DEVELOPMENT OF A DOUBLE ANTIBIOTIC ELECTROSPUN SCAFFOLD FOR ROOT CANAL DISINFECTION

by

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"We are what we repeatedly do. Excellence then, is not an act,
but a habit." – Aristotle

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INTRODUCTION

Dental caries and trauma in young children can often lead to pulpal necrosis, which clinically are challenging to treat due to immature teeth having undeveloped roots, increasing the risk for fracture.[1-4] In today's clinical practice, the endodontic treatment options for these teeth with pulpal necrosis are either apexification or regenerative-based procedures.[2]

With apexification, the main disadvantage is inhibition of further root development in both length and width.[5,6] This method allows for healing of any apical periodontitis and retention of the tooth but does not promote root end development and/or restore tissue vitality. On the other hand, the set of procedures involved in the so-called revascular or regenerative endodontics has been deemed as the ideal treatment.

The goal of regeneration is to encourage root development both in length and width and for the vitality to be restored through pulp-like tissue formation. Regenerative endodontics is done by disinfecting the root canal system (RCS) and inducing bleeding from the apical papilla. Stem cells are found in the apical papilla and laceration of this area triggers an inflow of blood, filled with stems cells, into the root canal system.[7-9] Over the past decade several publications have prompted the revitalization of a regenerative approach for endodontic treatment.[2,5,10-13] Additionally, multiple case reports of regenerative procedures have led to the resolution of apical periodontitis with radiographic increases in root width and length.[2,10-12,14,15] In the reported successful cases there is limited mechanical instrumentation and the main success relies on chemical disinfection of the root canal.[11-13]

Several *in-vitro* and *in-vivo* studies have shown the success of antimicrobial mixtures in chemical debridement of necrotic pulp tissue during regeneration.[11-13] Chemical debridement in regenerative endodontics has consisted of irrigation with NaOCl in addition to mixtures of different combinations of triple antibiotic paste (TAP), double antibiotic paste (DAP) or calcium hydroxide ($Ca(OH)_2$) as intracanal medicaments. These medicaments have shown to be successful at certain concentrations. However, at high concentrations they have been shown to be toxic to stem cells of the apical papilla.[16,17]

The regenerative protocol developed by the American Association of Endodontist involves using $Ca(OH)_2$ or TAP as intracanal medicaments.[18] When comparing the two, TAP-medicated teeth had significantly increased root wall thickness when compared with $Ca(OH)_2$.[6] Root canal system infections are polymicrobial and the microbial profile of infected immature teeth has been shown to be similar to primarily infected permanent teeth, with the most prevalent being *Actinomyces nausulundii*.[19] A combination of antibiotics (i.e., TAP, DAP) is needed to address the diverse bacterial composition. TAP and DAP have been shown to be highly effective against *E. faecalis* and *P. gingivalis*, common endodontic bacteria.[20-22]

In this study our focus was to develop a double antibiotic polymer-based scaffold containing ciprofloxacin and doxycycline. Doxycycline (DOX) is a long acting broad-spectrum bacteriostatic antibiotic that inhibits RNA translation. Ciprofloxacin's (CIP) mechanism of action is a broad-spectrum antibiotic that inhibits DNA synthesis. The effectiveness of an antibiotic requires direct contact with the microbe and is related to both the type and concentration of the antibiotic. In addition, they must reach the target

tissues in therapeutic concentrations.[17] Ultimately, the intracanal application of uniform controlled amounts of antibiotic allows for direct action, greater therapeutic effects and minimizes adverse effects of toxicity and systemic delivery side effects.[23]

In recent years, local drug delivery systems have been successfully developed through a nanotechnology based approached called electrostatic spinning.[23-28] Electrostatic spinning has been able to produce synthetic polydioxanone (PDS) fibrous scaffolds that are biocompatible and can support cell growth.[27] To the best of our knowledge, an electrospun polymer-based double antibiotic scaffold containing the drugs CIP/DOX has yet to be studied in regenerative endodontics as a root canal disinfectant. Thus, this study synthesized electrospun polymer-based scaffolds containing a specific concentration of CIP50% and DOX50%, and determined, *in vitro*, its mechanical properties, chemical composition through Fourier transform infrared spectroscopy (FTIR), and antimicrobial effectiveness, through agar diffusion, against multiple endodontic bacterium, i.e., *Enterococcus faecalis* (*E. faecalis*), *Streptococcus gordinii* (*S. gordinii), Porphyromonas gingivalis* (*P. gingivalis*) and *Fusobacterium nucleatum* (*F. nucleatum*)

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CLINICAL SIGNIFICANCE

This study has two areas of clinical significance in endodontics:

1. Continue to optimize regenerative endodontic disinfection through an electrospun scaffold.

2. Provide baseline research for the potential use of this scaffold as a root canal disinfectant against primary or persistent root canal bacteria.

PURPOSE

The purpose of this study is to synthesize antimicrobial active DAP electrospun scaffolds against the following pathogens: (*E. faecalis, S. gordonii, F. nucleatum and P. gingivalis*).

NULL HYPOTHESIS

The addition of CIP/DOX to the electrospun scaffolds will not eliminate or reduce antimicrobial action (*E. faecalis, S. gordonii, F. nucleatum and P. gingivalis*).

ALTERNATIVE HYPOTHESIS

The addition of CIP/DOX to the electrospun scaffolds will demonstrate elimination and or reduction of antimicrobial action (*E. faecalis, S. gordonii, F. nucleatum and P. gingivalis*).

REVIEW OF LITERATURE

"Yesterday is history, tomorrow is a mystery, today is a gift, which is why we call it the present" – Bil Keane.

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HISTORY OF ENDODONTICS

In order to understand the present and look to the future we must first look at the past. The first book written in English on dentistry was done by Charles Allen in 1687. The beginning of endodontics dates all the way back to the 17th century. However, the first description of dental disease and associated tooth pain could be traced back to a much earlier time. It was once believed the cause of tooth pain could be found in a worm that resided within the tooth which caused pain by burrowing inside of the tooth.[29] Drawings of the "worm theory" were illustrated by the Chinese in ancient history.[30] In 1728, Pierre Fouchard, challenged the worm theory in the *Surgical Dentist*. His book detailed accessing the pulp chamber to aid in removing the pulp allowing for abscess drainage followed by filling the access with lead.[31] In 1766, Robert Woofendale, wrote on relieving dental pain by "cauterizing the pulp with a hot instrument" and using oil of cinnamon, clove or turpentine on exposed pulp to aid with pain and "destroy the nerve.[31]

During the late 1700s and mid-1800s advancements in endodontics were accelerating. In 1838, Edwin Maynard, was credited with introducing the first root canal instrument, which was created with a watch spring.[32] In 1839, the *American Journal of Dental Science*, published John Bakers work as the first description of complete root canal therapy which involved removal of pulp tissue, cleaning and filling the root canal. In 1847, Edwin Truman, introduced gutta-percha as a filling material and twenty years

later G.A. Bowman used gutta-percha cones to obturate root canals.[33] In 1864, Sanford Christie Barnum, invented the rubber dam by shaping a thin rubber leaf to isolate the tooth when filling.[34] In 1867, Joseph Lister, documented the use of antiseptics to the medical community. Within the same year *Leptothrix buccalis* was identified as a "parasite" on teeth and in carious lesions. This led to the idea that caries could lead to pulpal changes. The "parasite" findings led to the use of antiseptic materials, to "sterilize" the pulp.[31]

