ANTIMICROBIAL PROPERTIES OF DRUG-CONTAINING ELECTROSPUN SCAFFOLDS

by

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INTRODUCTION

The field of tissue engineering (TE) has greatly evolved over the past decade.[1-3] The combination of life science principles and engineering technologies have been the foundation for the development of biological substitutes that recreate functional, healthy tissues and organs previously injured or lost due to trauma and disease.[2,4-6]

Dentistry has made significant progress towards implementing TE-based procedures to repair and regenerate tissues of vital importance to the oral maxillofacial complex. The use of tissue scaffolds, bone grafting materials, and oftentimes the local delivery of a great variety of cytokines and growth factors, have been able to some extent, to regenerate distinct dental-related tissues.[2,4,7-12]

Although the field of endodontics has been aware that healthy pulp tissue exists within the infected/inflamed pulp, resective therapy, i.e. pulpectomy/pulpotomy, has been the standard of care, and few attempts have been made to repair the infected pulp. In recent years the revascularization technique has changed the treatment of the necrotic immature tooth, and such teeth are now able to continue normal root development both in length and in width (maturogenesis).[13] In brief, the root canal system (RCS) is disinfected by using irrigation solutions with minimal to no instrumentation followed by the application of a triple antibiotic paste (TAP) consisting of metronidazole, ciprofloxacin, and minocycline. After 3 to 4 weeks, the tooth is re-entered and the TAP is removed with irrigation solution; then, the root canal space is dried and minimally instrumented beyond the apex to induce bleeding for the formation of a blood clot in the canal.[14] With bleeding, endogenously produced growth factors and stem cells from the

periapical tissues populate the fibrin-based natural scaffold inducing the regeneration process.[8,9] The blood clot serves as a natural scaffold (i.e., fibrin) that aids with new tissue formation and root maturogenesis.[14] The natural scaffold offers host acceptance, because it is the patient's own blood, and the scaffold also reduces the chances of an outside pathogen entering the canal space. In medicine, tissue engineering does not rely on blood clot formation because the distribution and composition of cells is unpredictable. This is a critical limitation to the blood clot. Due to these shortcomings, research is being done to investigate artificial scaffolds.[4,8]

Research is directed toward improving all areas of the revascularization process with scaffolds being of significant importance. This requires materials to act as scaffolds that could be more desirable than the natural blood clot because obtaining an adequate blood clot may be a problem given the variability in blood clot quality.[9,15-17] In general, the properties of an ideal scaffold (natural or synthetic) are biocompatibility, biodegradability, and the display of a three-dimensional porous structure. The latter mimics the extracellular matrix that is able to provide a spatially correct structure with adequate mechanical properties to support new pulp tissue formation, by supporting cell differentiation, growth, and sustained angiogenesis.[8] Furthermore, the scaffold should be able to degrade at a similar rate to that of new tissue formation.[18,19] Natural scaffolds include collagen, glycosaminoglycans, demineralized or native dentin matrix, and fibrin. Synthetic materials encompass biodegradable and biocompatible polymers such as poly(DL-lactide-*co*-glycolide) (PLGA), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(caprolactone) (PCL) among others.[3,7] These materials have proven to be

capable of supporting the growth of many different cell types[3,7,14] and may also control infection by facilitating the release of antimicrobial agents.[19,20]

High-voltage driven electrospinning is a facile, nanotechnology-enabled technique to fabricate synthetic nanofibrous scaffolds mimicking closely the morphological nanofeatures of native extracellular matrix (ECM). In the electrospinning process, a generated electrical field produces solid fibers from a polymer-based solution (Figure 1). Numerous researchers have proposed the use of the electrospinning method to fabricate biologically active scaffolds, i.e., scaffolds incorporated with therapeutic drugs and growth factors, aiming to assist in both the healing and regeneration processes. Another advantage of the drug delivery system in nanofibrous scaffold is the possibility of delivering uniform and highly controlled drug doses at the root canal system via the high surface area to volume ratio of the nanofiber system. The ability to incorporate drugs into a synthetic matrix may have implications for regenerative endodontics. Scaffolds could be made to incorporate antimicrobial properties in the form of antibiotics, and this might improve healing and regeneration of dental tissues (pulp and root).

The electrospinning technique was used to generate antimicrobial-containing scaffolds. Nano-microfibrous polymer-based scaffolds were obtained from a polydioxanone solution (PDS II). Metronidazole (MET), an antibiotic that is part of TAP for disinfection used in the revascularization method, was directly mixed at two distinct concentrations (5 wt % and 25 wt %) with the PDS solution and co-electrospun in order to obtain the different scaffolds. Metronidazole is known to be effective against gram-negative anaerobes. It has also been shown that MET is effective in the periodontal tissues.[21] The antibacterial properties of the manufactured scaffold were examined

against *Porphyromonas gingivalis*, a known microorganism associated with primary endodontic infections[22-24] and which served as the test bacterium for the antibacterial properties of the manufactured scaffold.

Antimicrobial properties of restorative materials have [25]been evaluated *in vitro* using various methodologies. The agar diffusion test (ADT) was the standard assay used in most of these studies despite its known limitations. Problems associated with the ADT include its qualitative nature, ability to measure only soluble components, inability to distinguish between bacteriostatic and bactericidal effects, and difficulties in comparing a large number of samples with a large number of variables.[26] Alternatively, the direct contact test (DCT)[27-29] has been used to quantitatively measure the antimicrobial effect of direct and close contact between the test microorganism and the material being tested. It is based on turbidimetric measurements of bacterial growth that is, more turbidity correlates with more bacterial growth. A 96-well microtiter plate was used and the results are independent of the solubility and diffusibility of the dental material being tested[26] We modified the well-known direct contact test to evaluate the antibacterial properties of the drug-containing electrospun scaffolds. The aim of this study was to evaluate the *in-vitro* property of a synthetic polymer scaffold to function as an antibacterial drug delivery device. The goal was to determine if there would be significant bacterial inhibition using a metronidazole-containing scaffold.

CLINICAL SIGNIFICANCE

Electrospinning is a nanotechnology that can fabricate synthetic scaffolds that mimic the morphological nanofeatures of the native extracellular matrix. For regenerative endodontics, the use of this technology is a novel idea and has not yet been done.

Utilizing the high surface area to volume ratio of the nanofiber system, it could be made into an effective matrix satisfying the ideal properties needed of a scaffold by controlling drug dose and concentration while supporting cell growth in a spatially stable porous environment ideal for the regeneration process.

HYPOTHESES

Null Hypothesis

P. gingivalis would be significantly inhibited by the drug release from the MET-containing PDS electrospun scaffolds.

Alternate Hypothesis

P. gingivalis would not be significantly inhibited by the drug release from the MET-containing PDS electrospun scaffolds.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Since the beginning of endodontics in the 17th century, there have been significant advances for the improvement of endodontic diagnosis, treatment, and outcomes.[30] Charles Allen in 1687 detailed the technique of dental transplants as being "taking out the rotten teeth or stumps and putting in their places some sound ones drawn immediately out of some poor body's head."[31] At that time the primary objective of endodontics was to relieve pain and preserve the tooth. Many times these crude attempts were unsuccessful, and this prompted new experimentation on technique and theory. Many theories were established as the cause of tooth pain. Pierre Fauchard is considered "the father of modern dentistry" and wrote the book *The Surgeon Dentist* which gave descriptions of various dental procedures. This book gave detailed descriptions of pulp chambers and canal anatomy of human teeth, the procedure of relieving an abscessed tooth by accessing the tooth and leaving it open for a period up to two months, and then filling the pulp chamber with lead foil. Pulp removal was done by using a small pin to extirpate the pulp. Fauchard's treatment for carious lesions involved the application of many oils left for a period of weeks. For persistent tooth pain that could not be addressed by his described methods, he mixed opium with the oils he used.[31,32]

In the 18th century, several ideas and concepts were introduced to preserve the pulp. An important attempt was made by Phillip Pfaff, a German dentist. He was the first to describe a pulp capping procedure. He cut a piece of metal (gold or lead) to fit over the exposed pulp.[31] Thomas Roders described the pulp capping procedure in more detail by

presenting 220 cases of pulp capping where 202 were considered to be successful. His criteria for success included: good patient health, no inflammatory tendencies related to the patient, no history of previous pain, and no other disease associated with the tooth in question.[31] Codman objectified pulp capping as obtaining secondary dentin at the site of pulp exposure. He was the first to define the objective of pulp capping as secondary dentin deposition.[33] However, a few years later G.V. Black reviewed 42 cases of pulp capping with a 10 year follow-up and concluded that only six were considered successful at five years.[32]

The 19th century brought advances in canal treatments in endodontics. Formalin was discovered and used to fix the pulp tissue.[30] John P. Buckley first introduced formocresol to the dental profession. It was made from a mixture of tricresol and formalin, although it would eventually be questioned due to the potential for toxicity. Formocresol was used for more than 50 years.[34-36] Also In the 19th century, the first instrument was made consisting of a watch spring and the concept of filing the canal; the rubber dam was introduced to dentistry and consisted of a thin sheet of rubber to isolate the tooth during gold foil restorations.[31, 37] Clarke Dubuqe introduced gutta-percha as an obturation material.[31] The technique consisted of placing hot gutta-percha into the root and delivering it to the apex with a hot churning instrument.[31,33] Another significant finding was by Rodgers who published an article in *Dental Cosmos* and suggested that pathogenic organisms may be the etiology of the diseased pulp.[38] Rodgers advocated that successful endodontic treatment was a direct result of pathogenic organism destruction. With the recent theory of pathogenic organisms in diseased pulps, Charles S. Tomes attributed this contamination to the lack of tooth vitality.[38] Toward the end of the 19th

century, W.D. Miller created the bacteriological basis for endodontic treatment and published it in a paper that discussed gangrenous tooth pulps as centers of infection.[38] This new concept justified pulp therapy and dentists began using toxic materials (phenol, tricresol-formol, paraformaldehyde, camphor, etc.) to kill bacteria. Eventually, many of these toxic materials would be evaluated and discontinued.

The 20th century brought significant advancements. One of the major ones in the development of endodontics was the discovery of x-rays by Konrad Wilhelm von Roentgen and a German dentist Otto Walkhoff.[30,31] This new technology would not be accepted by the American dental profession until 1910, but once accepted, it was a valuable diagnostic tool on a large scale. The first dental x-ray machine was available for use in 1913; it was a crude first edition and would later be improved in 1919 by the addition of the Coolidge tube, which improved concentrating the beam.[38,39] Edmund Kells transitioned the new 'x-ray' technology into practice with dentistry forever changing the profession.[40] Shortly thereafter, periapical radiolucencies were visualized and radiography became a part of diagnosing pulpless teeth.[40] In the early 1900s, techniques were introduced for root length measurement and obturation level so overfilling could be prevented.[34] This period was a critical time for advancement in endodontics; radiographs and safe local anesthetics were available, and this set the stage for rapid progress in all areas of endodontics.[41]

Endodontics had a setback when William Hunter, an English physician and pathologist, gave a lecture on focal infection, and for more than 20 years it blocked research and the teaching of endodontics.[30] His lecture, "The Role of Sepsis and Antisepsis in Medicine," theorized many physical ailments were a result of dental

treatment.[42] For the next 25 years no attempts were made to save devitalized teeth by most dentists. Teeth were extracted due to adherence to the focal infection theory. Nevertheless, there was a group of men who continued to improve their current procedures and administer treatment. These men included Coolidge, Grove, Prinz, Callahan, Reihn, and Johnson. Thanks to their efforts the principles of endodontics persisted. By the late 1940s the focal infection theory was debunked and endodontic treatment began to regain acceptance.[42]

In the 1920s Hermann used a calcium hydroxide paste for filling root canals and later in the 1930s he supported the use of calcium hydroxide for pulp capping, pulpotomy, pulpectomy, and for medication of infected canals.[38,43] His advocacy for calcium hydroxide came from the previous use of caustic medicaments. He deduced that these toxic materials would find their way into the surrounding dental tissues. He also demonstrated calcium hydroxide's effectiveness by severing a viable pulp and in response to calcium hydroxide a dentinal bridge was formed over the severed pulp.[38]

The 20th century brought many innovations and scientific reasoning to endodontics. The first obturation cement was suggested for use by U. G. Rickert.[44] He coated a gutta-percha cone with cement then placed the cone in the canal with pressure.[44] Shortly after this innovation, an instrument was designed to laterally condense the gutta-percha improving the lateral and apical distribution of the obturation material.[34] The same year Rickert introduced his root canal cement, and a Lentulo demonstrated his rotary paste inserter. It was a spiral wire made of flexible steel, when rotated counterclockwise, it carried medicaments to the apical area of the canal.[44] It was soon accepted and recommended for the delivery of Rickert's root canal cement.[44]

In the 1940s Adams and Grossman introduced antibiotic use in root canal therapy and were the first to use penicillin in pulp canal therapy.[34] Grossman recommended a non-aqueous preparation of penicillin for intracanal use. He hypothesized that it would be more stable than the aqueous form. He later used impregnated paper points in the canal to sterilize the canal space.[44,45] Intracanal antibiotic therapy was logical and promising at the time and created a shift in the management of periapical disease.[44] There was an increased focus on chemotherapeutic treatment and less emphasis on the mechanical cleaning and shaping.[44,46] However, resistant strains of microorganisms eventually surfaced, requiring the use of additional antibiotics.[44] Science and technology produced a variety of antibiotics, but it was later discovered that antibiotic therapy did not completely sterilize the root canal space. In the early 1950s, there was a shift back to emphasizing the importance of mechanical cleaning of the root canal system and not relying solely on drug therapy. This introduced the concept of chemo-mechanical cleaning of the canal system to reduce bacteria and obtain asepsis.[44,46] These important changes with science and drug therapy laid the foundation for the goals of endodontics.

