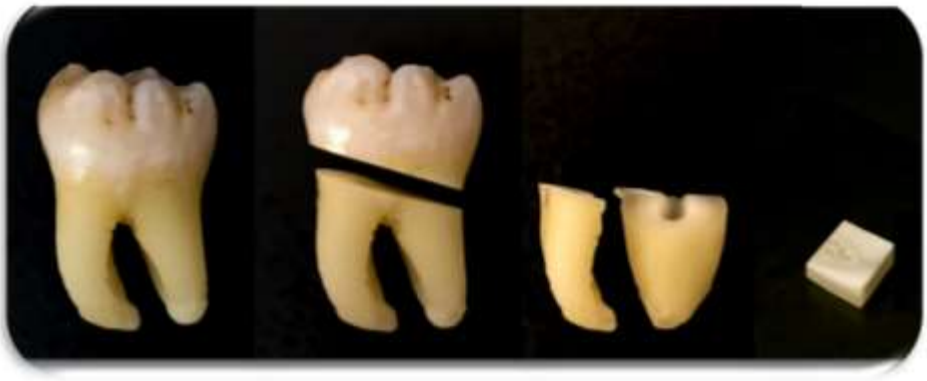

FIGURE 3. Example of sequential sectioning and final dentin sample.

FIGURE 4. High-speed saw used to initially section whole teeth.

FIGURE 5. Sectioned root mounted on acrylic plates using sticky wax.

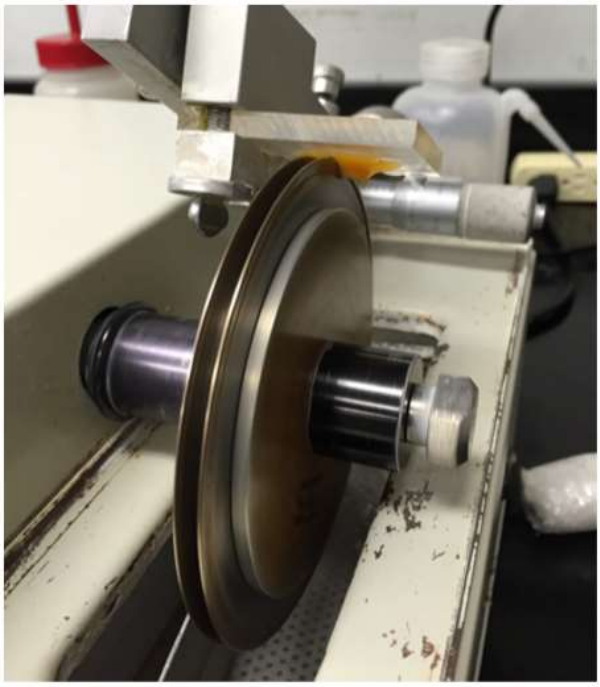

FIGURE 6. Low-speed saw used to section teeth into 4x4-mm samples.