Around the turn of the century drastic advances in medicine and dentistry were evolving. In 1895, William C. Roentgen discovered radiographs and by 1917 they were introduced to dentistry.[31,35] This invention allowed for evaluation of the endodontic treatment through radiographs. By the 1940s, 75 percent of dental offices were using an x-ray machine.[35]

Anesthetic use for dentistry started in the 1800s when Wells suggested the use of nitrous oxide during surgical procedures for general anesthesia. Other forms of anesthesia at this time were sulfuric acid, chloroform and cocaine as a topical. All these forms of anesthesia were limited for dentistry because of their major side effects. Alfred Einhorn, in 1905, saved many people from painful dental experiences through the development of procaine (Novacaine).[29] With radiographs and anesthetic becoming more common in dentistry endodontic treatment became a more pleasant experience for patients.

In 1967, the main principles of endodontic treatment discussed by Grossman.[36] These principles included confinement of instruments to the root canal; avoiding forceful entry of instrumentation; the use of biomechanical instrumentation and bathing the root canal system with antiseptic solution.[36] At first, many caustic materials were being used

as disinfectants including, sodium dioxide, metallic sodium or potassium, and 20-40% sulfuric acid. Ionization, at that time called electro-medication, was becoming more popular in an attempt to be less caustic.[31]

Endodontic therapy in the early 1900s began to gain acceptance in the dental community with the new technologies, materials, and techniques. The endodontic momentum took a detour in 1910 due to William Hunter, an English physician and pathologist, who lectured to the University of Montreal on focal infection theory. The lecture titled. "The Role of Sepsis and Antisepsis in Medicine," accused dentists of creating a "vertable mausoleum of gold over a mass of sepsis" that caused multiple system diseases.[37] This triggered the idea that system disease could be cured through tooth extraction. This farce made the practice, research and teaching of endodontics very difficult for over two decades. During this time many teeth were extracted that should have been saved. The endodontic detour tooth stopped, in the 1940s when laboratory research and clinical evidence showed devitalized teeth did not play a role in causation of system disease.[38] The focal infection theory lost credibility and faith was brought back to endodontic treatment.

Throughout the 1940s and the 1950s root canal therapy continued to take strides and inspired a small group of 20 dentists who met in Chicago to create an endodontic organization.[38]

The American Association of Endodontists was created with four goals in mind.[38]

1. Promote interchange of ideas on methods of pulp conservation and root canal treatment

2. Stimulate research studies, both clinical and laboratory

3. Assist in establishing local root canal study clubs

4. Help maintain a high standard of root canal practice within the profession by disseminating information through lectures, clinics, and publications

The association continued its growth, and created the American Board of Endodontics in 1956, the American Dental Association officially recognized endodontics as a specialty in 1963.[38] Since the AAE received support from the ADA, membership has grown over the past fifty two years to 7800 AAE members worldwide.

Endodontic Theory

The goal of endodontic therapy is to treat pulpal and periapical disease. When pulpal tissue is irritated by restorations, caries or trauma, a pulpitis occurs which can develop into pulpal necrosis. When bacteria enter the root canal space pulpal pathosis can lead to periapical disease. Kakehashi, Stanley, Fitzgerald demonstrated that when bacteria contaminates a tooth, endodontic problems will occur.[39] The major goal of endodontic therapy in primary or secondary infections is to eliminate microbial insult while maintaining natural form, function and esthetics of a tooth.[40] If this is not accomplished, bacterial byproducts that accumulate can travel through the root canal system into the periradicular tissue through accessory canals, the apical foramen or lateral canals, which can lead to inflammation and disease.[41,42] There are three phases of root canal treatment: chemomechanical preparation, microbial control, and obturation of the root canal.[43] Successful endodontics is directly related to reduction of bacteria in the root canal system through chemomechanical cleaning and the ability to seal the tooth to prevent recontamination.[39,40,43,44] Chemomechanical preparation of the tooth is achieved by first debriding the area with mechanical filing of the root system to allow for chemical

irrigants to reach the apical portion of the tooth. The standard of care requires treatment to be completed under rubber damn isolation to help further aid in asepsis.[40] Once chemomechanical debridement is complete three dimensional obturation to the cementodentinal junction or 0.5 mm to 1 mm from the radiographic apex is required.[44] After successfully cleaning and obturating the root canal system a final restorative seal is required to prevent bacterial recontamination.[40] All these factors contribute to a successful outcome in root canal therapy.

Microorganisms

Endodontic microorganisms can be classified into primary infectious bacteria and secondary infectious bacteria. In both primary and secondary infections there are a diverse group of bacteria present that exist in a community called a biofilm. A biofilm is a structural and dynamic organization of complex biologic systems that provide many advantages to bacteria for survival and virulence.[40]

Primary endodontic infections are composed of a mixed flora, predominately gram (–) anaerobic rods.[45,46] In 2014, Nagata showed that the microbial profile of infected immature teeth is similar to the microbial profile in primary infected permanent teeth with an average species of 2.13 per root canal.[19] The most prevalent species found in the root canals of immature teeth is *Actinomyces naeslundii*.[19] *A. naeslundii* is an anaerobic, Gram (+), facultative-rod shaped microbe that is found in the normal flora of oral caries, plaque, and primary endodontic infections.[40] *A. naeslundii* binds to salivary proteins, epithelial cells, and tooth surfaces.[47-49] Its major virulence factor that plays a key role in activating an inflammatory response is through the peptidoglycan layer (PGL) which accounts for 50 percent to 80 percent of its cell wall.[50-53] The PGL can activate the

innate immune system of the host cells causing a release of cytokines triggering inflammation.[50-53]

F. nucleatum another primary endodontic bacterium is a gram-negative, non spore forming spindle-shaped or fusiform rod.[54] It has frequently been found in patients with periodontal disease[54] and in patients with symptomatic apical periodontitis and acute apical abscess in primary infections.[55,56] This bacteria plays an essential role in biofilm formation and has been associated as a middle colonizer due to its ability to bind to several different Gram (+) and Gram (-) bacteria. Its main virulence ability is correlated to its ability to invade host tissue cells and modulate a host immune response.[54,57]

P. gingivalis has been found in around one half of primary endodontic infections, and is an obligate anaerobic gram (-), non-motile, coccobacilli rod.[55,56,58] In non-pathosis oral cavities, it is found in the gingival sulcus, dorsum of tongue, and tonsils.[58] Endodontic pathosis of primary root canal infections has shown *P. gingivalis* in chronic apical periodontitis, acute apical abscesses, and symptomatic/asymptomatic apical periodontitis.[56,58] *P. gingivalis* when grown on blood agar plates has been shown to form a black pigmentation due to its concentration of protoheme.[58] Its main virulence factors include lipopolysaccharide, fimbriae, capsule, lipoproteins, and vesicles to name a few.[56,58,59] Traditionally, these microbes have been eradicated from the root canal system by using a minimum of 1% NaOCl for 1 minute as an irrigant.[60]

Secondary endodontic infections are composed of a mixed flora, predominately Gram (+) facultative cocci (Enterococcus, Streptococcus, Staphylococcus[61]) with an average species of 1.3.[46] Secondary infectious microbes are not present during initial treatment and gain access through canal spaces during treatment, interappointment time

or coronal leakage.[40,55] Asepsis, a proper coronal seal during interappointment treatment and a final restoration are imperative to prevent secondary endodontic infections.