In 1943, 20 dentists convened with the intention to form an organization of endodontists.[47] Their organization continued to grow and in 1956 the American Board of Endodontics was created. The American Dental Association recognized endodontics as a specialty in 1963.[47]

THEORY OF ENDODONTICS

In 1965 Kakehashi, Stanley, and Fitzgerald showed that pulpal and endodontic problems are caused primarily by bacterial contamination.[48] The objective of endodontic therapy is the same for both primary and secondary infections; it is to eliminate microbial

insult to the pulpal and periapical tissues to maintain natural form, function, and esthetics.[49,50] Periradicular periodontitis is defined as inflammation and destruction of the periodontium that may or may not produce symptoms.[51] Bacterial byproducts that are found within the canal space can exit through the apex and lateral, or accessory canals that can produce periradicular inflammation.[52] Endodontic success is directly correlated with the reduction of bacteria in the canal space.[48,53] Bacterial reduction is achieved by cleaning and shaping of the canal system.[49,50,54] Modern endodontic practice is concerned with first opening the canals so that a reservoir exists to facilitate irrigation solution introduction into the canals for effective disinfection.[55,56] In his 1955 study, G.G. Stewart described three phases of root canal therapy: chemomechanical preparation, microbial control, and obturation of the root canal.[57] His study concluded that chemomechanical preparation was the most important part of endodontic treatment. As canal diameter is increased during mechanical preparation, the dentinal walls are cleaned by direct contact and debris and bacteria are removed. This enlargement also promotes irrigation solution delivery to the apical area. Grossman[58] later confirmed Stewart's findings that chemomechanical instrumentation is effective at removing bacteria and debris. In addition Grossman provided 13 principles that should be followed during root canal therapy:

1. Aseptic technique.
2. Instruments should remain within the root canal.
3. Instruments should never be forced apically.
4. Canal space must be enlarged from its original size.
5. Root canal system should be continuously irrigated with an antiseptic.

6. Solutions should remain within the canal space.
7. Fistulas do not require special treatment.
8. A negative culture should be obtained before obturation of the root canal.
9. A hermetic seal of the root canal system should be obtained.
10. Obturation material should not be irrigating to the periapical tissues.
11. If an acute alveolar abscess is present, proper drainage must be established.
12. Injections into infectious areas should be avoided.
13. Apical surgery may be required to promote healing of the pulpless tooth.

As Schilder[59] stated in 1967, the ultimate objective of endodontic therapy is the removal of diseased tissue and contents to reduce infection and inflammation of the periapical tissues. Schilder recommended chemomechanical preparation of the canal with instruments and antiseptics followed by a three-dimensional obturation to the cementodentinal junction or 0.5 mm to 1 mm from the radiographic apex. Pitt Ford[60] subsequently expanded on Schilder's objectives for obturation and gave three reasons for the importance of three dimensional obturation: First, obturation of the canal space would leave less space for bacterial colonization. Second, it prevents apical contamination and third, it prevents bacterial movement along the canal walls. Weine[61] argued that to achieve the objective of endodontics, it is necessary to completely debride the canal of bacteria and debris. Furthermore, he stated that when canals are properly cleaned and shaped, any method of obturation is adequate; rubber dams are mandatory to prevent contamination; all endodontically treated teeth need adequate restorations; and patients should be evaluated for healing. All these factors contribute to a successful outcome in root canal therapy.

MICROORGANISMS

A primary intraradicular infection is an infection of the necrotic pulp space and is the etiologic agent of apical periodontitis.[62] It is a mixed community of mainly anaerobic gram-negative and gram-positive bacteria. The gram-negative bacteria are frequently identified as *Fusobacterium, Dialister, Porphyromonas, Prevotella, Tannerella, Treponema, Campylobacter and Veillonella*. Gram-positive bacteria are identified as: *Parvimonas, Fillifactor, Pseudoramibacter, Olsenella, Actinomyces, Peptostreptococcus, Streptococcus, Propionibacterium and Eubacterium.*[63] Morphological studies have shown that the bacteria found in primary infections are diverse and includes cocci, rods, filaments and spirilla.[64,65] It is well-known that bacteria that colonize the root canal grow mainly in sessile biofilm communities adhering to the dentinal walls.[65] This gives bacteria uninhibited access to dentinal tubules and dentinal tubule infection reportedly occurs in 70 percent to 80 percent of teeth with apical periodontitis.[66]

Root canal infection is characterized by a change in bacterial species during different stages of the infection.[62] These changes in bacterial diversity are a result of changes in the environmental conditions especially with changes in oxygen and nutrient availability.[67] The initial infection involves mainly facultative bacteria, and after a few days or weeks, oxygen becomes limited due to metabolism of initial bacteria and reduced blood flow due to pulp necrosis. This creates an environment suitable for the anaerobic bacteria and they begin to dominate.[62]

Porphyromonas gingivalis is an asaccharolytic, non-motile, obligatory anaerobic, coccobacilli gram-negative rod.[68] It can be found primarily in subgingival plaque and can also be found on the tonsils and dorsum of tongue.[69] *P. gingivalis* has been shown to

occur in endodontic infections with chronic apical periodontitis, acute apical periodontitis, and acute apical abscesses.[41] The reported incidence of *P. gingivalis* ranges among the literature from 5 percent to 97 percent.[22,24] Clonal types of *P. gingivalis* can have varying degrees of virulence, and there is a high degree of heterogeneity reported for *P. gingivalis.* Different strains have been shown to differ in their tissue destruction.[22] Siqueira et al.[22] confirmed the findings of many other studies in that *P. gingivalis* is one of the most pathogenic species present in the human oral cavity. *P. gingivalis* produces many enzymes, proteins, and metabolic end products that are active against a wide range of host proteins and provides routes for evading host defenses.[68] The most common virulence factors of *P. gingivalis* are lipopolysaccharide, fimbriae, capsule, lipoproteins, outer membrane proteins, proteinases (collagenase, gingipains), fibrinolysin, phospholipase, alkaline and acid phosphatases, nucleases, hyaluronidase, chondroitin sulfatase, hemolysins, and toxic metabolites.[68,69] These virulence factors give *P. gingivalis* many mechanisms for self-preservation and disease pathogenesis to the host. Siqueira et al.[69] states that these virulence factors can further the disease process by acting as adhesins to promote bacterial aggregation to form biofilms; sustenins to maintain growth and survival; invasins to allow invasion of tissue; evasins to evade host defenses; aggressins to cause direct tissue damage; and modulins for indirect tissue damage. It is clear that the pathogenicity of *P. gingivalis* is diverse and not limited to a few virulence factors. These mechanisms for pathogenesis give insight to how *P. gingivalis* is able to invade root canal systems and cause infection.

A secondary infection results when bacteria that were not present in the primary infection are subsequently introduced into the root canal after root canal therapy.[70]

Bacteria responsible for secondary infections can gain access to the canal space during treatment, between appointments, or even after obturation via coronal leakage.[62,71] Whatever the avenue, it is most likely traced back to an oversight in asepsis.[62] The bacterial diversity of secondary infections is lower than for primary; the presence of *Pseudomonas aeruginosa, staphylococcus* species, *Escherichia coli, and Enterococcus faecalis* are characteristic of a secondary infection.[62] Gram-negative bacteria that are commonly involved in primary infections are usually eliminated.[72] Many studies have shown that *E. faecalis* is the most frequently found bacteria in secondarily infected canals.[73,74] Its persistence in canals is attributed to its ability to colonize the canal space and resist treatment procedures.[75] In general, most studies show that when bacteria persist, gram-positive bacteria are more frequent.

REGENERATIVE PROCEDURES

Regeneration of tissue back to its original and functional state has always been a goal for medicine.[76] Regenerative medicine is centered on three key components: stem cells, scaffolds, and growth factors to replace diseased or damaged tissues and restore physiological function. One of the key components of regenerative medicine is the use of a scaffold. The scaffold provides a three-dimensional environment for cells to attach and grow mimicking the extracellular matrix of the *in vivo* tissue.[76] A new biomedical field aimed at developing this approach is called tissue engineering. The goals of tissue engineering are regeneration of natural tissues and the creation of biological substitutes for defective, lost, or diseased tissue by utilizing stem cells, growth factors and scaffolds.[1,77,78]

With the recent advances in tissue engineering in medicine, there has been considerable effort in clinical studies and research towards regeneration of the pulp dentin complex in endodontics.[79] Dentistry is the practice of replacing lost or diseased tooth structure with a functional substitute of inert materials.[41] Regenerative efforts focus on the biologic replacement of diseased or damaged tissues. Early attempts of regenerating dental tissues began in the 1950s by B.W. Herman[80] when he described calcium hydroxide application for vital pulp therapy. Ostby[81] later attempted to determine if filling the canal with a blood clot would lead to regeneration. In the 1970s, Myers et al.[82] conducted similar experiments with generation of soft tissue observed, but only 0.1-1.0 mm of ingrowth was seen. The reason for poor results is that pulp tissue is different from periapical tissue. Therefore, pulpal regeneration from cells in adjacent tissue is unlikely.[76] Over the past several years, significant advancements have been made in dentistry with regenerative procedures; guided tissue and bone regeneration are areas that have seen significant improvements.

Stem Cells

All biological tissues have stem cell origin.[83] A stem cell is defined as a cell that can continuously divide and produce cells that can develop into many other types of cells and consequently different tissues.[4] Fortier[84] describes stem cells as embryonic (fetal) or adult (postnatal). These cells have different plasticities (potential for developing into specialized cells).[85] Stem cells can be further classified as totipotent, pluripotent, and multipotent with varying degrees of plasticity and source.[4] It is well known that embryonic stem cells have more plasticity than postnatal cells.[86] The more plasticity a cell has, the more potential for use in tissue engineering.[87] Although postnatal stem cells

have less plasticity, researchers have shown an increased interest due to ethical and legal issues.[4] Postnatal stem cells can be harvested from the umbilical cord, bone marrow, peripheral body fat, and many other tissues including the dental pulp.[4, 88] Dental pulp stem cells (DPSCs) are postnatal, multipotent stem cells.[12] Postnatal DPSCs are the most promising for endodontic tissue regeneration.[89] Stem cells can be further categorized by their source. Autologous cells come from the same person in whom the cells will be used. Some examples include bone marrow, adipose cells, the periodontal ligament, and umbilical cord to name a few. Allogeneic cells come from a donor of the same species. Some examples include blood transfusion and bone marrow transplants where the donor is not the same person as the recipient. Xenogeneic cells are cells from another species. An example would be animal cells that have been used in cardiovascular implants.[90] The dental pulp is known to have regenerative capabilities.[91,92] The pulp cells have the potential to differentiate into odontoblasts after carious exposure by responding to growth factors released from the surrounding dentin.[11] In the dental pulp, stem cells can be found concentrated in the perivascular region and the cell-rich zone next to the odontoblastic layer.[41] The pulp consists of fibroblasts, extracellular matrix, and collagen. To form this complex organization of loose connective tissue, several cell types are needed. Gronthos et al.[89] reported that at least five different types of postnatal mesenchymal stem cells have been identified in the formation of the dentin-pulp complex: 1) dental pulp stem cells (DPSC), 2) stem cells of human exfoliated deciduous teeth (SHED), 3) stem cells of the apical papilla (SCAP), 4) dental follicle progenitor cells (DFPC), 5) bone marrow derived mesenchymal stem cells (BMMSC). It has been found that the younger the patient is, the better the chance of having autologous stem cells available.[4]

Growth Factors

According to Smith,[92] growth factors are peptide molecules that transmit signals between cells functioning as stimulators or inhibitors of growth as well as modulators of cell differentiation. Growth factors can be classified based on their actions on cells. Autocrine growth factors act on the same cell where it was produced; juxtacrine growth factors act on neighboring cells, and paracrine growth factors act on cells in distant tissue.[77] These molecules are essential for cell activity and signal changes in tooth morphogenesis and differentiation by interacting with specific receptors on cells which causes a cascade of intracellular changes.[77,92] While there is much understanding of cytokines and growth factors involved with inflammation and immune responses, growth factors and signaling molecules related to pulpal repair and regeneration have only recently been identified.[92] The current understanding and role of identified growth factors is incomplete and a growing question in research. Regenerative endodontics relies on the release and use of growth factors to induce regeneration of tissue into the canal space. Recently, several growth factors have been identified and studied in relation to pulpal regeneration, transforming growth factor beta (TGF-*B*), bone morphogenic protein (BMP), and vascular endothelial growth factor (VEGF). TGF-*B* signals odontoblast differentiation, mineralization of pulp tissue, anti-inflammatory effects, and promotes wound healing.[4, 92] TGF has an overall effect of stimulating dentin matrix production, which is critical for root length and width development. TGF is found in the dentin matrix because it is secreted by odontoblasts in the matrix. BMP is another important growth factor in the regeneration procedure. BMP induces differentiation of odontoblasts, which results in mineralization of deposited bone critical to continued root

development.[4,92] VGEF is another important growth factor involved in the regenerative process; in wound healing vascularity is a critical factor for success. VGEF regulates angiogenesis by inducing chemotaxis, proliferation, and differentiation of cells for vascular ingrowth and dental pulp cells.[4,92] Other reports showing growth factors embedded in dentin are released during dentin demineralization with EDTA.[9,92] Dentin inherently has many growth factors associated with it. Demineralization of dentin can expose dentinal tubules; with this dissolution of tissue growth factors are released into the environment of the canal space.[4] The effectiveness of this source of growth factors is unclear, but studies show EDTA to very effective at releasing these growth factors.[9] Once these growth factors are released, they are capable of signaling the repair and regeneration of the pulp dentin complex.[93] While it is known that growth factors have a positive effect on successful regeneration, a complete knowledge of all growth factors involved and their distribution and concentrations is still unknown.