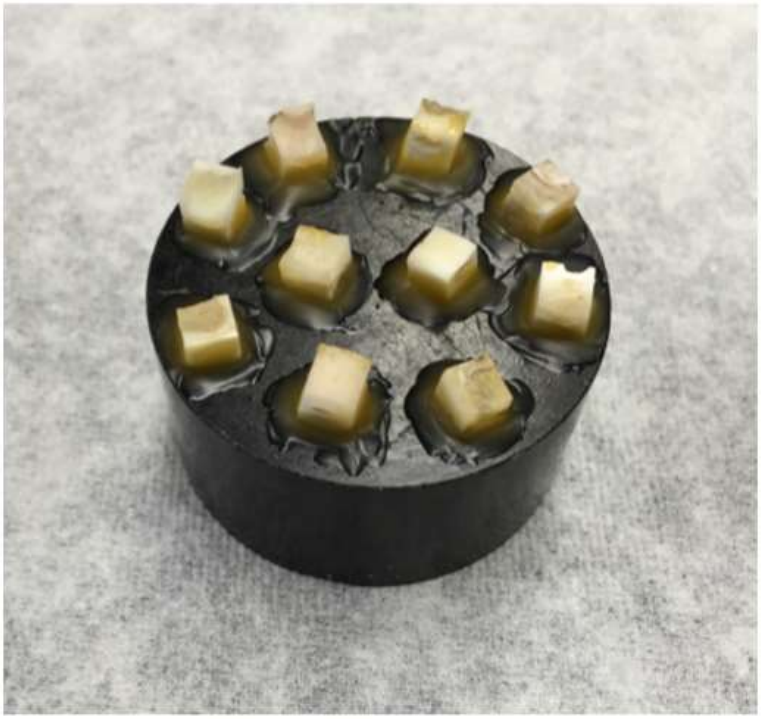

FIGURE 7. 4x4-mm dentin samples mounted on polishing jig.

FIGURE 8. Dentin polishing unit.

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

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

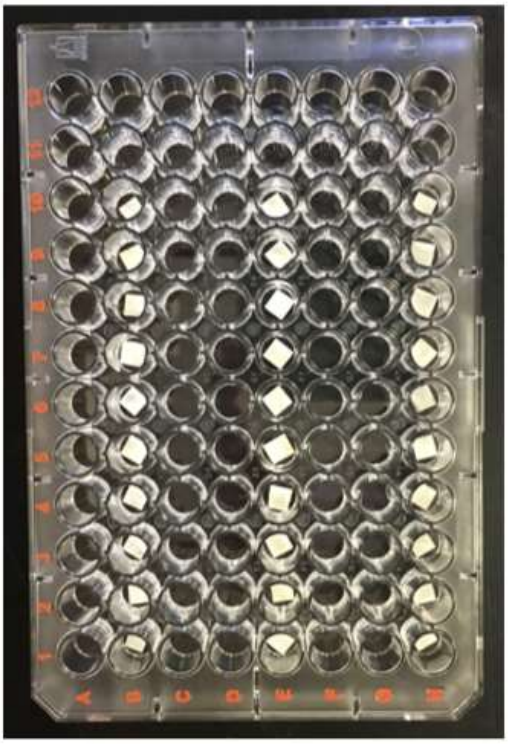

FIGURE 11. Dentin specimens placed into sterile 96-well microtiter plate.

FIGURE 12. Inoculated dentin specimens with BHI-YE broth.

FIGURE 13. DAP paste placement.

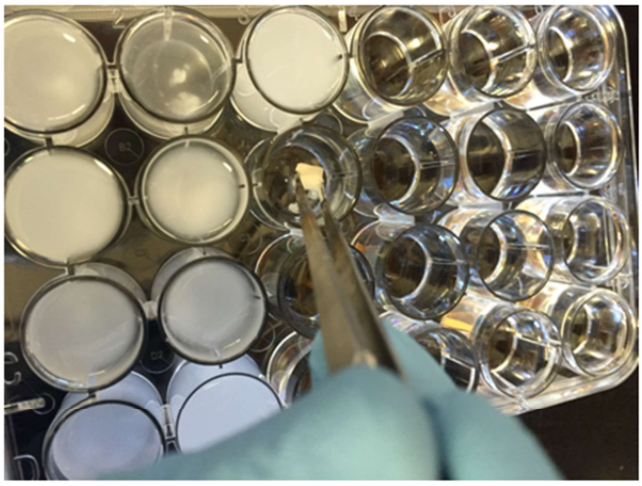

FIGURE 14. Washing of dentin specimens to remove experimental pastes.

FIGURE 15. Sonication (A) and vortexing (B) to detach biofilm from dentin specimens.