E. faecalis is a facultative anaerobic gram positive cocci that can occur singular, in pairs or as short chains.[62] It is present in the oral cavity in individuals who have an endodontic history[63]. It has been isolated in low prevalence in primary endodontic infections and high prevalence in persistent endodontic infections.[62] *E. faecalis* is the most frequently isolated microbe in persistent endodontic infections due to its ability to invade dentinal tubules and resist the high pH of calcium hydroxide.[40,55,61,64,65] *E. faecalis* has many virulence factors which include: lytic enzymes, cytolysis, aggregation substance, pheromones, and lipoteichoic acid.[66] *E. faecalis* is a master of survival in the root canal system and is able to form a biofilm that helps it survive by becoming 1000 times more resistant to phagocytosis, antibodies, and antimicrobials than non-biofilm organisms.[62,67]

S. gordonii a non-motile gram (-) cocci has been associated with gene transfer resistance with *E. faecalis*.[64] As well, Streptococcus species have been shown to be in high association with persistent endodontic infections.[68] It has been shown that 3% sodium hypochlorite is an effective irrigant in destroying these microbe from the root canal system.[69]

Treatment of the Immature Tooth

Apexification

Traditionally, immature permanent necrotic pulps were treated with calcium hydroxide to induce apexification at the root apex.[70] Traditionally, after calcium hydroxide the apical area was sealed with gutta percha. Modern day apexification, is

done in one appointment, sealing the apical portion with MTA allowing healing of apical periodontitis without continued development and subsequently a thin fragile root presides.[70] Long-term calcium hydroxide has been shown to decrease root strength to only half in a year.[3] Both these methods are proven treatment modality, which requires less patient compliance. The risks and benefits should be given to the patient as an alternative treatment option.

An ideal alternative to apexification is regenerative-based procedures. The goal of regeneration is to encourage root development both in length and width and for the vitality to be restored of the pulp dentin complex. Regenerative endodontics is done by disinfecting the root canal system (RCS) and inducing bleeding from the apical papilla. Stem cells are found in the apical papilla and laceration of this area triggers an inflow of blood with stem cells, into the root canal system.[7-9] Over the past decade several publications have prompted the revitalization of a regenerative approach for endodontic treatment.[2,5,10-13] Additionally, multiple case reports of regenerative procedures have led to the resolution of apical periodontitis with radiographic increases in root width and length.[2,10-12,14,15] In the reported successful cases there is limited mechanical instrumentation and the main success relies on chemical debridement of the root canal.[11-13]

Regenerative treatment

Regeneration of tissue to normal form and function has always been a goal in medicine; this process is called tissue engineering.[71,72] There are three major biological requirements that must be present for tissue engineering: a cell source, a physical scaffold, and the presence of signaling molecules that allows for differentiation of cells.[2]

The field of tissue engineering has gained ground in regeneration of the pulp dentin complex in endodontics over the last decade.[73]

Stem cells are one of the three integral parts of regeneration and are the cell source of tissue engineering in endodontic regeneration. Stem cells are defined as lonogenic cells capable of self-renewal and multi-lineage differentiation.[74] Gronthos in 2000 found a major reservoir of stem cells, in the apical papilla (SCAP), periodontal ligament (PDLSC), stem cells from human exfoliated deciduous teeth (SHED), dental pulp stem cells (DPSC), and dental follicle stem cells (DFS).[74,75] Endodontic regeneration stem cells are post natal and have been shown to be the most promising[74]. Mesenchymal stem cells are delivered into the root canal system in regenerative procedures by stimulating the apical portion of the tooth.[8] In necrotic immature teeth, stem cells located in the apical papilla are viable following necrosis.[7,71] The dental pulp has proven to have regenerative properties to assist in regeneration.[76,77] Stem cells are concentrated in the perivascular region of the cell-rich zone next to the odontoblastic layer.[40,72] The five mesenchymal stem cells described by Gronthos are essential for pulp fibroblasts, extracellular matrix, and collagen to regenerate.[40,74,75] However, SCAP's hold a great potential in regenerative endodontics and are believed to be the main source of undifferentiated cells in the process of root development. They have greater proliferation rates than dental pulp stem cells and have previously differentiated to odontoblastic-like cells resulting in new production of dentin in vivo.[7,78,79] The apical papilla represents an enriched source of stem cells and have been of particular interest in regenerative endodontics due to their location and ability to go from a quience to an activated state.[9,40,74]

Growth factors are the signaling molecules in regenerative endodontics. They are polypeptides that promote proliferation, migration, and differentiation of a variety of cells.[40] In regenerative procedures several growth factors have been identified. The growth factors include transforming growth factor beta (TGF-B), bone morphogenic protein (BMP) and vascular endothelial growth factor (VEGF). Current sources of growth factors for endodontics are found in platelets and dentin. TGF found in dentin stimulates dentin formation allowing for development of root length and width.[77,80] BMP induces differentiation of odontoblasts allowing for mineralization during root development. VEGF regulates angiogenesis through chemotaxis induction, proliferation, and differentiation of cells present in wound healing.[73,80] It has been shown that the application of dexamethasone as a growth factor greatly increased differentiation of stem cells to odontoblastic like cells.[14] In regenerative endodontics, it has been demonstrated that ethylenediaminetetraacetic acid (EDTA) induces odontoblastic/osteoblastic differentiation when dentin pulp stem cells are in direct contact with EDTA-treated dentin.[81] The EDTA-treated dentin provides a reservoir of growth factors being released which signals repair and regeneration.[82,83]

Scaffolds are the third part of the trifecta of tissue engineering. It acts as an extracellular matrix that allows for transport of nutrients, oxygen, and metabolic waste.[76] Scaffolds should be biocompatible, non-toxic and have optimal physical features and mechanical properties.[84] There are two types of scaffolds; natural (platelet-rich-plasma, autologous blood clot) and/or synthetic. Synthetic polymers allow for precise control over physicochemical properties such as degradation rate, porosity, microstructure and mechanical properties.[76] Electrospun synthetic scaffolds have gained popularity due to

their excellent mechanical properties and ability to add antibiotics into their microstructure.[85-88] Electrospun fibers have been shown to be very similar to the fibers of the extracellular matrix which allows for cell migration, distribution of nutrients and architecture of the dentin-pulp complex.[85,88] Polydioxanone (PDS) sutures are used for scaffold fabrication. The PDS sutures are resorbable, and degrade to fifty percent in three weeks and at six months are completely absorbed.[85,88] The scaffold is infused with antibiotics to help maintain an antimicrobial environment within the root canal system to allow for healing to occur.[88,89] By localizing the delivery of the antibiotic the negative systemic side effects of antibiotics are limited.[88] Drug release can be manipulated to allow for different rates of drug delivery.[23,27,88] The electrospun scaffolds with antibiotics address two major parts of regenerative endodontics by providing a scaffold and allowing for disinfection. Another major advantage is the reapplication of dressings is not as prevalent and this will limit potential bacterial recontamination.