Scaffolds

Tissue engineering is a multidisciplinary field which has the potential to produce an artificial, functional, and immunologically tolerant organ tissue that grows in the patient.[78] This technology could give a permanent solution to a diseased or damaged organ. A critical component of tissue engineering involves a three dimensional substrate for the ingrowth of cells.[4,11,14,17,78, 90,94,95] These three-dimensional substrates are known as scaffolds. Scaffolds provide an environment for cell growth, differentiation, and angiogenesis to allow for integration with the host tissue.[96] Scaffolds function to provide a spatially correct positioning of the cell for appropriate growth conditions similar to the extracellular matrix.[4,11,97] Hutmacher[98] outlined the properties of a scaffold for tissue

engineering. First, the scaffold should be porous for tissue and vascular integration. Second, it must be biodegradable at a rate of tissue formation. Third, the scaffold must allow cellular attachment for differentiation and proliferation. Fourth, the mechanical properties of the site being implanted must be adequate. Fifth, the scaffold should not elicit any adverse reactions. Finally, it should be easily shaped into different sizes and shapes. Potential materials with these characteristic are natural polymers, synthetic polymers, ceramics, and combinations of these materials.[99]

Scaffolds can be categorized as either natural or synthetic. Examples of natural scaffolds include collagen, glycosaminoglycans, demineralized or native dentin matrix, and platelet rich plasma or (PRP).[41] When implanted, natural scaffolds start a host-tissue response that initiates the necessary biological responses to restore structure and function of the tissue being grafted.[96] Naturally occurring scaffolds also prevent many of the complications with foreign body reactions and are more readily recognized by the body.[96] The pulp space represents a significantly different environment for tissue engineering. Natural scaffolds present some challenges. Collagen gel has mainly been used as a scaffold material for the canal space. Collagen presents some shortcomings; it has shown severe contraction after placement resulting in undesirable mechanical properties, lack of dentin attachment, unstable healing, and insufficient regeneration of dentin-like tissue.[76,100] Other studies have shown success when using collagen as a scaffold material.[101] Its success in the literature is controversial. In the current pulpal regeneration procedure, a natural scaffold is used, which is a blood clot. The blood clot has limitations that need to be overcome for future progress of this exciting new treatment. A critical limitation to the blood clot revascularization approach is that the concentration and

composition of cells trapped in the fibrin clot are unpredictable.[4] Therefore, there could be variation in treatment outcome; the use of effective concentrations and compositions of cells is critical to success.[4]

Synthetic scaffold examples include polylactic acid (PLA), polyglycolic acid (PGA), polylactic-coglycolic acid (PLGA), hydroxyapatite, and bioceramics.[41] Polymer has been used in medicine since the discovery of polymers.[77] Nylon sutures were introduced in the 1940s and by the mid-1940s, they were significantly represented in the medical literature.[77] Since then, polymer use has expanded to prosthetics, grafts, implants, and artificial lenses. Polymer *in-vivo* use has been well-documented and accepted in medicine.[77] Currently, polymers are being applied in the field of tissue engineering and used to assist in the regeneration of three-dimensional structures.[1,77,78] Research has shown significant promise with synthetic polymer scaffolds and their clinical use and may have the most promise for creating replacement tissue.[2,18,102,103] Human dental pulp cells and fibroblasts have been shown to successfully adhere, proliferate, and produce extracellular matrix when seeded on polymer scaffolds *in vitro*.[3,7,14] Polymer scaffolds have many desirable properties for tissue engineering. When the polymer diameter is reduced to nanometers, the surface area increases dramatically and still remains functional with excellent mechanical and degradation properties.[104] Once in place, tissue formation relies on mechanical properties of the scaffold for cell growth and transmits them appropriately to surrounding tissue.[105] Degradation is essential for functional tissue regeneration and, ideally, the rate of degradation should mirror the rate of new tissue formation.[18,19,105]

Scaffold Fabrication Methods

Electrospinning has gained attention because of the ability to manipulate many polymers and also for creating fibers with crucial properties for scaffold use. Electrospinning can increase fiber surface area, create ideal surface morphologies, and has excellent mechanical properties.[106] Thus, scaffolds used for tissue engineering have been researched in the electrospinning field.[106-108] Non-woven electrospun nanofibers are very similar to the fibers in the extracellular matrix (ECM), which is advantageous for cell migration, distribution of necessary nutrients, and architecture.[106] Electrospun nanofibers are now an important part in vascular, neural, and tendinous tissue engineering.[106]

Polydioxanone (PDS) is a colorless, bioabsorbable, monofilament polymer that was made specifically for sutures. The PDS used for electrospinning in this study was obtained from Ethicon, Inc. (Somerville, NJ). As a suture, it has high flexibility independent of filament diameter, excellent shape memory, high strength, slow absorption rates, and low inflammatory response when compared with Vicryl and Dexon.[109] Upon degradation, 50 percent of its strength is reduced after three weeks, and by six months it is completely absorbed.[109] Electrospinning forms pores that are crucial for diffusion of molecules, cell integration and migration into the scaffold.[109] With these desirable properties, it has promise for tissue engineering uses.

There has been increasing interest in the incorporation of drugs into electrospun fibers.[106] An ideal scaffold should also maintain an antimicrobial environment to reduce contamination and promote healing.[19] Many drugs can be incorporated into electrospun polymers through electrospinning.[106] Local delivery of drugs by electrospun scaffolds

could significantly reduce systemic involvement of the drug while still delivering local effects.[106] The morphological features of electrospun polymers allow for potential and efficient drug release. Currently, these polymeric nanofibers are under investigation for application in drug use.[106,110,111] The electrospinning technique allows the molecular structure and bioactivity to remain intact[106] and drug release can be manipulated to be rapid, immediate, or delayed.[106,112] In the case of regenerative endodontics, an antibiotic dressing is applied and changed if needed.[4,14,113-116] Reapplication or changing of antibiotic dressings is an inconvenient process and increases risk of contamination. In the endodontic revitalization procedure the TAP must be removed and sometimes changed. Eliminating this step could improve treatment outcomes.

In this study, metronidazole was chosen because of its efficacy against obligate anaerobes, which are known endodontic pathogens.[22,24,113,117-119] In the present study, a 5.0- wt % MET and 25-wt % MET mixture were electrospun into two different scaffolds. Studies have shown that therapeutic doses of antibiotics have been successfully delivered with polymer scaffolds.[18,19,21] This has not been attempted in an endodontic application.

Regenerative Endodontics

Regenerative endodontics is defined as a biologically-based procedure designed to replace damaged, diseased, or missing dentin and root structures, as well as cells that restore the normal physiological functions of the pulp dentin complex.[4,14] Hemorrhage into a surgical or wound site resulting in a blood clot has been the foundation of creating an environment for healing. Ostby tested this principle in endodontics in the 1950s to investigate pulpal regeneration.[81] In the 1970s, Myers conducted similar experiments and was successful in soft tissue formation. However, it was limited to very small distances

into the canal and, furthermore, connective tissue ingrowth was not enhanced by blood or blood substitutes.[82] Histological findings have shown that with complete loss or degeneration of pulp tissue, the canal space is invaded by periapical tissue.[120,121] In the 1990s pulp-tissue regeneration was revisited with modern tissue engineering technology advancements.[76] Later *in-vitro* reports showed pulp cells were capable of induction to become odontoblast-like cells and deposit dentin like hard tissue.[88,122] Gronthos et al.[89] reported *in vivo* evidence of pulp cells depositing dentin in mice.[123] This and other research introduced the possibility of applying this technology clinically. Murray et al.[4] listed several regenerative endodontic techniques: (a) root canal revascularization via blood clotting; (b) postnatal stem cell therapy; (c) pulp implantation; (d) scaffold implantation; (e) injectable scaffold; (f) three-dimensional cell printing and; (g) gene delivery.[4] These procedures are based on tissue engineering principles that include cells, growth factors, and scaffolds. In the past, clinically acceptable results have been obtained through apexification procedures using long-term calcium hydroxide treatment. However, calcium hydroxide may negatively affect dentin with long-term use.[9] When presented with a necrotic immature tooth, endodontic therapy cannot prevent susceptibility to fracture in the future.[124,125] The advantage of regenerative endodontics over apexification is that continued root maturation is seen along with the ingrowth of vital tissue.[126] As regenerative endodontic procedures improve, they may become the standard of care, replacing previous techniques; and with improved knowledge and techniques, they may be applied to mature teeth in the future.[9,127]

Clinical Regenerative Endodontic Procedures

Apexogenesis

Apexogenesis is aimed at maintaining vital pulp tissue in the canal system to sustain continued root formation.[41] Unlike apexification, apexogenesis allows the root to continue normal development resulting in increased thickness and length of root structure. In the first 24 hours, the inflammation is located superficially in the pulp and after that, it moves more apically.[128] Clinically, a large or deep pulpotomy is done to remove inflamed and infected pulp tissue, allowing healthy tissue to remain in the canal. Apexogenesis can be accomplished in single-rooted anterior or posterior multirooted teeth. The tooth is accessed; a deep pulpotomy and hemostasis are completed, and the remaining pulp is treated with a dressing on the coronal aspect. In most cases, this dressing is some form of calcium hydroxide. Calcium hydroxide has been the dressing of choice for many years; however, it has some disadvantages. Studies have shown it can be unpredictable and also incorporates irregularities to the developing reparative dentin, which can be an avenue for bacterial contamination.[129] An alternative capping material is mineral trioxide aggregate (MTA). MTA has been shown to induce hard tissue formation in pulp tissues.[128] Studies have shown that MTA has the ability to induce a thicker dentinal bridge, less pulpal necrosis, and less pulpal inflammation.[128,129] However, MTA comes with some disadvantages; namely, it is more costly and has a delayed setting time. After successful dressing placement, the coronal access is sealed with a restoration. The patient is recalled, and the root is monitored for continued root development and dentin bridge formation beneath the dressing material. Apexogenesis is indicated when a tooth is

vital, or the apex is not completely formed, or there is a desire to maintain the tooth. With improved dressing materials, apexogenesis has become a more predictable procedure.

Apexification

Apexification is a procedure to form a calcified barrier at the apex of a nonvital permanent tooth; this barrier serves as a matrix that obturation material can be condensed against. Clinically, the canal is accessed and disinfected with minimal instrumentation to avoid weakening the thin dentinal walls; working length is determined radiographically. After debridement, calcium hydroxide is placed into the canal and access is sealed. The patient is then recalled at three-month intervals to determine apical barrier formation. After adequate barrier formation, the canal is obturated and a permanent restoration is placed in the access. The most important steps of the procedure are effective debridement of the canal and sealing the access. Apexification is not an attempt to reintroduce vital tissue into the canal space and has disadvantages. The long-term use of calcium hydroxide can reduce root strength.[130,131] In a large case series using apexification, the chief reason for tooth loss was root fracture.[41] Apexification is only capable of root end closure; no additional root structure is increased by length or thickness. It is considered the treatment of last resort.