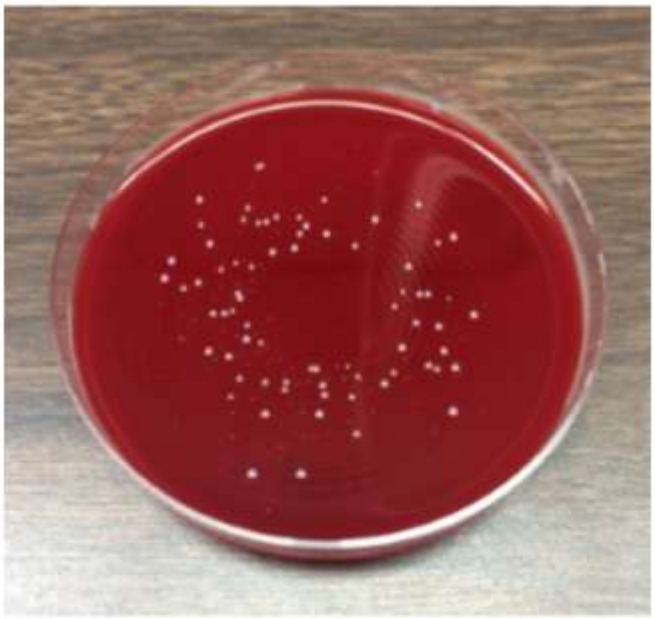

FIGURE 16. Spiral plating of the dilutions of the detached biofilm cells from the treated dentin samples.

FIGURE 17. Blood agar plates incubated in an anaerobic Gas Pak chamber.

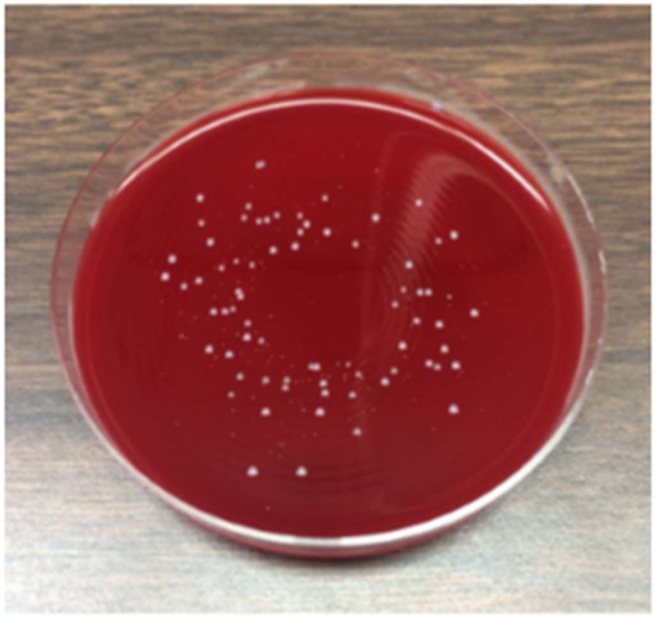

FIGURE 18. Example of bacterial growth from immature biofilm treated with methylcellulose containing zirconium oxide.

FIGURE 19. Digital colony counter.

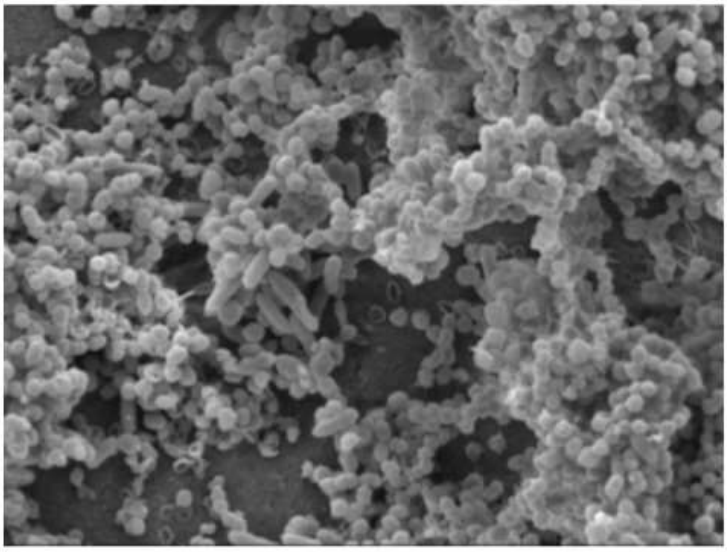

FIGURE 20. Scanning electron microscopic image of 3-week old bacterial biofilm formed by polymicrobial bacteria from an infected root canal of an immature tooth with necrotic pulp.