Considerations for regenerative procedures updated by the AAE 4/2015 are:[18]

Case Selection:

- Tooth with necrotic pulp and an immature apex.
- Pulp space not needed for post/core, final restoration.
- Compliant patient/parent.
- Patients not allergic to medicaments and antibiotics necessary to complete procedure (ASA 1 or 2).

Informed Consent

- Two (or more) appointments.
- Use of antimicrobial(s).

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

First Appointment

- Local anesthesia, dental dam isolation and access.
- Copious, gentle irrigation with 20 ml NaOCl using an irrigation system that minimizes the possibility of extrusion of irrigants into the periapical space (e.g., needle with closed end and side-vents, or EndoVac™). Lower concentrations of NaOCl are advised [1.5% NaOCl (20 mL/canal, 5 min) and then irrigated with saline or EDTA (20 mL/canal, 5 min), with irrigating needle positioned about 1 mm from root end, to minimize cytotoxicity to stem cells in the apical tissues. Dry canals with paper points.
- Place calcium hydroxide or low concentration of triple antibiotic paste. If the triple antibiotic paste is used: 1) consider sealing pulp chamber with a dentin bonding agent [to minimize risk of staining] and 2) mix 1:1:1 ciprofloxacin: metronidazole: minocycline to a final concentration of 0.1 mg/ml.
- Deliver into canal system via syringe
- If triple antibiotic is used, ensure that it remains below CEJ (minimize crown staining).
- Seal with 3mm to 4 mm of a temporary restorative material such as Cavit™, IRM™, glass ionomer or another temporary material. Dismiss patient for 1 week to 4 weeks.

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

- Assess response to initial treatment. If there are signs/symptoms of persistent infection, consider additional treatment time with antimicrobial, or alternative antimicrobial.
- Anesthesia with 3% mepivacaine without vasoconstrictor, dental dam isolation.
- Copious, gentle irrigation with 20 ml of 17% EDTA.
- Dry with paper points.
- Create bleeding into canal system by over-instrumenting (endo file, endo explorer) (induce by rotating a pre-curved K-file at 2 mm past the apical foramen with the goal of having the entire canal filled with blood to the level of the cemento–enamel junction). An alternative to creating a blood clot is the use of platelet-rich plasma (PRP), platelet rich fibrin (PRF) or autologous fibrin matrix (AFM).
- Stop bleeding at a level that allows for 3 mm to 4 mm of restorative material.
- Place a resorbable matrix such as CollaPlug™, CollaCote™, CollaTape™ or other material over the blood clot if necessary and white MTA as capping material.
- A 3 mm to 4 mm layer of glass ionomer (e.g., Fuji IILC™, GC America, Alsip, IL) is flowed gently over the capping material and light-cured for 40 s. MTA has been associated with discoloration. Alternatives to MTA should be considered in teeth where there is an esthetic concern.

- Anterior and Premolar teeth - Consider use of CollaTape/CollaPlug and restoring with 3 mm of RMGI followed by bonding a filled composite to the beveled enamel margin.
- Molar teeth or teeth with PFM crown - Consider use of CollaTape/CollaPlug and restoring with 3 mm of MTA, followed by RMGI or alloy.

Follow-up

- Clinical and Radiographic exam
  - No pain, soft tissue swelling or sinus tract (often observed between first and second appointments).
  - Resolution of apical radiolucency (often observed 6 months to 12 months after treatment)
  - Increased width of root walls (this is generally observed before apparent increase in root length and often occurs 12 months to 24 months after treatment).
  - Increased root length.
  - Positive pulp vitality test response
- The degree of success of regenerative endodontic procedures is largely measured by the extent to which it is possible to attain primary, secondary, and tertiary goals:
  - Primary goal: The elimination of symptoms and the evidence of bony healing.
  - Secondary goal: Increased root wall thickness and/or increased root length (desirable, but perhaps not essential)

- Tertiary goal: Positive response to vitality testing (which if achieved, could indicate a more organized vital pulp tissue)

Irrigation

Irrigation is defined as washing out a body cavity or wound in water or a medicated fluid.[29,40] It has been well documented that after mechanical rotary instrumentation that 35 percent of the root canal system remains un-instrumented.[90,91] Irrigation of the canal system is an integral step in root canal therapy and compliments debridement by aiding in disinfection of the un-instrumented spaces. The ideal characteristics of an endodontic irrigant should:[40,92,93]

1. Be an effective germicide and fungicide.
2. Remain stable in solution and be nonirritating to periapical tissues.
3. Have prolonged antimicrobial effect.
4. Be active in the presence of blood, serum, and protein derivatives of tissue
5. Have low surface tension.
6. Not interfere with repair of periapical tissues.
7. Not stain tooth structure.
8. Be capable of inactivation in a culture medium.
9. Not induce a cell-mediated immune response.
10. Be able to completely remove the smear layer, and be able to disinfect the underlying dentin and its tubules.
11. Be nonantigenic, nontoxic, and noncarcinogenic to tissue cells surrounding the tooth.
12. Have no adverse effects on the physical properties of exposed dentin.

13. Have no adverse effects on the sealing ability of filling materials.
14. Have convenient application.
15. Be relatively inexpensive.

For endodontic effectiveness irrigation solutions must be: antimicrobial, dissolve tissue and be biocompatible.[94] In regenerative procedures the current AAE clinical considerations recommend lower concentrations of NaOCl (1.5%, 20 ml/canal, 5 min) and then with saline (20 ml/canal, 5 min), with a safe end needle 1 mm from the root end at the initial appointment and 17% EDTA at the second appointment.[18] The side vented needle or use of an EndoVac will help in preventing extrusion of irrigant minimizing cytotoxicity to stem cells of the apical tissue.[18] In regenerative procedure the preservation and promotion of stem cells is vital to success. The irrigation regimen for traditional nonsurgical treatment is different due to the focus of stem cell preservation in regenerative procedures.