Revascularization

The necrotic immature tooth presents potential complications for treatment. Standard cleaning and shaping with endodontic files are troublesome due to the thin dentinal walls. This is because instrumentation would further weaken the tooth.[126] Obturation is difficult as the apical seal is unpredictable due to the large immature apex.[41] Apexification has historically shown success; however, the long-term prognosis has not

been well-documented.[126,132,133] Even if an apical barrier is successfully formed, there is an increased risk of fracture. It has been shown that up to 30 percent of immature teeth can fracture after treatment.[133] Composite resin has been used to clinically strengthen the roots of immature necrotic teeth. This has internally increased the strength, but it eliminates the option for retreatment.[134] This would imply an ideal solution to the immature necrotic tooth would be to regenerate mineralized tooth structure. The goal of the revascularization procedure is to replace the damaged or diseased pulp tissue in the canal space.[4,41] Choen et al.[41] describe the clinical procedure as follows: during the first appointment, the tooth is isolated and accessed; working length is determined and irrigated with 20 ml of NaOCl; the canal is then dried with paper points and a triple antibiotic paste is placed in canal space. Bacteria present in the necrotic canal and deep layers of canal walls have been reported to be predominately obligate anaerobes.[118] Metronidazole has a wide spectrum of bactericidal action against oral obligate anaerobes, even in the necrotic pulp.[118,119,135] Hoshino et al.[113] found that 99 percent of bacteria in carious lesions and infected root dentin were eradicated in the presence of metronidazole. However, metronidazole did not kill all the bacteria, which suggests more antibiotics may be needed. Sato et al.[119] reported that ciprofloxacin, metronidazole, and minocycline can sterilize root dentin. In a later study, Sato et al.,[118] using the same triad of antibiotics, found that, *in vitro*, no bacteria were recovered in test tooth samples after 48 hours. They also found that triple antibiotic paste was able to penetrate 1 mm into dentin after 48 hours and was therefore an effective medicament for sterilization of the root canal. After antibiotic placement, the tooth is then sealed with a sterile sponge and a temporary restorative material. After 3 weeks to 4 weeks, the patient returns for the second

appointment. At this appointment, the tooth is isolated and re-accessed. Again, the canal is irrigated with 20 ml of NaOCl to remove the antibacterial paste, and according to Hargreaves,[9] a 10-ml rinse with EDTA is used to partially demineralize dentin and release embedded growth factors in dentin. Yamauchi et al.[126] suggested that partial dentin demineralization could help the adherence of mineralized tissue to the inner dentin walls. The canal is then dried with paper points; a large K file is placed a few millimeters beyond the apical foramen, and the apical tissue is lacerated. Bleeding was induced into the canal up to 3 mm from the CEJ. This blood clot serves as a scaffold for the regeneration of pulpal tissue. It has been shown that in humans, inducing bleeding into the canal space after disinfection can promote mineralized tooth structure thickening and lengthening.[136] Thibodeau et al.[137] concluded in a recent study that although the blood clot was not quantified, it was useful to induce tissue formation in the previously necrotic canal space. Murray et al.[4] have shown the blood clot contains vital components for regenerative endodontics. He also states these components are unpredictable and incomplete. Platelets are a major component of the blood clot. They secrete growth factors and have necessary serum proteins including fibrin, fibronectin, and cell adhesion molecules for osteoconduction.[126] Platelets are also known to function as a matrix for bone, connective tissue, and epithelial cell migration in wound healing.[138] It is accepted that the blood clot serves as a natural scaffold for stem cell migration and new tissue formation in the canal space.[139] After successful bleeding into the canal space, a piece of Colla-Plug is then placed on top of the bleeding to serve as a matrix to condense MTA. About 3 mm to 4 mm of MTA is placed and the access is sealed with a restoration. As apical development continues, Yamauchi et al.[126] found two types of hard tissue

deposition after histological study. The first was called dentin-associated mineralized tissue (DAMT), which they describe as "mineralized tissue with relatively uniform thickness devoid of vasculature, and some portion is anchored to the dentin wall and some detached."[126] The second was called bony islands (BI) located in the inner lumen and independent of the dentin wall. They reported that BI was distinct from DAMT and BI "appeared as mineralized matrix islands that contained many embedded cells, blood vessels, and bone marrow-like tissues."[126] Yamauchi et al.[126] also concluded that the use of a scaffold was necessary to increase mineralized tissue formation.

In regenerative endodontics scaffolds, stem cells, and growth factors are essential for regeneration. However, there first needs to be an aseptic environment to facilitate the function of the three components. Irrigation solutions place a critical role in this area.

IRRIGATION

Canal irrigation is an essential step in non-surgical root canal therapy. It is responsible for the debridement and disinfection of the root canal system.[140] This requires that the irrigation solution dissolves organic tissue and that the method of delivery is able to reach uninstrumented canal spaces.[141] It has been shown that 35 percent to 53 percent of the canal system remains uninstrumented.[142,143] The irrigation solution will help remove debris from the non-instrumented anatomy, have an antibacterial action, and dissolve of organic debris.[141,144] Harrison[145] outlined that irrigation solutions must have certain properties for endodontic effectiveness: antimicrobial properties; dissolution of tissues; adjunctively debride the root canal system; biocompatibility. Irrigation solutions are used in the regenerative procedure and are beneficial to successful outcomes. Presently, there is not a set standard for an appropriate irrigation regimen and thus

various irrigation regimens have been reported in recent case reports of the revascularization procedure.[146] The irrigation regimen is based on known bactericidal and bacteriostatic properties of the different irrigation solutions. An irrigation regimen for the regenerative procedure should be based on the antimicrobial effectiveness and the ability to promote stem cell survival. This is a different consideration for irrigation solution use than what is used in traditional non-surgical endodontic procedures.

Sodium Hypochlorite

NaOCl was effectively used for wound irrigation during World War I and was later implemented in endodontics by Coolidge.[41,147] It is a potent antimicrobial agent and effectively dissolves organic tissue such as pulp and collagen. NaOCl is available in many concentrations ranging from 0.5-percent to 6.0-percent.[148] It kills bacteria quickly even at low concentrations. Lower concentrations are effective on tissue that is primarily necrotic.[149] Higher concentrations give both necrotic and vital tissue dissolution and enhanced antimicrobial effectiveness.[150] Siqueira et al.[151] showed that NaOCl has a superior effect on root canal microorganisms in comparison with sterile saline. Vianna et al.[152] conducted an experiment using gram-negative anaerobic rods isolated from primary apical periodontitis and showed that the bacteria were killed within fifteen seconds using NaOCl in varying concentrations of 0.5-percent to 5.0 percent. Conversely, Estrela et al.[153] in a systematic review, concluded that NaOCl and CHX are not effective in completely eliminating *E. faecalis* when PCR and other culture techniques were used. Regardless of the controversy, it is still the main irrigation solution used in endodontic therapy today. Its tissue-dissolving properties are related to the duration of exposure, concentration, and temperature.[141,154] The bactericidal rates for NaOCl are doubled for

every 5°-C rise in temperature.[144] NaOCl has many of the necessary properties of a root canal irrigation solution and is the best one available for non-surgical endodontics.[155,156] In the regenerative procedure, a reduced concentration of 2.5-percent is recommended. Trevino et al.[146] reported the inclusion of 6-percent NaOCl decreased cell viability when combined with EDTA. The cytotoxic effects of NaOCl are well known and relied upon for traditional non-surgical root canal therapy. In contrast with the principles of regenerative endodontics, stem cell viability is necessary for success.[4]

Chlorhexidine

Since the 1950s, chlorhexidine (CHX) has been used for disinfection applications and treatment of various infections in humans and animals.[41] More recently, in dentistry, CHX is used intraorally for periodontics and cariology to prevent disease processes.[157, 158] CHX is available in various concentrations of 0.12 percent to 2.0 percent. CHX is a broad spectrum antimicrobial that is effective against gram-positive and gram-negative bacteria and yeasts.[41] CHX's effectiveness comes from its ability to bind to the negatively charged surface of bacteria damaging the outer cell wall making the cell permeable to the environment.[159,160] At low concentrations, CHX is bacteriostatic by affecting cell metabolism; at high concentrations it is bactericidal and acting as a detergent.[41] In endodontics CHX is used as an irrigation solution based on its substantivity and extended antimicrobial effect, which comes from binding to hydroxyapatite.[41] CHX has been shown to be effective against *E. faecalis* biofilms in root canal systems.[161] Because of the substantivity of CHX, it can remain on dentinal walls for extended periods of time reducing bacterial colonization.[162] It has been shown that 2-percent CHX is a better endodontic irrigation solution than 0.12 percent. CHX's antimicrobial activity is directly

related to the concentration.[163] Unlike NaOCl, CHX does not possess tissue-dissolving properties that are essential to endodontic treatment.[41] CHX has been advocated as a final rinse in the irrigation regimen due to its desirable properties, especially in endodontic retreatment.[164] Chlorhexidine (0.12 percent and 2 percent) have been reported in the literature as irrigation solutions in the regenerative procedure due to their antibacterial activity; however, they have some limitations. They do not dissolve tissue like NaOCl; when combined with NaOCl they have been known to form parachloroanaline-like (PCA) precipitates; and they are cytotoxic to stem cells.[146,165] Trevino et al.[146] in an *in-vitro* study examined different irrigation solution regimens and concluded CHX was detrimental to the stem cells of the apical papilla (SCAP) survival and that the irrigation regimen should be limited to the use of 17-percent EDTA and NaOCl. Thus, the use of CHX in the regenerative application is not advocated.

EDTA

Shahravan et al.[166] conducted a systematic review and meta-analysis on the smear layer's effect on sealability of canal obturation and concluded that smear layer removal improves the fluid-tight seal of the root canal system. Thus, smear layer removal is recommended. Ethylenediamine Tetra-Acetic Acid (EDTA) is used as an irrigation solution in endodontics because of its property to chelate and remove the mineralized part of the smear layer that is present on instrumented canal walls.[41] Seventeen-percent EDTA is the concentration most commonly used in endodontics. This concentration (17 percent) can remove the smear layer in approximately one minute.[41] The effect of EDTA is considered to be self-limiting, and when organic debris is present, EDTA can have decreased effectiveness.[167] When combined with NaOCl, however, there is a synergistic

effect allowing for the removal of the entire smear layer and allowing deeper penetration into dentinal tubules.[168, 169] Trevino et al.[146] along with other authors have reported that EDTA promoted SCAP survival, possibly by promoting the release of dentinal growth factors that were imbedded into dentin during dentinogenesis.[4,126]

OBTURATION

Endodontic obturation is an essential part of clinical endodontics to prevent infection of the periapical tissues by sealing the canal space.[170] Many materials have been advocated for obturation over the last 100 years. Most techniques use a core material and a sealer to achieve a fluid tight seal; the most common core material used is gutta-percha.[41] In 1940, Grossman[171] identified the criteria for an ideal obturation material: 1) easily introduced; 2) liquid or semisolid and becomes solid; 3) capable of apical and lateral sealing; 4) no shrinkage; 5) resistant to moisture; 6) bacteriostatic; 7) does not stain tooth structures; 8) biocompatible with periapical tissues; 9) easy removal; 10) sterile when introduced into canal; 11) radiopaque. These principles are dependent on proper cleaning and shaping to have maximum effect. A recent meta-analysis of the literature compared the success of root canal therapy related to the obturation length from the apex. It found that success rates were better when obturation was short of the apex.[172] A recently published meta-analysis by Peng et al.[173] found obturation with warm gutta-percha resulted in a higher rate of overextension than obturation with cold gutta-percha, indicating warm gutta-percha obturation is more technique sensitive. In the case of the necrotic immature tooth, conventional obturation techniques are inadequate to fulfill Grossman's criteria. The thin walls increase the risk of fracture, and the blunderbuss apex makes obtaining a hermetic seal very difficult with traditional materials.[9] In regenerative

endodontics, the goal is to render the canal aseptic and regenerate the pulp-dentin complex to continue root maturation.[4, 9,174,175] If the procedure is successful, no obturation is necessary because there has been successful regeneration of vital tissue into the canal space; the access is simply sealed to prevent re-infection. Gillen et al.[176] found the most predictable outcome of periapical healing comes with good quality endodontics and good quality coronal restoration.

DIRECT CONTACT TEST

Antimicrobial properties of restorative materials have[25] been evaluated *in vitro* using various methodologies. The agar diffusion test (ADT) was the standard assay used in most of these studies despite its known limitations. Problems associated with the ADT include its qualitative nature, ability to measure only soluble components, inability to distinguish between bacteriostatic and bactericidal effects, and difficulties in comparing a large number of samples with a large number of variables.[26] Its use in endodontic research is no longer accepted due to the lack of any kind of standardization of the media and testing materials.[177]

The direct contact test (DCT) has been described in the literature by Weiss et al.[178] as a test based on the turbidimetric determination of bacterial growth in a 96-well microtiter plate. Bacterial growth in the microwells is monitored at 590 nm at 37°C and recorded every 30 minutes over a 12-hour period by a spectrophotometer. The DCT is a quantitative and reproducible method that simulates contact of the test organism with the material being tested.[179] A temperature controlled spectrophotometer is used which allows for standardization of measurements for a large number of specimens, which is an advantage. The DCT allows monitoring of bacterial growth in all microwells and makes

it possible to measure the bactericidal effect of the materials. It makes it possible to directly calculate the exact numbers of surviving bacteria after each contact time.