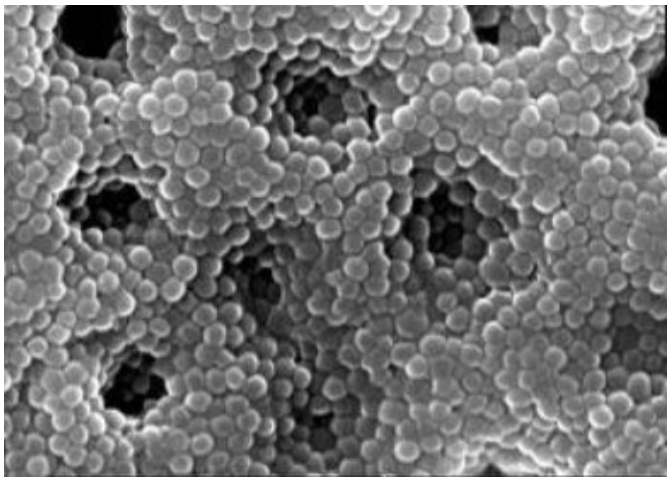

FIGURE 21. Scanning electron microscopic image of 3-week old bacterial biofilm formed by bacteria obtained from a standard strain of *E. faecalis.*

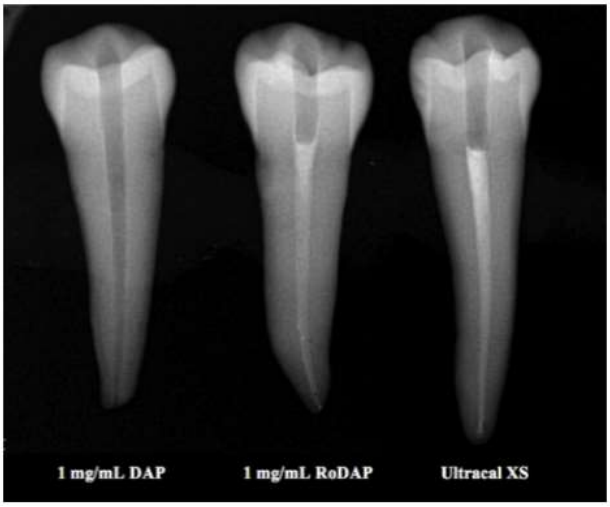

FIGURE 22. Radiograph demonstrating similar radiopacity of 1.0-mg/mL RoDAP compared with Ultracal XS when 30% w/v zirconium oxide was added to DAP as a radiopacifying agent.

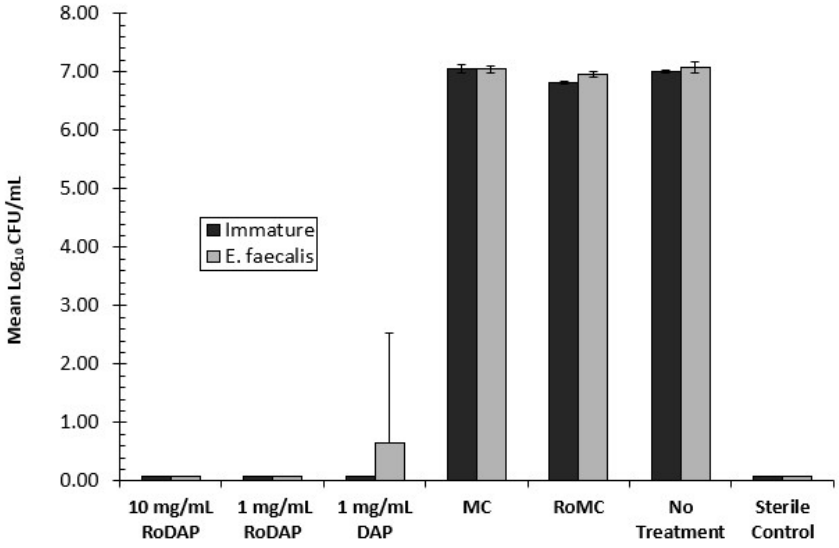

FIGURE 23. Graph representing the direct antibacterial effects of the tested medicaments against a polymicrobial bacterial biofilm from an immature tooth with pulpal necrosis and an *E. faecalis* biofilm (mean of the $\log_{10}$ CFU/mL ± SEM).