Sodium Hypochlorite (NaOCl)

NaOCl was introduced by Coolidge to dentistry in 1919.[95,96] To date, NaOCl is the most commonly used irrigation in endodontics due to its phenomenal antibacterial activity and capability to dissolve organic tissue.[40,97,98] Concentrations of NaOCl vary from 0.5 percent to 8 percent in endodontic treatment. A lower concentration, such as 1.5 percent, dissolves mainly necrotic tissue and minimizes vital tissue destruction[99]. NaOCl is hypertonic, bactericidal, has a high pH of 11, and has a minimal effect on the dentin smear layer.[40,98] The free chlorine in NaOCl breaks down proteins into amino acids.[40] Hypochlorous acid is the active antibacterial property of NaOCl which disrupts oxidative phosphorylation activities.[100] When acting against enterococci, NaOCl has been shown to

deactivate (kill) the microorganism within 30 minutes at 0.5 percent and 2 minutes at 5.25 percent when in direct contact with the bacteria.[101] When acting against actinomyces, 1 min at 1.0-percent solution has been shown to deactivate the organisms.[101] However, Estrela et al. noted NaOCl with CHX has also been shown to not completely eliminate *E. faecalis* when a PCR culture technique was used.[102] CHX has been shown to be detrimental to stem cells of the apical papilla (SCAP) survival and is not currently recommended for regeneration procedures.[103] Sterilization of the root canal system is an unattainable goal and the focus remains on disinfection. Heating NaOCl has shown to enable lower concentrations to have a similar effect as higher concentrations at different temperatures. When looking at heating the solution consideration must be given on the effects on the periodontal ligament, osteoblasts, and stem cells.[40,104] NaOCl is currently the staple in endodontic irrigation due to its enhanced proteolytic activity, cost effectiveness and wide use in endodontics.[40,99]

Ethylenediamenetetraacetic Acid (EDTA)

Ferdinand Munz first described EDTA in 1935.[40] As a chelating agent it rapidly sequesters di- and tricationic metal ions, such as Ca^{2+} and Fe^{3+}.[40] Over time, EDTA will cause bacterial death when there is direct exposure of EDTA to surface proteins.[40] EDTA cannot work alone, first NaOCl must remove the organic debris prior to its effects.[105] EDTA prepared dentin surfaces have shown to remove the dentin smear layer and improve the fluid tight seal when sealing the root canal system.[106] Current regenerative protocol considers using 17-percent EDTA, which is a common percentage in endondontics.[18] The concentration recommended can remove the dentin smear layer within one minute and is self-limiting after 7 hours.[40,107] The chelation of EDTA has

shown to detach biofilms, by removal of divalent cations from adhering to the dentin walls which saline cannot provide.[40] In regenerative endodontics, it has been shown that EDTA induces cell attachment and odontoblastic/osteoblastic differentiation when dentin pulp stem cells are in direct contact with EDTA-treated dentin.[81] It has also been reported that EDTA aids in SCAP survival.[103,108] When testing EDTA and chlorhexidine in regards to stem cell survivability, EDTA had an 89-percent cell survivability compared to 0 percent with chlorhexidine.[103] Chlorhexidine is not currently recommended for regenerative procedures due to its effects on stem cells.

Intracanal Medicaments

Cases requiring multiple visits use intracanal medicaments to aid in limiting surviving bacteria from proliferating between appointments.[40,109,110] For regenerative treatment, the two main intracanal medicaments that are recommended for clinical consideration are calcium hydroxide and triple antibiotic paste.[18]

Calcium hydroxide: $Ca(OH)_2$

In 1920, Hermann introduced $Ca(OH)_2$ for endodontic use.[37,40] Calcium hydroxide has been shown to be antimicrobial by inactivating and detoxifying lipopolysaccharide (LPS) endotoxins.[100,111,112] LPS inactivity is important which will limit any stimulated inflammatory responses to the periapical tissue. Calcium hydroxide has a basic pH and is effective when in direct contact with bacteria.[113] The hydroxyl ions create free radicals that react with bacterial DNA by inhibiting DNA replication and cell activity[113]. Increased pH (12.5) alters enzyme activity disrupting cellular metabolism and structural proteins.[113] *In vitro,* $Ca(OH)_2$ in direct contact for 24-hours showed complete killing of

enterococci.[40,114,115] Pure calcium hydroxide should be avoided due to its effects on OPG/RANKL ratio and inhibition of hard tissue formation.[116] Calcium hydroxide should be mixed with sterile water or saline to create a slurry or paste and should have a consistency throughout the working length of the tooth. There are limitations associated with calcium hydroxide including its antimicrobial effectiveness against endodontic pathogens and the inability to completely remove calcium hydroxide with saline, NaOCl, or EDTA.[40,117,118] In regenerative endodontics calcium hydroxide has been approved, is widely available and has been supported in case series reports.[2,5] As well it has been shown to increase SCAP survival and proliferation.[16,119]

Triple Antibiotic Paste (TAP)

Endodontic infections are polymicrobial, which no single antibiotic can fully eliminate all the bacteria.[70] Hoshino first used TAP to disinfect teeth and found that it was effective in eradicating bacteria from infected root dentin.[120,121] The three antibiotics used were 200 mg ciprofloxacin, 500 mg metronidazole, and 100 mg minocycline.[121] TAP'S effectiveness has been shown when tested in clinical studies on dogs' teeth the CFU counts were decreased to zero when treated with NaOCl and TAP in two weeks of treatment.[122] The concentration of the antibiotics has been shown to determine the effects on SCAP. In high concentrations (1 mg/mL, 10 mg/mL, and 100 mg/mL) it has detrimental effects on SCAP, however in low concentrations (0.1 mg/mL, and 0.01 mg/mL) there is no detectable effect on SCAP.[16] The main advantages of TAP are its effectiveness, supported case series and non-toxicity at low concentrations to SCAP. However, the disadvantages are staining, it not being widely available and potential for

drug allergies and resistance. If the paste is used it is important to let the parents know that it is not FDA approved and must be made by a compounding pharmacy.

Double Antibiotic Paste (DAP)

Ciprofloxacin has been shown to be effective in treatment of periodontitis and oral recommendation for dosage is 500 mg every 12 hours for 8-10 days. It's mechanism of action inhibits DNA-gyrase and promotes break down of double stranded DNA. Contraindications include previous hypersensitivity reactions to quinolones.[123]

Doxycycline has been shown to be effective in treatment of periodontitis. It's mechanism of action inhibits protein synthesis by binding with the 30S and possible the 50S ribosomal subunit (s) of susceptible bacteria. Patients should not use during pregnancy or if they have had a previous tetracycline reaction.[123] Current research is being done on the effects it has on discoloration of the teeth.