SUCCESS OF ENDODONTIC THERAPY

The ultimate goal of endodontics is to prevent or heal the disease apical periodontitis.[180] Endodontic outcomes should be defined in reference to healing and disease as follows:

1. Healed: Both the clinical and radiographic presentations are normal.
2. Healing: Reducing radiolucency with reduction in clinical symptoms.
3. Disease: Radiolucency has formed or remained with no change in size. clinical signs or symptoms are present, even if the radiographic presentation is normal.
4. Functional retention: The clinical presentation is normal, while radiolucency may be absent or present.[181]

Healing of endodontically treated teeth has been addressed in many studies, but even comprehensive review of those studies show inconsistencies and outcome variability.[182,183] Because of the variability in the data included in these studies, the outcomes can be misleading. Clinical studies provide varying levels of evidence and must be compared against a well-defined criteria to differentiate them according to their levels of evidence.[184] In 2002, Friedman[182] published a paper on the prognosis of initial treatment and used the accepted guidelines for appraisal of studies.[185] Friedman grouped the appraisal criteria into four parameters: cohort at inception and end-point of study, treatment, outcome assessment, and data analysis. Friedman included studies for review that included three of the four parameters. The results were divided into several categories. The first regarded treatment outcomes in teeth presenting with no apical

periodontitis. These were teeth that might have been diagnosed with irreversible pulpitis, necrosis, or inadequate obturation. Large percentages of these teeth maintained periapical health after 10 years. It was concluded that these teeth have a 92-percent to 98-percent success rate after initial treatment and orthograde retreatment. The second category was about treatment outcomes in teeth with apical periodontitis; these were teeth with either a primary or secondary infection. There was more variability reported in this section. The success rates 10 years post-operative ranged from 73 percent[186] to 90 percent[187] for initial treatment and 74 percent[188] to 86 percent[189] for retreatment. Friedman[181] states that seven studies from initial treatment and one from retreatment showed 88 percent of teeth were considered functional with signs of disease healing. A subsequent study by Friedman et. al.[190] estimated that 5 percent of the teeth included in the study were functional with disease present, but without symptoms. The percentage of functional teeth included with endodontic successes approximates 95 percent.

Epidemiological studies have shown 97 percent of teeth that were treated by NSRCT were clinically present at an eight-year recall. In this study Delta Dental reviewed an insurance claim pool of 1,400,000 root canal treated teeth. It was determined that 97 percent of those teeth were clinically present after an eight-year follow-up.[191] In a similar study, a pool of 44,000 patients showed a 94-percent retention rate for endodontically treated teeth after a three-and-a-half year follow up.[192] There has been some controversy regarding success of one-visit or two-visit root canal therapy. In a recent systematic review by Sathorn et al.,[193] single-visit root canal treatment appeared to be slightly more effective than multiple visits, i.e. a 6.3-percent higher healing rate. However, the difference in healing rate between these two treatment regimens was not

statistically significant. In summary, NSRCT and surgical endodontics have high success rates. To maintain this high success rate, certain preoperative variables and treatment techniques need to be taken into consideration to determine successful treatment outcomes.

Initial NSCRT has been shown to be successful; however, failures do occur. When failures occur, it is because of persistent infection or secondary infection. Many factors can influence a negative outcome, inadequate chemo-mechanical debridement, missed canals, irregular anatomy, or poor asepsis protocol.[194] This pathology can be addressed with retreatment or surgical treatment of the periradicular tissues. A systematic review by Torabinejad et al.[194] concluded the literature reports a decreased success rate of 13 percent to 36 percent on non-surgical retreatment cases with many factors influencing the outcomes of the various studies reviewed. Torabinejad et al.[194] concluded there are improved outcomes for non-surgical retreatment with increased recall time and that non-surgical retreatment is a successful alternative to extraction. In a study by Imura et al.,[195] 2000 retreatment cases treated by a specialist were evaluated, and the success rate was 85.9 percent. Overall, the literature supports good success rates for non-surgical retreatment in endodontics when thoroughly treated cases are completed.

The literature determining the success of endodontic surgery is plagued with the same challenges as for conventional root canal therapy. Variability in success may be attributed to case selection, repeat surgery, quality of previous treatment, and surgical technique.[181,183,196-198] Because of these differences, success of endodontic surgery has been reported to be 31 percent[199] to 91 percent.[198] After a review of the literature by Friedman et. al.,[181] the functional success rate of teeth after surgery is 86 percent[199] to 92

percent.[200, 190, 201, 202] Friedman et al.[183] concluded that the tooth with apical periodontitis remains asymptomatic 91 percent to 97 percent of the time. Recent systematic reviews by Setzer and Tsesis emphasize the use of microsurgical techniques, which increased surgical success rates to 97 percent.[203]

Surgical endodontics is indicated when practitioners are presented with these situations: failure of NSRCT; retreatment cannot resolve the problem; teeth restored with posts; full coverage restorations for which retreatment risks the removal of excessive tooth structure, and when there are persistent symptoms.[204] Since the introduction of microsurgical techniques, there has been a marked increase in success rates of surgical endodontics.[205, 206] Several reports have been published reporting the success of surgical endodontics. Rubenstein et al.[207] performed 94 surgical cases with microsurgical techniques over a period of 14 months. The cases were followed for 12 months and they found a success rate of 96.8 percent with an average healing time of 7.2 months. Rubenstein et al.[208] followed up his short-term study with a 5-year-to-7-year recall and found a success rate of 91.5 percent. Christiansen et al.[209] found similar short-term results as Rubenstein using microsurgical techniques. Tsesis et al.[206] conducted a meta-analysis of the literature and concluded that microsurgical endodontics had a successful outcome of 91.6 percent at one year. Surgical endodontics is a predictable conservative approach to maintaining a healthy dentition after NSRCT and retreatment failure.

Regenerative endodontics is a new treatment and only case reports currently exist in the literature at this time. The predictability of the success of this procedure has not been determined. Only case reports, case series, and controlled animal studies are available for review at this time.[4,14,126,136, 210] However, recent case reports have

demonstrated that revascularization of necrotic immature infected root canals is possible *in vivo.*

MATERIALS AND METHODS

PDS*II (polydioxanone) suture was obtained from Ethicon (Somerville, NJ) and was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol, HFIP (Sigma Aldrich) under stirring conditions. Drug-containing polymer solutions were prepared with 5.0-wt % and 25.0-wt% metronidazole. The polymer based solutions were mixed together and homogenized under vigorous stirring (Figure 3). For each polymer/mixture, the solution was drawn into a 5-ml plastic syringe with a blunt-tip needle. This loaded syringe was then placed in an electrically grounded (Figure. 1) syringe pump (PHD2000, Harvard Apparatus, MA) and the needle connected to a DC voltage power supply (Model ES60P-20W/DAM, Gamma High Voltage Research Inc., FL).

An electrically grounded aluminum foil collector sheet was adequately distanced from the tip of the spray needle (Figure 1). The tip of the electrically charged needle and the grounded collector sheet were fully enclosed within a clear Lexan box in order to minimize influence from air currents. An exhaust hose pulling a weak vacuum was attached to the Lexan box to help remove solvent vapors during electrospinning. The drug-containing fibers were co-electrospun at optimized conditions (i.e., voltage, distance, and flow rate). As control groups, drug-free (0.0 wt %) PDS scaffolds were also obtained (Figure 9). The obtained fibrous scaffolds were dried in a desiccator at room temperature for at least 48 h to remove the residual solvent.

MODIFIED DIRECT CONTACT TEST (MDCT)

The MDCT (Figure 8) was used to assess the antimicrobial effect of the drug-containing scaffolds by turbidimetric determination of bacterial growth. Given the

antibiotic was in the scaffold, we indirectly brought the bacteria into contact with the scaffold in the broth. It was expected the antibacterial effect would be a result of the antibiotic being released from the scaffold into the surrounding broth. Bacterial growth is observed through changes in absorbance. The effect of the antibiotic was determined by turbidimetric analysis of bacterial growth at 37°C for testing to determine bacterial growth. The microplate reader uses a light source that transilluminates the sample with a predetermined wavelength, and a light detector on the opposite side of the well measures the quantity of the light transmitted through the sample. A smaller amount of light transmitted correlates with a higher density of bacterial cells. The electrospun scaffolds were collected onto an aluminum foil sheet and subsequently cut into 4 mm diameter sections (Figure 10). The aluminum foil was separated from the scaffold and the scaffold was placed at the bottom of the microwell in the flat bottom microtiter plate. *P. gingivalis* was cultured for 48 hours in Brain Heart Infusion (BHI) broth containing yeast extract and 0.25 ml of vitamin K at 37°C in anaerobic conditions (Figure 12, 17). Bacterial growth was confirmed by turbidity in the broth. One hundred ninety (190) µl of the non-inoculated BHI with yeast extract was added to each well and 10 µl of the 48-h bacterial suspension (approximately 10^6 bacteria) was placed in the microwells (Figure 13). The prepared microplates were incubated under anaerobic conditions for 48 h. After the 48-h incubation period the microplates were removed from the GasPack (Figure 16) and the scaffolds were carefully removed with sterile instruments to prevent cross-contamination of the microwells. Spectrophotometry was done by measuring the optical density at 595 nm using an endpoint reading with the spectrophotometer set to 37°C.

Controls consisted of:

1. Negative control: Untouched walls of microwell with non-inoculated medium (n = 8 wells).

2. Negative control: Walls of microwell coated with scaffold with non-inoculated medium (n = 8).

3. Positive control: Untouched microwell with inoculated medium (n=8).

Experimental groups consisted of:

4. PDS scaffolds with no antibiotic and inoculated with *P. gingivalis* media (n = 8).

5. PDS scaffolds containing 5.0-wt % MET and inoculated with *P. gingivalis* media (n = 8).

6. PDS scaffolds containing 25-wt % MET and inoculated with *P. gingivalis* media (n = 8).

Each experiment was conducted three times.

STATISTICAL ANALYSIS

Comparisons among the groups for differences in optical density as a measure of bacterial growth were made using mixed-model ANOVA, with a fixed effect for group and a random effect for experimental run. Pair-wise group comparisons were performed using Tukey's multiple comparisons procedure to control the overall significance level at 5 percent. The analyses were performed using the ranks of the data.

RESULTS

Optical density (OD) is a measurement of the turbidity of a solution. In our experiment uninoculated BHI+YE broth showed a significantly lower OD at 595 nm than all other groups ($p < 0.0001$) indicating that the broth had no turbidity at the time of the reading, which confirmed no bacterial growth in our negative controls (Figure 4 to Figure 6).

BHI+YE+*Pg* and BHI+YE+*Pg* + control scaffold (no antibiotic) showed a significantly higher OD at 595 nm confirming positive bacterial growth in our positive controls. This growth was significantly more than growth measured in the scaffolds containing 5.0 wt % and 25 wt % ($p < 0.0001$ and $p < 0.0001$), respectively. Less bacterial growth was seen in the groups containing the MET incorporated into the scaffold indicating that the scaffold was effective at inhibiting bacterial growth (Figure 4 through Figure 6).

However, the 5.0-wt % and 25-wt % MET were not significantly different from each other ($p = 0.21$) indicating that the higher concentration of MET used in this study was not more beneficial than the lower concentration. The concentrations used in this study were well above the MIC for metronidazole against *Pg* reported in the literature to be 0.122 µl.[211]

FIGURES

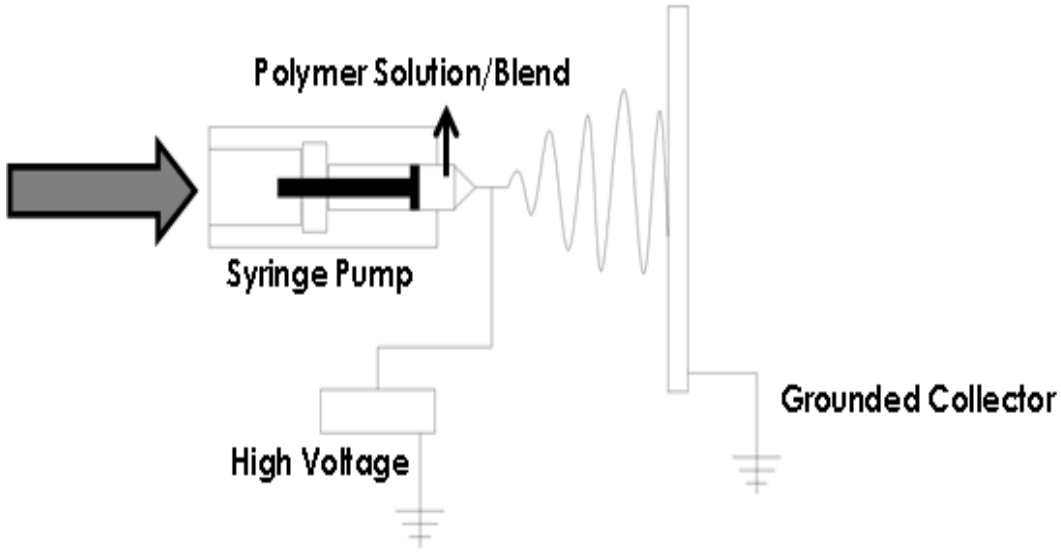

FIGURE 1. Schematic of the electrospinning set-up used to obtain the nanofibrous structures. The PDS solution and PDS solution + metronidazole were loaded into the syringe. The solution was expressed through the needle at a predetermined rate with a specific voltage attached to the needle. The electrospun polymer was collected on a grounded collector with aluminum foil at a predetermined distance.