TABLE I

Comparison of the antibacterial effects of radiopaque and non-radiopaque 1 mg/mL DAP (standard deviation) of single species *E. faecalis* and polymicrobial immature biofilm

Type of treatment	***E. faecalis* Biofilm Mean log_{10} (SD)**	**Immature Biofilm Mean log_{10} (SD)**
1 mg/mL radiopaque DAP	0	0
1 mg/mL non-radiopaque DAP	0.66 (1.87)	0
No treatment	7.08 (0.09)	7.00 (0.02)

*Different upper case letters indicate significant differences between biofilms from *E. faecalis* 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

TABLE II

The comparison of antibacterial effects of the different radiopaque medicaments against an *E. faecalis* bacterial biofilms or a biofilm from an immature tooth with pulpal necrosis.

Type of treatment	*E. faecalis* Biofilm Mean $\log_{10}$ (SD)	Number of positive samples	Immature Biofilm Mean $\log_{10}$ (SD)	Number of positive samples
10 mg/mL RoDAP	0.00 (0)	0/8	0.00 (0)	0/8
1 mg/mL RoDAP	0.00 (0)	0/8	0.00 (0)	0/8
Ro-methylcellulose	6.96 (0.04)A	8/8	6.82 (0.03)Aa	8/8
No treatment	7.00 (0.02)	7/7	7.00 (0.02)a	7/7
Sterile control	0.00 (0)	0/8	0.00 (0)	0/8

*Different upper case letters indicate significant differences between biofilms from *E. faecalis* 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.

TABLE III

Comparing the *E. faecalis* vs. polymicrobial immature biofilm effect for each treatment

Type of treatment	p-value
10 mg/mL RoDAP	1.0
1 mg/mL RoDAP	1.0
1 mg/mL DAP	1.0
Methylcellulose (MC)	0.46
RoMC	0.01*
No treatment	0.08
Sterile control	1.0

*Significant difference between the *E. faecalis* biofilm and polymicrobial biofilm from an immature tooth within the given experimental group

DISCUSSION

The field of regenerative endodontics has changed the mindset and vision of the endodontic community. It has provided another avenue in which to treat an immature tooth with a necrotic pulp. For decades an immature tooth with short, thin radicular dentin in conjunction with blunderbuss apices has challenged endodontists. Young children and adolescents who would lose these teeth due to lack of treatment options were left with critical problems namely the loss of dentition as well as the loss of alveolar bone adjacent to the missing tooth. Regenerative endodontic procedures have been shown to be a safe and predictable procedure in which to treat these individuals in hopes to maintain their dentition. These procedures focus on regenerating the pulp-dentin complex by enabling the body through tissue engineering practices to not only resolve clinical signs and symptoms of infection, but to take a stalled underdeveloped tooth root and provide an avenue for it to mature. Despite the difficulty and underlying complex biological nature of the procedure, the disinfection stage encompasses three main tissue engineering principles, stem cells, scaffolds and growth factors. However, without adequate disinfection the process of regenerating the pulp-dentin complex will not occur. An adequate disinfection process balances the effects of antibacterial properties while maintaining viability of stem cells and growth factors. Proper disinfection will provide an environment that is conducive to regeneration of host tissues within the tooth.

The American Association of Endodontics has support and recommended the use of intracanal medicaments during the disinfection phase of regenerative endodontics. Their current guidelines suggest the usage of either antibiotic pastes or calcium

hydroxide. In the past triple antibiotic paste (TAP) was used successfully in regenerative endodontic procedures but cause unwanted discoloration of tooth structure. [23, 37] More recently, there has been a growing interest in the use of a double antibiotic paste (DAP) in which minocycline, the antibiotic responsible for discoloration when TAP is used, has been excluded.[113] The use of TAP or DAP at low concentrations has been shown to have superior direct and indirect antibacterial effects while maintaining the viability of stem cells needed to facilitate the regenerative process.[41,46]