Scanning Electron Microscope (SEM)

Developed in the 1950s, SEM uses electrons instead of light to develop an image. The SEM's has distinct advantages over traditional microscopy such as: a larger depth of field, higher resolution and a greater control over the degree of magnification. A larger depth of field allows for more of the specimen to be in focus. A higher resolution allows for greater magnification of images. With the SEM electromagnets are used instead of lenses to allow for more control in the degree of magnification.[124]

Prior to using the SEM, the samples must be prepared by removing all the water from the sample and a sputter coat must be formed on the sample. If water is left on the sample the vacuum would vaporize the water. All non-metal samples must be coated with

a thin layer of metal by using a "sputter coater". The sputter coater uses an electron field and argon gas which allows gold atoms to fall and settle onto the surface of the sample producing a thin gold coating.[124]

After preparing the samples the sends a beam of electrons are sent through a vertical path through the microscope, which is in a vacuum. Once the beam hits the sample, electrons and X-rays are ejected from the sample. A detector collects these X-rays and electrons and converts them to the final image. [124]

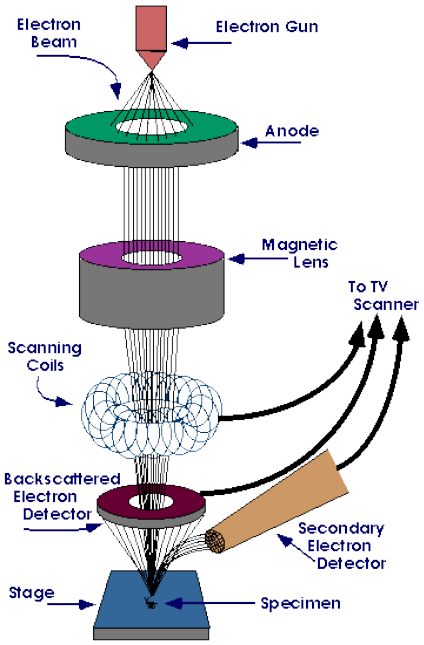

Image courtesy of Iowa State University[124]

Agar Diffusion

Diffusion assays allow for a substance to diffuse through agar gels seeded with a test organism to view its biological activity.[125] This measurement is how the bacterial growth is inhibited not necessarily the killing of bacteria. Once the agar plates with the bacteria have been inoculated with the antimicrobial test specimen a clear zone of inhibition may form. The ability for the antibiotic to diffusion through the bacterial lawn is related to size and action of the antibiotic. Larger molecular weight antibiotics will not

be able to diffusion as easily when compared to smaller molecular weight antibiotics. Doxycycline, a bacteriostatic drug, has a molecular weight of 462.46. Ciprofloxacin, a bacteriocidal drug, has a molecular weight of 385.8. The zone of inhibition is in a circular shape and the diameter is then measured in millimeters to provide a numerical value to the biological activity. The mean inhibitor concentration obtained from agar diffusion is considered the gold standard in antibiotic susceptibility testing against bacteria.[125,126]

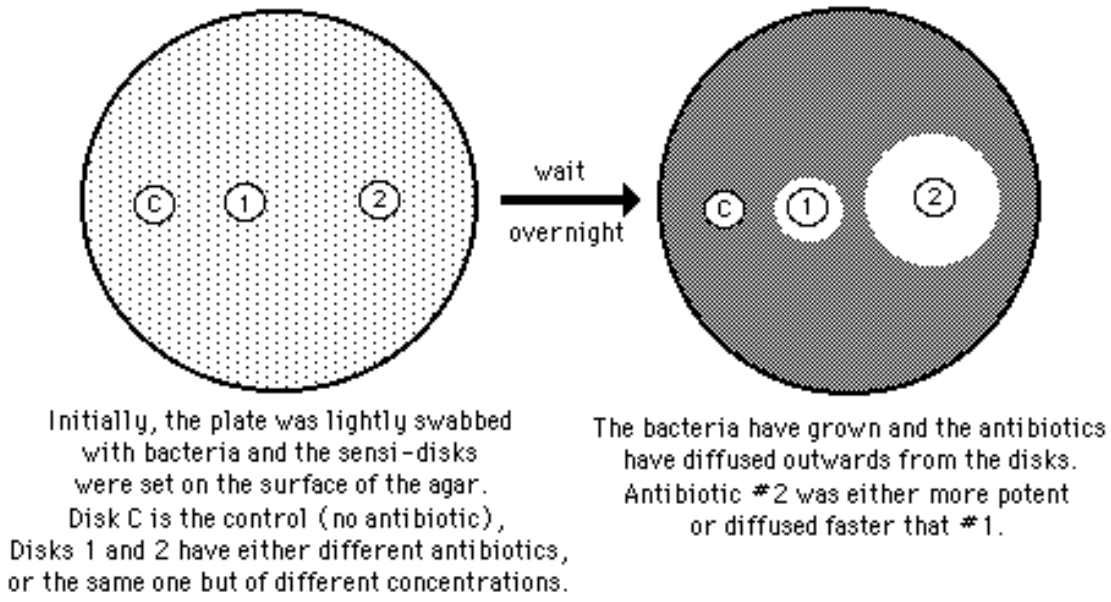

Image taken from: http://www.science-projects.com/apdiffuz.htm

MATERIALS AND METHODS

ELECTROSPUN POLYMER SCAFFOLD

Polymer solutions containing distinct concentrations/ratios of CIP (i.e., 50wt%) were electrospun into fibers based on optimized electrospinning parameters.[23] In brief, Polydioxanone (PDS), a monofilament synthetic absorbable suture material (PDS, Ethicon Inc., Somerville, NJ, USA) was cut into pieces and placed into glass vials containing dichloromethane (Sigma-Aldrich, St. Louis, MO, USA). Dichloromethane was added to remove the violet dye.[27,127] The non-violet, clear PDS was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma-Aldrich) at a 10wt% concentration with vigorous stirring.[27,28] Ciprofloxacin (CIP) and Doxycycline (DOX) (Sigma) was added to the dissolved PDS. The concentration of CIP 50%wt (300mg CIP), DOX 50%wt (300mg DOX) and CIP50%:DOX50% (300mg CIP: 300mg DOX) were added by directly mixing the two compounds with the PDS solution and vigorously stirred, to obtain the distinct PDS-CIP, PDS-DOX, PDS-CIP/DOX based scaffolds via electrospinning. The solution of dissolved PDS with respective antibiotic concentrations was electrospun into fibers through a custom made apparatus as described in previous studies by Bottino and co-workers.[27,28] Pure PDS (control) or PDS-DOX, PDS-CIP, PDS-CIP/DOX solutions were individually loaded into 5-mL plastic syringes (Becton-Dickinson, Franklin Lakes, NJ, USA) having a metallic 27g blunt-tip needle and electrospun under ideal parameters (2 mL/hr., 18-cm distance, and 15 kV). The fibers were collected at room temperature (RT) on the aluminum-foil-covered rotating mandrel. The electrospun scaffolds (EMATS),

were dried in a desiccator at RT for at least 48 hrs for removal of any remaining solvent.[23,27]

FIBER CHARACTERISTICS: MORPHOLOGICAL (SEM) AND CHEMICAL (FTIR)

Scanning electron microscopy (JSM-5310LV;JEOL,Tokyo,Japan) was performed to assess scaffold structure and fiber morphology. Samples taken from each electrospun scaffold were mounted on an Al stub and sputter-coated with Au-Pd prior to microscopy. Image J software (1.44i; National Institutes of Health, Bethesda, MD) was used with SEM images to determine fiber diameter of 50 single-fibers per each image obtained (3 images/group) at the same magnification (X5000); the average fiber diameter was reported as a mean ± standard deviation. Fourier transform infrared spectroscopy (Jasco FT/IR-4100; Jasco, Easton, MD) using attenuated total reflection was conducted to confirm antibiotic incorporation.[23]

Mechanical Properties

Uniaxial tensile testing (eXpert 5601; ADMET, Norwood, MA) was used to evaluate the scaffolds (15mm x 3 mm, n=16/group, hydrated n = 8, dry n = 8) under both dry and hydrated conditions (24 h in PBS at 37℃). Stress-strain curves of each sample were collected and the following properties were evaluated: Tensile strength (TS), Young's modulus (YM), and elongation at break (E Break).[23,27] The results were reported as a mean ± the standard deviation.