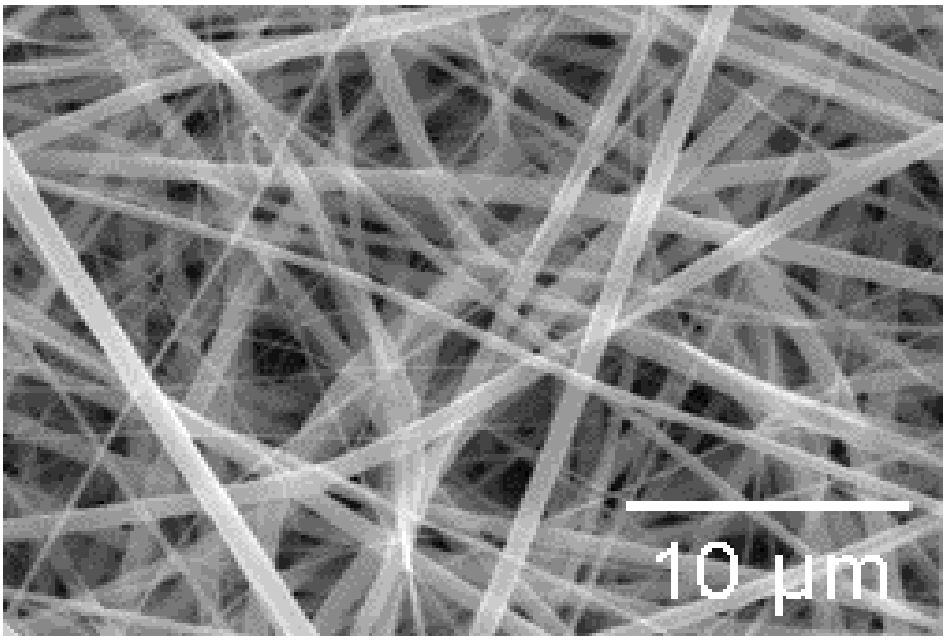

FIGURE 2. Representative scanning electron microscopy (SEM) images of an electrospun scaffold containing metronidazole. The image shows the individual distribution of each electrospun nanofibers and their spatial relationships at a measurement of 10 µm.

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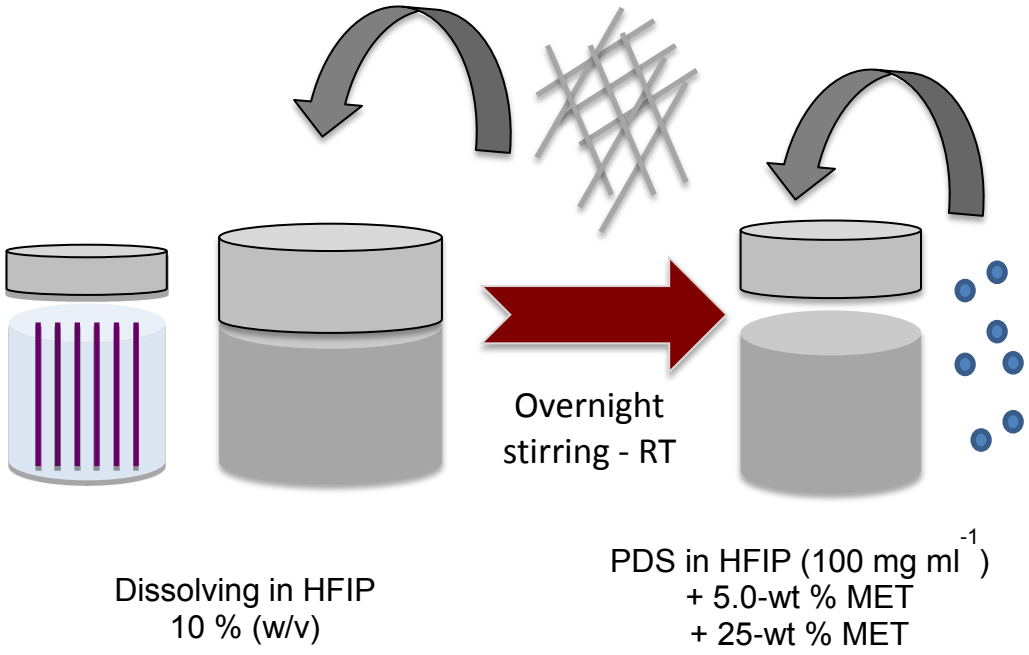

FIGURE 3. Clear PDS II® pieces were dissolved overnight in HFP under stirring conditions. PDS solution was then loaded with 2 distinct amounts of metronidazole-MET (i.e., 5.0 wt % and 25 wt % respective to the total polymer weight). The 3 different solutions (i.e., control, PDS +5.0 wt % MET and PDS + 25 wt % were individually loaded into 5-ml plastic syringes fitted with a 27-gauge stainless steel needle and electrospun at optimized conditions.

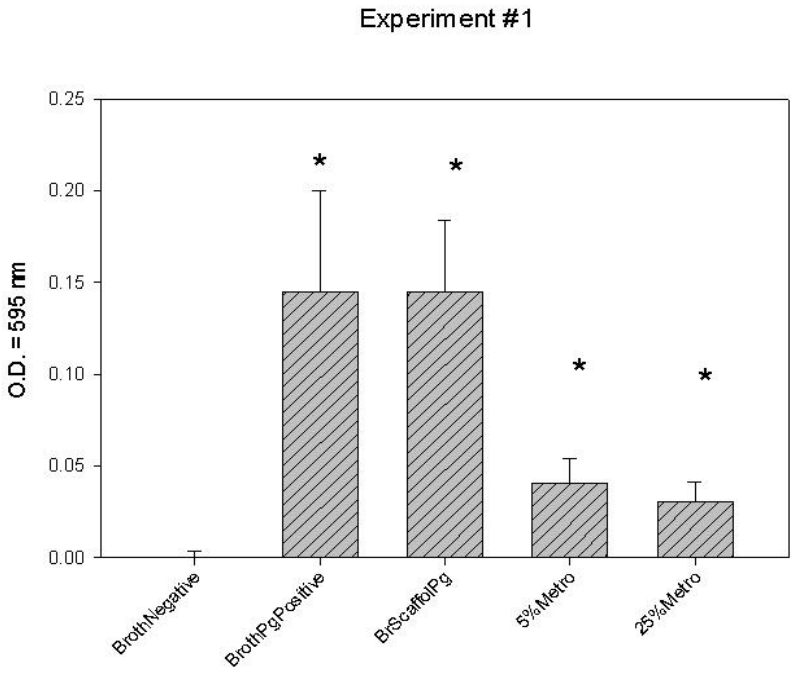

FIGURE 4. Graph representing experiment #1. The x-axis shows optical density measured at 595 nm and y-axis shows experimental groups tested. No bacterial growth was measured in the negative control group. Significant growth was measured in broth + *Pg* and broth + control scaffold + *Pg* groups. Groups containing 5.0% wt. and 25% wt. showed significant *Pg* inhibition compared with positive control groups.

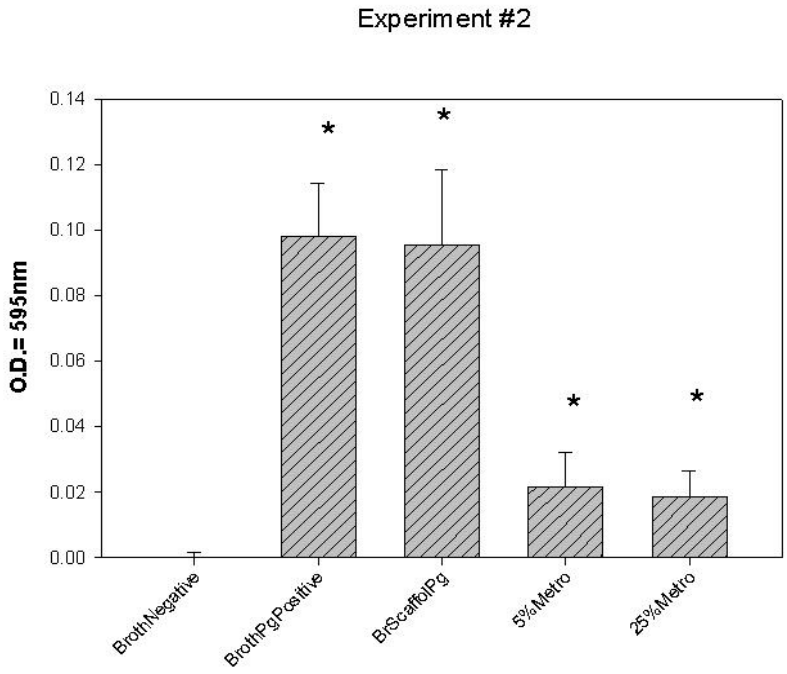

FIGURE 5. Graph representing experiment #2. The x-axis shows optical density measured at 595 nm and y-axis shows experimental groups tested. No bacterial growth was measured in the negative control group. Significant growth was measured in broth + *Pg* and broth + control scaffold + *Pg* groups. Groups containing 5.0% wt. and 25% wt showed significant *Pg* inhibition compared with positive control groups.

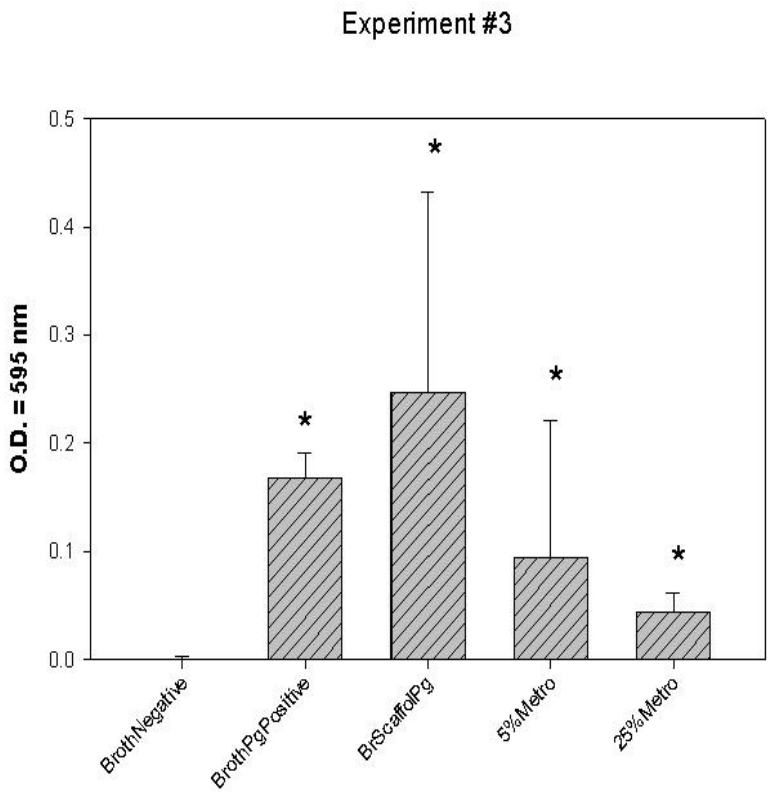

FIGURE 6. Graph representing experiment #3. The x-axis shows optical density measured at 595 nm and y-axis shows experimental groups tested. No bacterial growth was measured in the negative control group. Significant growth was measured in broth + *Pg* and broth + control scaffold + *Pg* groups. Groups containing 5.0% wt. and 25% wt. showed significant *Pg* inhibition compared with positive control groups.

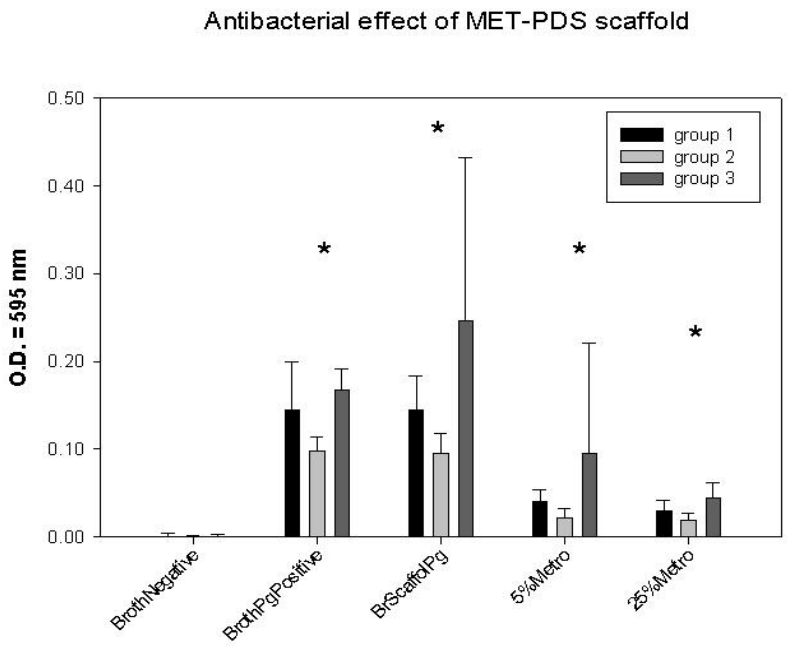

FIGURE 7. Graph combining all three experiments. The x-axis shows optical density measured at 595 nm and y-axis shows all three experimental groups tested. Group 1 (black) represents experiment #1. Group 2 (grey) represents experiment #2. Group 3 (charcoal) represents experiment #3. The results of all three experiments were compared, and the negative controls in all three groups showed no *Pg* growth. All three groups showed *Pg* growth in the positive control groups. The groups with the metronidazole containing scaffold at 5.0% wt and 25% wt showed significant *Pg* inhibition in all three groups.