A major limitation in both the TAP and DAP preparations is the lack of radiopacity and therefore they cannot be visualized within the canal system, unlike other medicaments such as calcium hydroxide. When treating immature teeth with open apices it is important to carefully manage the apical area so as not to over- or under-apply the antibiotic paste which could lead to harmful effects and compromise results. In order to overcome this difficulty our study focused on the use of radiopaque DAP and to test it direct antibacterial effects against a known multispecies biofilm from microorganisms obtain from an immature tooth with a necrotic pulp as well as a single species biofilm obtained from an *E. faecalis* isolate. By so doing, a clinician can visualize radiographically the placement of radiopaque DAP within the canal system while understanding the direct antibacterial effects of the medicament. Historically there have been a variety of radiopacifying agents used in the dentistry such as; barium, zirconium, bismuth, and zinc. All of which are salts of heavy metals. Recent studies completed at IUSD have focused on using barium sulfate ($BaSO_4$) in DAP preparations and have found that the opacifier had no effect on the antibacterial properties of DAP. The focus of our study was to examine the radiopacifier, zirconium oxide, to see if it affected the

antibacterial properties of DAP. Currently the FDA approved zirconium oxide to be used in many endodontic products from sealers to root end filling materials such as Biodentine, Endosequence root repair material, Endosequence Bioceramic sealer, and AH Plus sealer. It has been shown to improve the physical and biological properties as well as the biocompatibility of these popular bioceramic materials.[149-151] Specifically, zirconium oxide has been shown to reduce the initial and final setting times of bioceramic cements,[152] as well as having the ability help induce fibroblast proliferation and accelerate the reduction of inflammatory processes.[153] When compared to bismuth oxide, zirconium oxide increased the compressive strength of Portland cement as well as increasing its biocompatibility.[151] Another study suggested that zirconium itself has antibacterial properties.[50] In the present study, it was shown that the radiopaque methylcellulose (RoMC) group had significantly lower immature biofilm colony count than methylcellulose (MC) as well as the no treatment group. In addition, *E. faecalis* biofilm colony count was significantly higher than immature biofilm counts for RoMC, with no significant differences between biofilms for any other group. Therefore, the addition of zirconium oxide may have an additive antibacterial effect against a three-week old multispecies biofilm obtained from an immature tooth with necrotic pulp.

In the past antibiotic paste medicaments were prepared in such a way as to add the antibiotic to sterile water and hand mix until a desired consistency was achieved. This undoubtedly resulted in a higher concentration of the antibiotic within the paste with poor handling characteristics. As described previously, studies have shown that higher concentrations of antibiotics in the medicament can have excellent direct antibacterial properties but caused decreased viability of stem cells needed for regeneration of the

pulp-dentin complex. To optimize the handling characteristics of the antibiotic paste a methylcellulose delivery system was employed. Methylcellulose has the ability to provide the paste with predictable stability and viscosity to increase its handling characteristics. Methylcellulose is currently used on the market with calcium hydroxide mixtures and has been shown to improve the physical and chemical properties of the paste by, providing more durability, increased longevity of the alkaline nature of the medicament, and allowing the paste to diffuse more readily with slower ionic dissolution.[154,155] The addition of methylcellulose to antibiotic pastes was shown to not alter its antimicrobial effect or cause detrimental effects to dental pulp stem cells proliferation and attachment.[38,156] Within our study DAP in combination with methylcellulose delivery system provided adequate ease of delivery and predictability of placement as well as ease of removal from dentin.

To date the AAE recommends the use of antibiotic pastes at low concentrations to balance the effect of disinfection with that of toxicity to stem cells. The guidelines suggest using antibiotic pastes at concentrations ranging from 0.1 mg/mL to 1.0 mg/mL during the disinfection process of regenerative endodontic procedures.[145] Knowing these recommendations, we utilized 1.0-mg/mL radiopaque DAP (RoDAP) within our experimental groups and found it had excellent direct antimicrobial effects against both an immature and *E. faecalis* biofilm. This finding was similar to previous studies that 1.0-mg/mL DAP was able to completely remove and eradicate biofilms.[38] Also found in our study and similar to previous studies was that higher concentrations of 10 mg/mL RoDAP had a tremendous direct antibacterial effect and completely eliminated biofilms. Therefore, the clinician must remember that regenerative endodontic procedures require a