Antimicrobial Testing

Agar diffusion testing against Enterococcus faecalis (Ef), Streptococcus gordinii (Sg), Porphyromonas gingivalis (Pg) and Fusobacterium nucleatum (Fn) was used to evaluate antimicrobial efficacy of the scaffolds. Square-shaped scaffolds (15mm x 15mm; n=4/group; CIP50% 14mg±0.3mg; DOX50% 9.8±0.5mg; CIP50%/DOX50% 13.7 ±0.1mg; PDS 8.4±0.5mg) were cut, weighed and placed in vials with 10 mL sterile phosphate-buffered saline (PBS, pH = 7.4; GIBCO BRI, Grand Island, NY). 1 mL aliquots of the elevated material in PBS were collected (1 day, 4 days, 7 days, 14 days; n=4/group) and 1 mL fresh PBS was added to vial to replace the volume of the aliquots. Samples were frozen (-20℃) until all were collected for antimicrobial testing (agar diffusion).

Bacteria were cultivated in Brain Heart Infusion (BHI) broth supplemented with 5 g yeast extract/L and 5% v/v vitamin K+ hemin (BHI-YE; Becton, Dickinson and Company) at 37℃ in an anaerobic GasPak jar for 24 h.[23,27] Each bacterium was swabbed over the entire surface of blood agar plates to create a bacterial lawn. 10 µL of each suspension was dropped on cultured blood agar plates containing the bacterial lawns, and the inhibition zones (in mm) were measured after 24 hrs of incubation for gram (+) and 5 days of incubation for gram (–) bacteria.[23,27] Each bacterium and sample (1 day, 4 day, 7 day, 14 day) was done in triplicate with a positive control (0.12% chlorhexidine) and negative control (PDS, antibiotic-free scaffold). Statistical analysis was completed with a two-way ANOVA with significant of $P < 0.05\%$.

RESULTS

FIBER MORPHOLOGY AND SCAFFOLD DIAMETER

Fiber morphology and scaffold diameter were evaluated under a SEM. Fig. 34 A-D shows SEM images of the pure PDS fibers, and fibers containing CIP50%, DOX50% and CIP/DOX50% displaying the fiber diameter distribution. Pure PDS fibers were demonstrated to be smooth with a wide range of diameters (Fig. 34A) compared to the more uniform drug containing scaffold diameters (Fig. 34B-D). The pure PDS fibers showed a median diameter of 933 nm with a gradual bell shaped curve distribution over its wide range (Fig. 34A). The antibiotic-containing scaffold of CIP50% had a median fiber diameter of 958 nm with less variability (Fig. 34B). DOX 50% had a median fiber diameter of 862 nm with a balanced fiber diameter distribution (Fig. 34C). CIP50%/DOX50% had a median fiber diameter of 1,159 nm which was larger than pure PDS and single-antibiotic containing scaffolds (Fig. 34D).

SCAFFOLD MECHANICAL PROPERTIES

Tensile strength was determined based on the stress-strain curve from the mechanical testing of each scaffold. The tensile strength is the maximum stress the fibers withstood before failing or breaking. The dry samples showed greater tensile strength in comparison to the wet samples, significance of $p < 0.05$ (Fig. 35). Of the dry scaffolds tensile strength measurements (in MPa) were in ascending order CIP/DOX50%, CIP50%, PDS, DOX50%. DOX50% wet showed to have the highest tensile strength and CIP50%-DOX50% wet showed to have the lowest tensile strength when comparing the different groups (Fig. 35). The dry DOX50% scaffold demonstrated a significantly higher tensile

strength and no significant difference were found amongst the other three groups (Fig. 35).

Elongation at break percentage, also known as fracture strain, is the ratio of changed length and initial length after breakage of each fiber. The dry samples exhibited greater elongation at break percentage in comparison to the wet samples (Fig. 36). Elongation at break (in %) of dry CIP/DOX50% showed a significant lower percentage when compared to the other three (Fig. 36). However, no significant difference was found amongst the other groups. Elongation at break (in %) wet DOX50% & CIP/DOX50% showed a significant lower percentage when compared to CIP50% & pure PDS. However, no significant difference was found amongst pure PDS and CIP50% (Fig. 36).

Young's Modulus of Elasticity, or the elastic modulus is the measurement of the fibers resistance to deforming elastically and was calculated based on the slope of the stress-strain curve in the elastic region of the fibers graph. Fig. 37 shows the Elongation at break (%) of each scaffold tested. The data illustrate that a significant difference was found in dry CIP/DOX50% when compared to pure PDS and no significant difference was found between CIP50% and DOX50%. The wet scaffold showed a greater modulus (MPa) when compared to the other three scaffolds (Fig. 37). CIP50%-DOX50% showed the highest dry young's modulus and lowest wet young's modulus.

SCAFFOLDS CHEMICAL COMPOSITION

Fourier transform infrared spectroscopy (FTIR) is a technique that obtains an infrared spectrum of absorption, emission, photoconductivity of the fibers and compares it to the individual antibiotic. FTIR analysis (Fig. 38) confirmed the presence of the antibiotics in each scaffold. Pure DOX in Fig. 38 shows an absorbance peak between

3,000 & 3,500 (cm^{-1}), which is marked with the orange arrow. A similar peak was noted in the scaffolds that contain DOX and are indicated with an orange arrow in Fig. 38. Pure CIP exhibited a characteristic absorbance peak at 1,000-1,500 (cm^{-1}) and identified with a purple arrow. Similar peak characteristics are found in the CIP containing scaffolds and also marked with a purple arrow (Fig. 38). These results confirmed the presence of each drug in their respective scaffolds. The peaks highlighted below in Fig. 38 show the presence of each drug.

AGAR DIFFUSION

Enterococcus faecalis (E.f)

All antibiotic-containing scaffolds inhibited the growth of E.f except for the negative control (pure PDS, Fig. 39). The bacterial inhibition of CIP50% on E.f demonstrated maximum inhibition zones at day 7. CIP50% exhibited the greatest inhibition against E.f. Statistical significance was found between all groups at each elution time interval. In ascending order, CIP50%, CIP/DOX50%, DOX50% provided the greatest inhibition at day 14 and all except DOX50% were comparable to the positive control (0.12%CHX) (Fig. 39). DOX 50% showed to have a similar significance to PDS non-drug containing scaffold after day 1 against E.f. CIP50% and CIP/DOX50% were compared to the positive control (0.12% chlorhexidine) with no significant difference.

Streptococcus gordonii (S.g)

All antibiotic-containing scaffolds demonstrated inhibition of growth against S.g, except for the negative control (pure PDS, fig. 40). The bacterial inhibition of CIP50% on S.g exhibited maximum inhibition at day 4 and was statistically significant (Fig. 40).