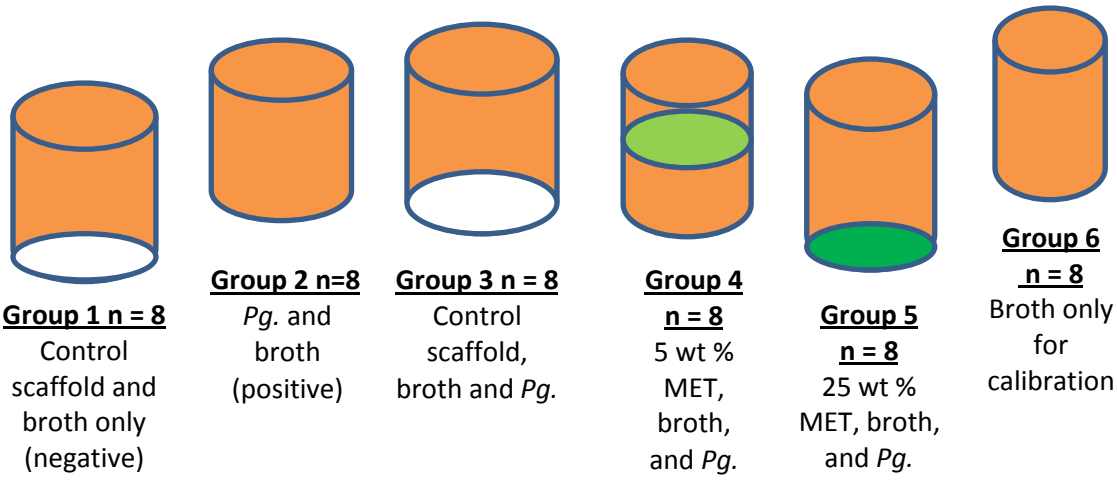

FIGURE 8. Representation and explanation of experimental groups placed in microwells in this study.

FIGURE 9. Electrospun scaffold on aluminum foil after being collected on aluminum foil during the electrospinning process. This shows an unused section of scaffold used in this experiment.

FIGURE 10. A biopsy punch 4 mm in diameter was used to cut all the experimental scaffolds from the aluminum foil collector. After the cutting, the aluminum foil was removed and the scaffold was placed in an air tight container and stored in a desiccator until used in the experiment.

FIGURE 11. A 96-well microtiter plate used for our experiment obtained from Fisher Scientific.

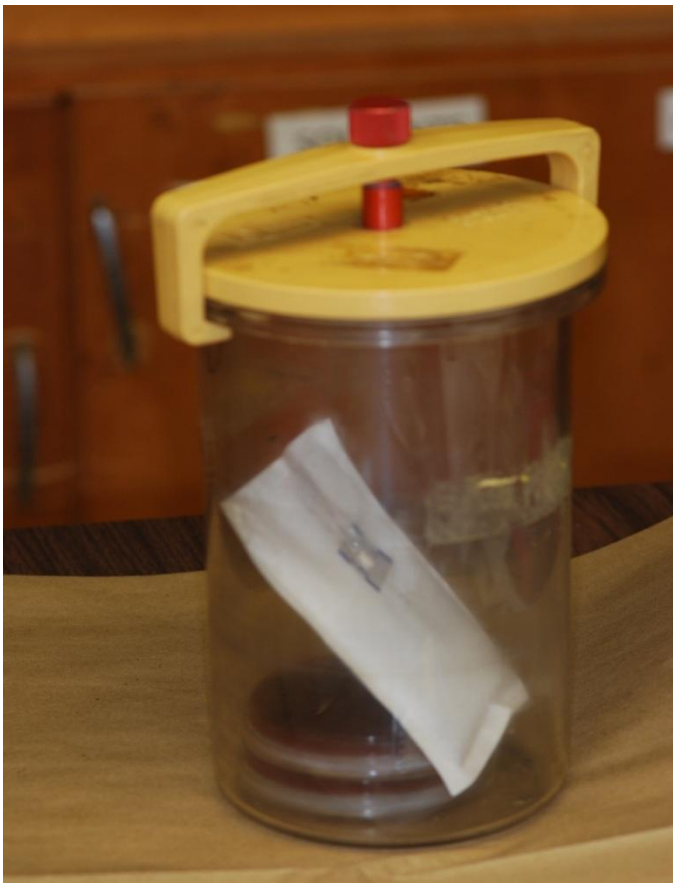

FIGURE 12. GasPack used to maintain anaerobic conditions during cultivation of *Pg.* After assembly the GasPack was placed in an incubator for 48 hours.

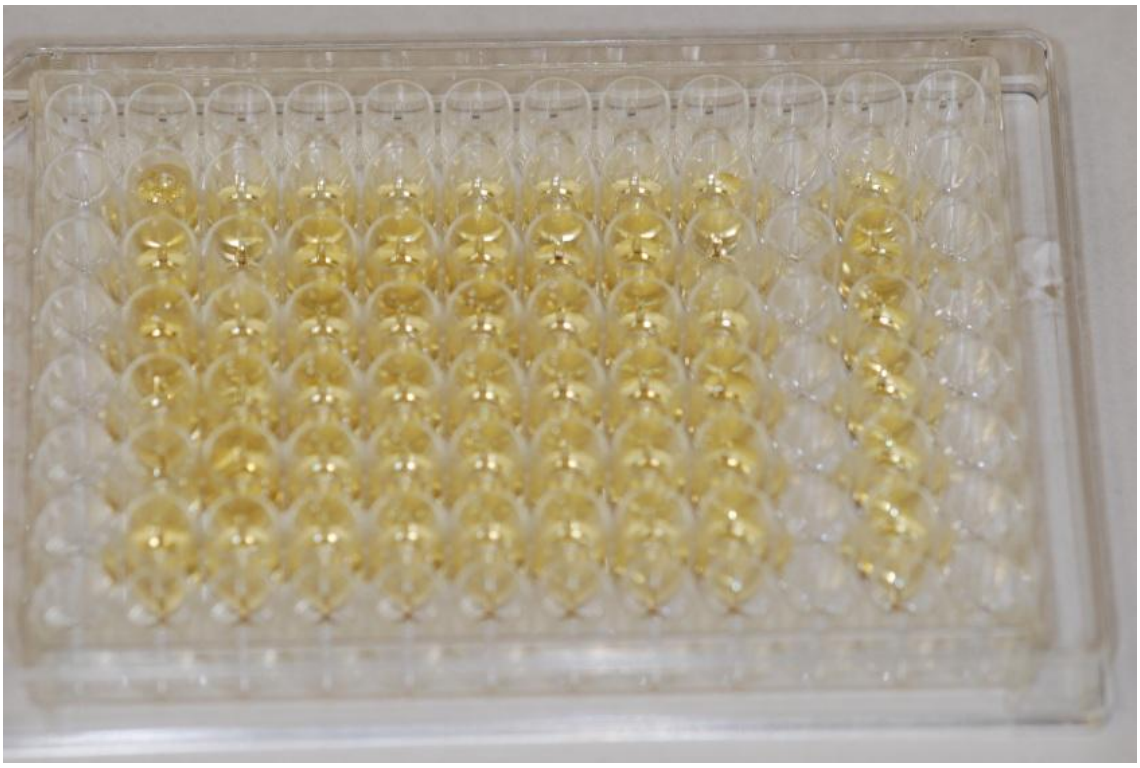

FIGURE 13. Prepared microplate with all experimental groups present as represented in FIGURE 8.

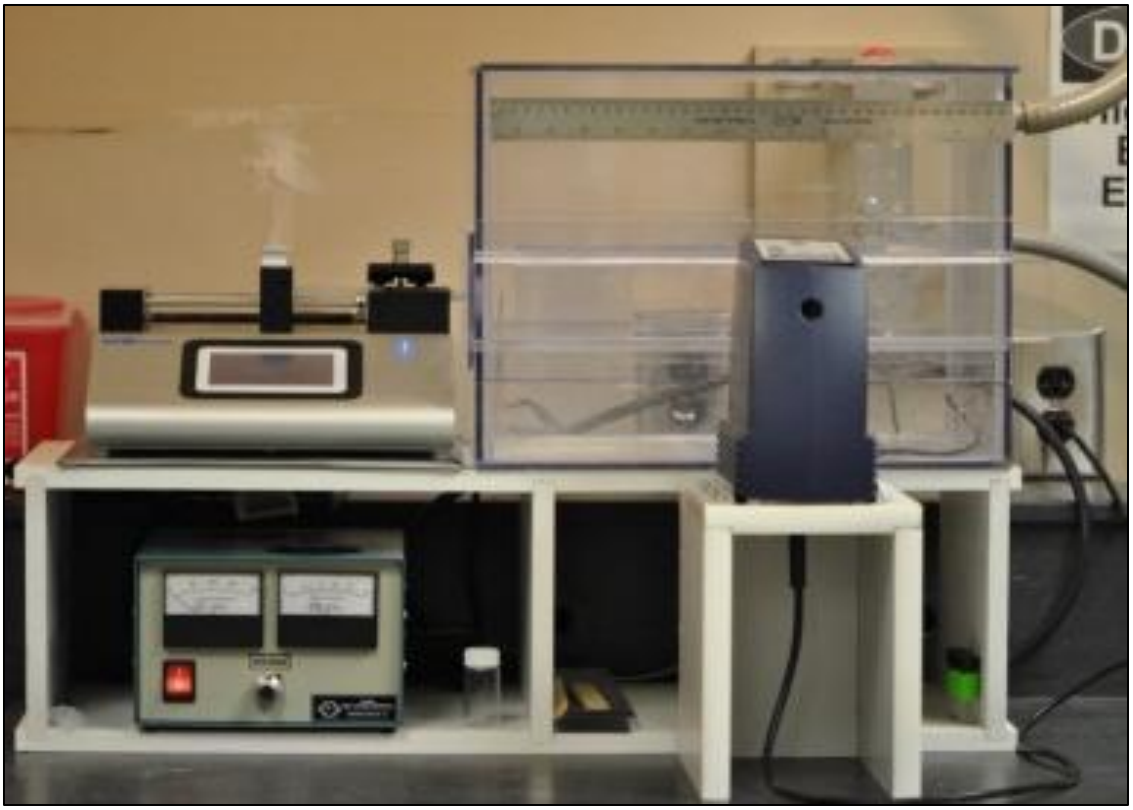

FIGURE 14. Image of electrospinning apparatus used in this project located in the dental materials department of IUSD.

FIGURE 15. A 10-mm zone of inhibition seen around cyanoacrylate on cultured plate indicating that cyanoacrylate has antimicrobial properties and is not a suitable material to use in the microwells of this experiment.

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FIGURE 16. Microplates in GasPack container to maintain anaerobic conditions. GasPack container was sealed for 48 hours and incubated before spectrophotometer reading.

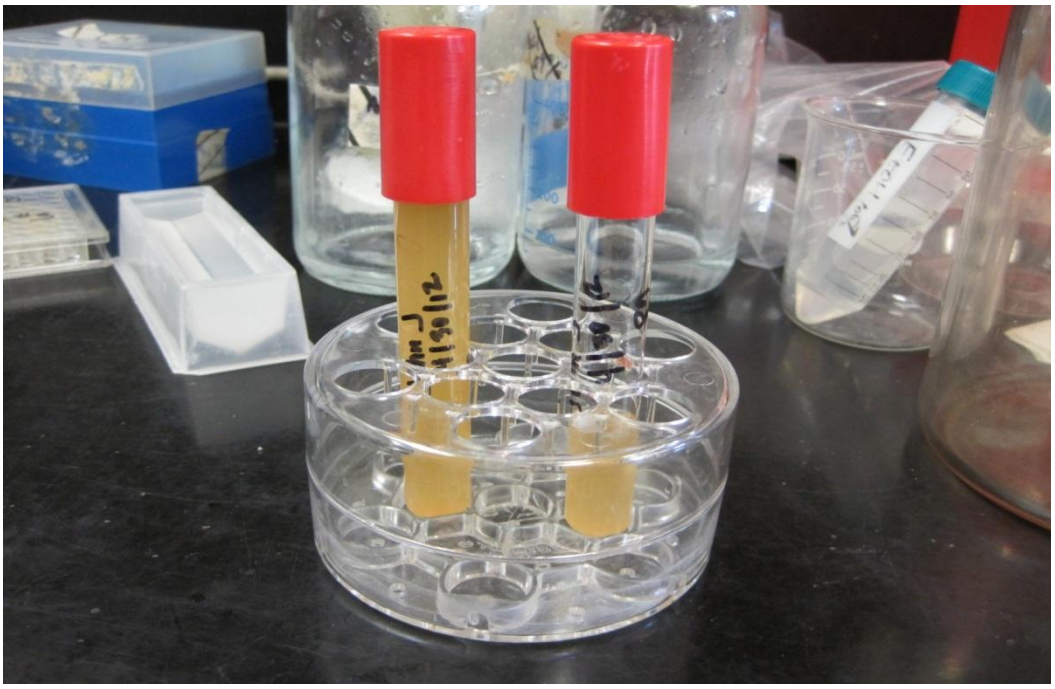

FIGURE 17. Prepared *Pg* in BHI + YE broth after 48 hour incubation in GasPack to maintain anaerobic conditions. Visual turbidity confirms the growth of *Pg*. These bacteria were then used in experimental microwells.