fine balance of disinfection and biocompatibility. Though the desire and most important step in regenerative endodontic procedures is disinfection it cannot come at the cost of destroying viable stem cells. Studies have shown a detrimental effect on stem cells of the apical papilla (SCAP) with higher concentrations of DAP, specifically SCAP survival was reduced with the use of 10-mg/mL of DAP medicament.[109] In general, it has been demonstrated that stem cell survival when it comes to antibiotic pastes in dose dependent. Continuing with that theory, DAP at concentration of 1.0-mg/mL did not have detrimental effects on survival or proliferation of SCAP.[39,108,156] Our study tested the antibacterial properties of RoDAP and found it to have similar properties as reported in previous studies. Future studies will need to show RoDAP at varying concentrations and its effect on dental pulp stem cells.

Our study focused on two separate biofilms; one was multispecies being obtained from an immature tooth with necrotic pulp and the other against a single species *E. faecalis* biofilm. *E. faecalis* has historically been a difficult bacterial species to disinfect in endodontic infections due to its many virulence factors and its ability to survive in the root canal system during long nutritional deprivation.[24] It has been shown that *E. faecalis* can survive in the root canal system as a single organism rather than a polymicrobial community[25] and is capable of forming its own biofilm in the presence of intracanal medicaments.[26] It has been shown to be capable to eluding the effects of common irrigants and medicaments such as sodium hypochlorite and calcium hydroxide.[46,122] *E. faecalis* is one of the most frequently isolated species from failed root canal treatments and resistant infections with a prevalence ranging from 24 percent to 77 percent.[24,157] Therefore this our study focused on the direct antibacterial effects of both an immature

multispecies biofilm and the tough to eradicate, difficult endodontic pathogen, *E. faecalis* biofilm. It was shown that 1.0-mg/mL RoDAP had significantly lower colony count than MC, RoMC, and the no treatment group for both biofilms. The study showed that there was no bacterial colony growth obtained on blood agar plates from the RoDAP group, concluding that RoDAP has a direct antibacterial effect against both an immature and *E. faecalis* biofilm. This finding is similar to previous studies of Alaa et al who found that *E. faecalis* DAP at low concentrations was more effective than calcium hydroxide against an *E. faecalis* biofilm and that DAP could be considered an effective antibacterial substitute for TAP.[46]

In this study, the application of medicament remained for 1 week. The AAE guidelines call for the disinfection medicaments to be within the canal system for 1-4 weeks. Our treatment time of 1 week in our study fell within the lower range of the current recommendations and guidelines presented by the AAE. Studies have shown and suggested more specific time tables for treatment with medicaments in regenerative endodontic procedures, even though 1 week has been found to eliminate bacteria.[156] Jenks et al found that with varying concentrations of DAP ranging from 5.0-mg/mL to 500 mg/mL DAP was shown to have substantial and significant residual antibacterial effects against a single species *E. faecalis* biofilm when compared to one-week treatment.[158] Regardless of treatment time, the primary goal of the disinfection stage is to see a clinical reduction or elimination of the signs and symptoms of disease. This should be the ultimate deciding factor for the length of time for treatment.

There are limitations in this study that need to be addressed. Though the use of a multispecies isolate obtained from an immature tooth with pulpal necrosis provides a

mean for testing direct antibacterial properties of various antibiotic pastes, the isolate is only from one individual. Even with the addition of a second studied *E. faecalis* biofilm, studies have shown that a wide range of microbial diversity exist from tooth to tooth and that one tooth with similar preoperative diagnosis could have unique microbial make ups.[159] Thus, further studies that have more diverse bacterial sample sizes need to be conducted to help confirm our results. Another limitation with the immature isolate is that during conception and retrieval of the isolate from an immature tooth with necrotic pulp it is very difficult to do a procedure while maintaining an anaerobic environment. Therefore, one could conclude that obligate anaerobes may have been selected because of the presence of oxygen. Maintaining an anaerobic environment throughout the length of the study my need to be examined in future studies. Another drawback with the use of zirconium oxide RoDAP is to examine its use over longer treatment times at concentrations between 0.1-mg/mL and 1.0 mg/mL to see if it has residual antibacterial properties. Though shown in previous studies, another investigation to consider would be the direct application of zirconium oxide alone against different species of endodontic microorganisms compared to other radiopacifying agents to see if these opacifiers have inherent antibacterial properties.