DISCUSSION

Polydioxanone was successfully electrospun with metronidazole to form a synthetic polymer scaffold. The results show that the scaffold is capable of inhibiting bacterial growth *in vitro*. Our results confirm results from other studies that incorporated antibiotics into electrospun fibers.[1,19,106] The present study is the first report that we are aware of introducing an electrospun scaffold containing antibiotics to be used as a drug delivery device for the endodontic regeneration procedure.

PDS II (polydioxanone) is a colorless bioabsorbable monofilament polymer commonly used as a suture material.[212] In this study PDS II was selected because it has many desirable properties for a scaffold material. Also, polymer scaffolds have been reported in the literature to perform as functional scaffolds for pulp fibroblast seeding, proliferation, and differentiation.[213] PDS biodegrades into water and carbon dioxide, which are easily disposed of by the body resulting in a low inflammatory response and host acceptance.[212] When scaffold remnants persist, they can negatively affect the outcome by creating a foreign body response.[1] PDS II naturally has a slow biodegradation time, which can be manipulated by electrospinning. It is important to not have the scaffold degrade too early as this has negative effects on mechanical properties and stability.[1] Currently, synthetic electrospun polymeric nanofibers are under investigation for drug delivery devices in medicine and other applications for tissue engineering.[106]

The agar diffusion test (ADT) is the standardized method to test for antibiotic susceptibility.[178,214] With this test antibiotic-containing discs are placed on agar plates

containing bacteria. When a clear zone around the plate is present, bacterial inhibition is represented, and the effectiveness of the antibiotic can be tested against specific microorganisms. ADT is not a predictable test for the antimicrobial effectiveness of endodontic disinfecting agents or intracanal medicaments because of the differences between the infected root canal system and the agar. The DCT is an alternative method to test antimicrobial activity of restorative material and endodontic sealers.[178,179,214]

The selection of antibiotics to treat an infection should be based on the most current knowledge available to successfully treat an infection. Since the majority of bacteria located deep in dentinal tubules are obligate anaerobes, metronidazole was selected as the test drug.[118] It is well established in the literature that metronidazole is effective and has a wide spectrum against gram-negative anaerobes.[21] Intraorally, metronidazole has been shown to have bactericidal action against oral obligate anaerobes, anaerobes from infected pulps, and in 99 percent of bacteria in carious lesions.[118] Metronidazole exerts its effect by deactivating critical enzymes intracellularly once inside the bacteria and is bactericidal.[215]

Currently, the regenerative endodontic procedure uses a triple antibiotic paste (TAP) that is introduced into the canal for disinfection.[4,9,17,136,216] Recent case reports and studies have demonstrated the effectiveness of TAP when used as a canal disinfectant in the regenerative procedure; however, it is still unknown what the effective concentrations of the antibiotics are for this procedure. Currently, a mixture of 200 mg of ciprofloxacin, 500 mg metronidazole, and 100 mg of minocycline is reported in the literature.[217] Drug sensitivity is usually determined by finding the MIC (minimum inhibitory concentration); however, so many bacterial species are found in endodontic lesions and in the oral cavity,

it would be impossible to determine the MIC for every bacteria present.[119] The reported MIC for metronidazole is 0.122 µl for *P. gingivalis.*[211] A reduced known effective concentration would be beneficial to reduce the overuse of antibiotics. This was addressed by starting with specific concentrations of metronidazole in the scaffolds (5.0 wt % and 25 wt %) that are well above the MIC for metronidazole. The MIC in this study was found to be 0.134 µl, which is not significantly different than a previous study which was 0.125 µl.[218] In addition, whether higher levels of antibiotics incorporated into the scaffold would be of any benefit was evaluated. The results indicated there was no significant difference between the 5.0-wt % and 250-wt % concentrations; both effectively inhibited bacterial growth. These results confirm the reported effectiveness in the literature of an electrospun scaffold capable of drug release.[1,19,106] An electrospun scaffold with antibiotic incorporated could be used as a drug delivery device for canal disinfection.

For the TAP to be effective, it must be in contact with dentin.[135] It is clinically impossible to confirm that the TAP is adapted to the canal walls since it is not radiopaque. This also presents a problem on the required second visit to remove the paste. It is difficult to determine if all the paste is removed. A recent report found that TAP is not toxic by histological examination; however, the vehicle used to deliver the TAP (propylene glycol) may be difficult to remove from the dentin surface potentially altering the regeneration potential. The second visit to remove the TAP also introduces risk of recontamination if good isolation is not obtained.

A better understanding of effective antibiotic concentrations and or the use of single or multiple antibiotics, distribution, and vehicles for delivery is necessary for efficient disinfection would improve the suggested current treatment protocol.

Other scaffolds have been investigated to improve the regenerative procedure, the most recent being an injectable hydrogel scaffold. Hydrogels are three-dimensional and can be delivered by syringe. In theory, they would provide a substrate for cell proliferation and differentiation. However, progress has been halted by their ability to support cell survival; the mechanical properties are not ideal and degradation is rapid.[4] Platelet rich plasma (PRP) is also currently being investigated as a scaffold material. It fulfills much of the criteria for a scaffold: it is rich in growth factors, biodegradable, forms a three-dimensional matrix, and is autologous.[9] Research is now focusing on making them more rigid to support cell growth. Polymer electrospun scaffolds are currently attractive for tissue engineering due to their desirable properties; they have already been approved by the FDA for patient use and their ability to incorporate bioactive molecules.[219]

Many of the current challenges with the regenerative procedure could be addressed with a drug incorporated electrospun polymer scaffold. As shown in the current study, it can serve *in vitro* as a drug delivery device, using specific predetermined amounts of antibiotic. A unique feature of polymeric nanofibers is the high surface area of the fibers arranged in a interconnecting structure that allows controlled drug release.[106] As the scaffold degrades over time, the drug is released giving an ideal dose in time.[219] Also, the drug release can be manipulated to be rapid, intermediate, delayed, or modified depending on the polymer used.[110] These advantages can improve drug delivery, reduce

appointments, and improve drug adaptation to the canal wall in the regeneration procedure.

In a pilot study, the scaffold was attached to the microwell wall with OptiBond and cyanoacrylate. By attaching the scaffold to the microwell, it would not interfere with the optical density reading. However, by attaching the scaffold with the aluminum foil still attached only one side would be available for MET release. OptiBond was unable to secure the scaffold to the wall during the sterilization process. Cyanoacrylate was used, and it successfully secured the scaffold to the wall for the duration of the experiment. However, upon interpreting the results, the cyanoacrylate control showed bacterial inhibition (Figure 15). It was impossible to determine what kind of efficacy the scaffold had against bacteria while using cyanoacrylate. Subsequently, it was decided to separate the scaffold from the aluminum foil and place the scaffold in the microwell unbounded to any wall. The scaffold was carefully removed with sterile instruments and the plate read. This proved to be the best way to test the scaffold's *in-vitro* effectiveness.

Although metronidazole is effective against obligate anaerobes, even at high concentrations, it has been shown that it cannot eliminate all the bacteria in a necrotic root canal.[118] However, when combined with multiple drugs such as the TAP, it has been reported that TAP can sterilize a root canal.[119,135] Future studies could incorporate other antibiotics, individually and combined, into the scaffold for bacterial testing. Other bioactive molecules such as growth factors could be included to improve the scaffold's tissue regenerative properties.

Future studies could include the incorporation of multiple antibiotics, evaluation for interim root canal dressing, incorporation of bioactive molecules, and animal studies for effectiveness of the scaffold.

SUMMARY AND CONCLUSIONS

Our null hypothesis was accepted as *P. gingivalis* was significantly inhibited by the PDS electrospun scaffold.

The PDS scaffold used in this study can function as an antibacterial drug delivery device against anaerobic bacteria. The 5.0-wt % should be considered for use to avoid excess antibiotic use since there was not a statistical difference from the 25-wt %.

This scaffold could potentially improve the regenerative endodontic procedure by providing a predictable scaffold for growth, sustained antibiotic release, controllable drug concentrations, and eliminating a step in the regenerative procedure.

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ABSTRACT

ANTIMICROBIAL PROPERTIES OF DRUG-CONTAINING ELECTROSPUN SCAFFOLDS

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Endodontic treatment of the infected immature tooth has undergone a dramatic change. Conventional endodontic treatment can control infection, but root development usually remains impaired. A novel regenerative endodontic procedure, the revascularization method, can now control the infection and enable such teeth to continue root development. This is done by creating a fibrin-matrix scaffold in the antibiotic treated root canal space (RCS). Dental stem cells and growth factors have been able to continue root development in such an environment. The fibrin-matrix scaffold is

dependent on the induction of a blood clot into the RCS, and this cannot always be predictably induced. PDS is a biocompatible material that can be electrospun to provide a matrix for cells and growth factors and perhaps improve on the blood clot induced fibrin scaffold by incorporating metronidazole as an adjuvant antimicrobial. A metronidazole containing electrospun PDS scaffold was examined *in vitro* using a turbidimetric test, the modified direct contact test. This scaffold significantly inhibited growth of an anaerobic primary endodontic pathogen *Porphyromonas gingivalis*. This scaffold may improve the treatment of the infected immature tooth by providing a designed matrix for root regeneration while serving simultaneously as an antibiotic drug delivery device to disinfect the RCS.

The aim of this study is to evaluate *in vitro* the property of a synthetic scaffold to function as an antibacterial drug delivery device.

PDS*II (polydioxanone) suture was obtained from Ethicon, INC. (Somerville, NJ) and was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol, HFIP (Sigma Aldrich). Three different scaffolds were electrospun onto an aluminum foil background; (1) control scaffold with no antibiotic incorporated, (2) scaffold with 5.0-wt % metronidazole incorporated, and (3) 25-wt % metronidazole incorporated. All scaffolds were cut using a 4-mm diameter biopsy punch under aseptic conditions and removed from foil, control scaffold (n = 64), scaffold containing 5.0-wt % metronidazole (n = 32), and scaffold containing 25-wt % metronidazole (n=32). Experimental scaffolds were placed in a 96-well sterile flat bottom microtiter plate. *Porphyromonas gingivalis* a known primary endodontic pathogen was grown in 5 ml of BHI + YE with 0.25 µl of vitamin K with incubation at 37°C under anaerobic conditions for 48 hours. Microplates were sterilized

before inoculation with *Pg* with 400 µl of 70-percent EtOH for a minimum of 30 minutes then pipetted out. After sterilization the microwells were washed with 400 µl of sterile water and pipetted out. Group 1 (negative control) microwells (n = 8) contained control scaffold and 190 µl of broth only. Group 2 (positive control) microwells (n = 8) contained 190 µl of broth and *Pg* only. Group 3 microwells (n = 8) contained control scaffold, 190 µl of broth, and 10 µl of *Pg* inoculum. Group 4 microwells (n = 8) contained scaffold with 5 wt % metronidazole, 190 µl of broth, and 10 µl of *Pg* inoculum. Group 5 microwells (n = 8) contained scaffold with 25 wt % metronidazole, 190 µl of broth, and 10 µl of *Pg* inoculum. Group 6 contained 190 µl of uninoculated broth for spectrophotometer calibration. Sterile microplate lids were used to isolate microwells from the surrounding environment. Microplates were incubated at 37°C under anaerobic conditions for 48 hours. After 48 hours the microplates were read by using an endpoint reading in the spectrophotometer. This was repeated four times.

Comparisons among the groups for differences in optical density as a measure of bacterial growth were made using mixed-model ANOVA, with a fixed effect for group and a random effect for experimental run. Pair-wise group comparisons were performed using Tukey's multiple comparisons procedure to control the overall significance level at 5 percent. The analyses were performed using the ranks of the data. Broth had significantly lower OD than all other groups ($p < 0.0001$). Broth+Pg and Broth+Pg+Scaffold had significantly higher OD than 5-wt % Metro ($p < 0.0001$) and 25-wt % Metro ($p < 0.0001$), but Broth+Pg and Broth+Pg+Scaffold were not significantly different from each other ($p = 0.97$). 5-wt % Metro and 25-wt % Metro were not significantly different from each other ($p = 0.24$).

From the results of our study, we concluded that the 5.0-wt % and 25-wt % metronidazole containing scaffolds significantly inhibited bacterial growth and could be effectively utilized for the endodontic regeneration procedure.

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