SUMMARY AND CONCLUSION

In summary, the purpose of this study was to investigate the direct antibacterial effect of 1.0-mg/mL and 10-mg/mL zirconium oxide infused radiopaque DAP (RoDAP) on radicular dentin containing biofilms formed by a multispecies isolate obtained from an immature tooth with pulpal necrosis and single species *E. faecalis* isolate. A key reason for this study was to evaluate the use of a zirconium oxide radiopacifier in combination with DAP to better understand their antibacterial properties and utilize them in regenerative endodontic procedures.

The results from this study show significant direct antibacterial effects against both biofilms when treated for 1 week with 1.0-mg/mL RoDAP and 10-mg/mL RoDAP. Thus, our null hypothesis that 1.0-mg/ml and 10-mg/mL concentrations of RoDAP will have no significant antibacterial effect on the formation of multispecies biofilms from bacterial isolates from an infected immature tooth with necrotic pulp as well from a single species *E. faecalis* biofilm was rejected. Our alternative hypothesis was not rejected – that 1.0-mg/ml and 10-mg/mL concentrations of radiopaque DAP will demonstrate a significant antibacterial effect on the formation of multispecies biofilms from bacterial isolates from an infected immature tooth with necrotic pulp as well as a single species *E. faecalis* biofilm. With the addition of a radiopacifier to the DAP preparation, it can be stated that the precise application of RoDAP, confirmed radiographically, and its direct antibacterial properties may be beneficial for intracanal disinfection during regenerative endodontic procedures.

In conclusion both 1.0-mg/mL and 10 mg/mL RoDAP demonstrated significant antibacterial effects against bacterial isolates from an immature tooth with a necrotic pulp as well as an *E. faecalis* isolate.

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ABSTRACT

THE ANTIBACTERIAL EFFECT OF A RADIOPAQUE DOUBLE ANTIBIOTIC PASTE AGAINST BOTH AN ESTABLISHED MULTISPECIES AND A SINGLE *ENTEROCOCCUS FAECALIS* BIOFILM

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For regenerative endodontic procedures (REPs) to be successful an elimination of bacteria from the root canal system must be accomplished. Many different medicaments with antibacterial properties have been used to obtain complete disinfection. Double antibiotic paste (DAP) containing a mixture of ciprofloxacin and metronidazole has been shown to be a promising intracanal medicament. The addition of a radiopaque filler such as zirconium oxide to DAP may affect the antibacterial properties of DAP as well as allow precise placement and radiographic visualization of its position in the canal system. The aim of the proposed study was to evaluate the direct antibacterial properties of zirconium oxide radiopacifier combined with DAP (RoDAP) against a multispecies

biofilm from a bacterial isolate from an infected immature tooth with a necrotic pulp and a known single species biofilm.

4x4 mm radicular dentin specimens (n = 112) obtained from human extracted teeth were used prepared and sterilized prior to use. A multispecies clinical bacterial isolate from an immature tooth with a necrotic pulp and a single species *Enterococcus faecalis* isolate were obtained. These bacterial isolates were used to inoculate dentin slabs and grown for 3 weeks. The dentin slabs were treated for 1 week with 1.0-mg/mL and 10-mg/mL RoDAP, 1.0-mg/mL DAP, and two placebo pastes consisting of methyl cellulose (MC) and methyl cellulose combined with zirconium oxide (RoMC), respectively, as well as two no-treatment controls. Following treatment, the grown biofilm was detached and spiral plated. The plated biofilm cells were cultured for 24 hours and each group examined using a colony counter to determine bacterial numbers (CFUs/mL). Data analysis, using a 5.0-percent significance level was conducted using one-way ANOVA followed by pair-wise group comparisons.

Both 1.0-mg/mL and 10 mg/mL RoDAP demonstrated significant antibacterial effects against bacterial isolates from an immature tooth with a necrotic pulp as well as an *E. faecalis* isolate. The precise application of RoDAP confirmed radiographically with its direct antibacterial properties may be beneficial for intracanal disinfection during REPs.

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