CIP50% provided significant inhibition when compared to DOX50% and CIP/DOX50% at all time intervals. DOX 50% had the greatest inhibition at day 1. When compared to the negative control (0.12% chlorhexidine) no significant difference was found between CIPDOX 50% and CIP50%.

Fusobacterium nucleatum (F.n)

All antibiotic-containing scaffolds inhibited the growth of F.n, except for the negative control (fig. 41). The bacterial effect of DOX50% against F.n demonstrated maximum significant inhibition when compared to the other scaffolds at all intervals except day 14.

Porphyromonas gingivalis (P.g)

All antibiotic-containing scaffolds inhibited the growth of P.g, except for the negative control (pure PDS, fig. 42). DOX50% and CIP/DOX50% against P.g demonstrated maximum inhibition at day 1 with no statistical significance between the two (Fig. 42). CIP/DOX50% exhibited the greatest inhibition and statistical significance at days 4, 7, and 14 in comparison to the other two groups. When compared to the negative control (0.12% chlorhexidine) significant differences were found in all groups at each interval.

FIGURES

FIGURE 1. PDS sutures in the manufacturer's packaging and vial containing sutures.

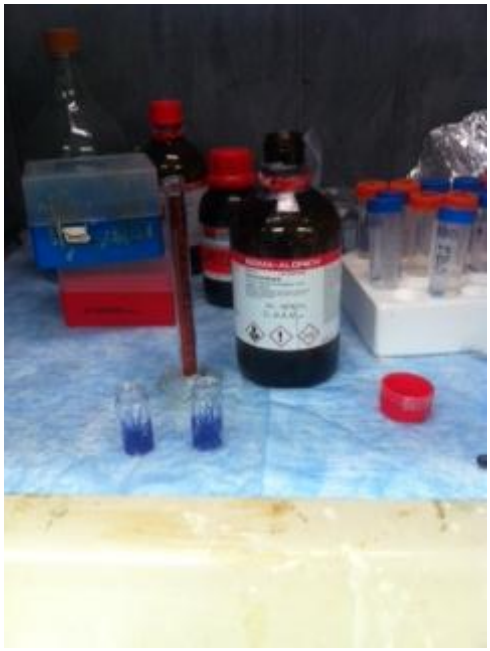

FIGURE 2. Dichloromethane being added to vials containing PDS suture filaments to remove purple violet dye.

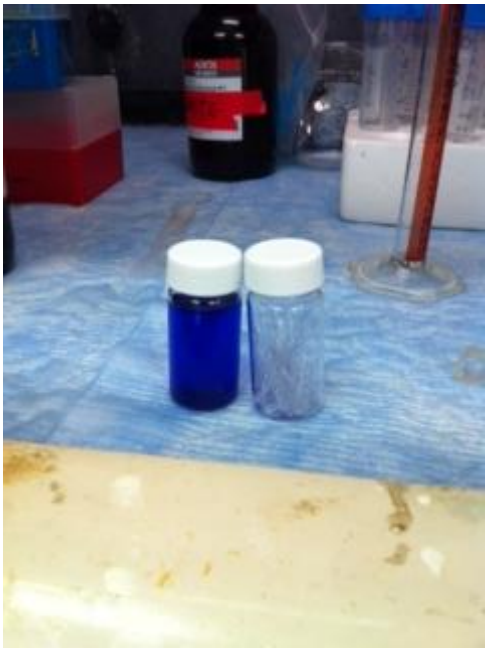

FIGURE 3. PDS after dichloromethane treatment.

FIGURE 4. Vacuum drying of PDS suture filaments and electrospun scaffolds.

FIGURE 5. Electrospinning machine.

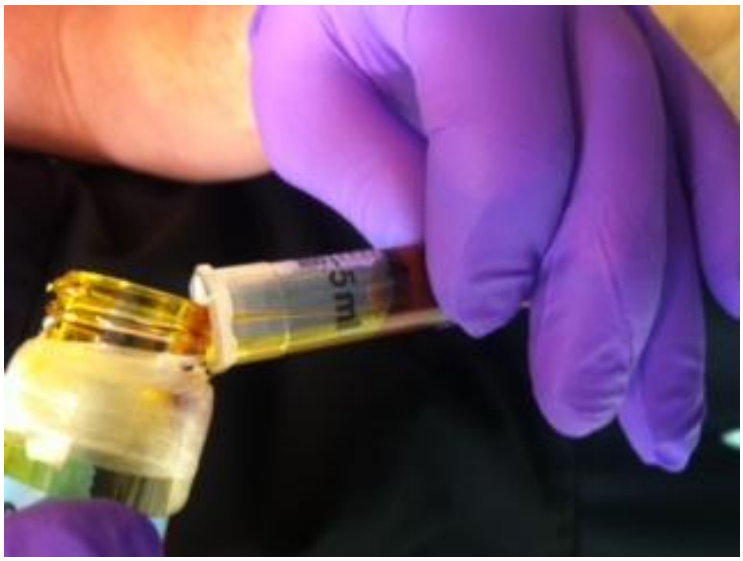

FIGURE 6. Adding antibiotic-containing PDS solution to a plastic syringe.

FIGURE 7. Antibiotic-containing PDS solution plastic syringe prepared for electrospinning device.

FIGURE 8. Settings for electrospinning.

FIGURE 9. Electrospinning of scaffold on mandrel during process.

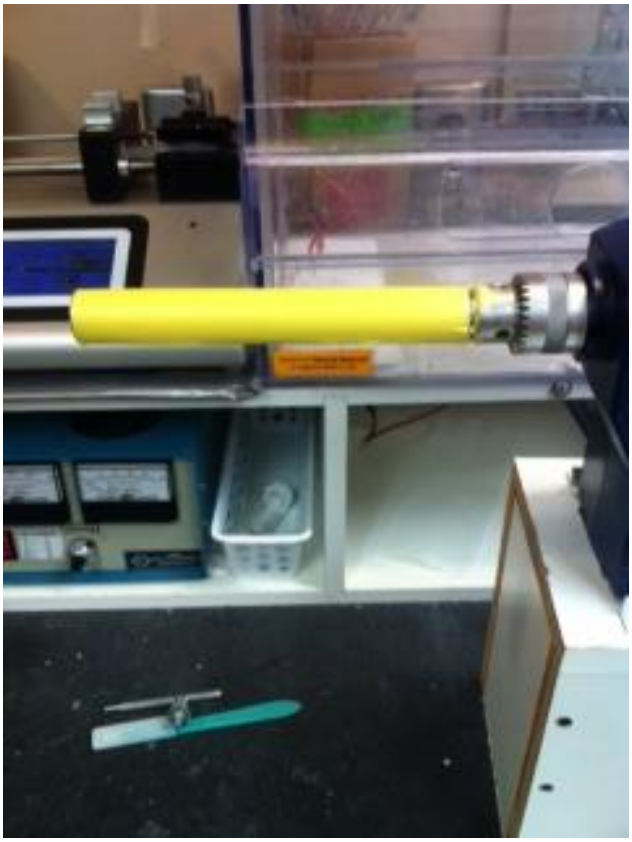

FIGURE 10. Electrospinning of scaffold on mandrel finished.

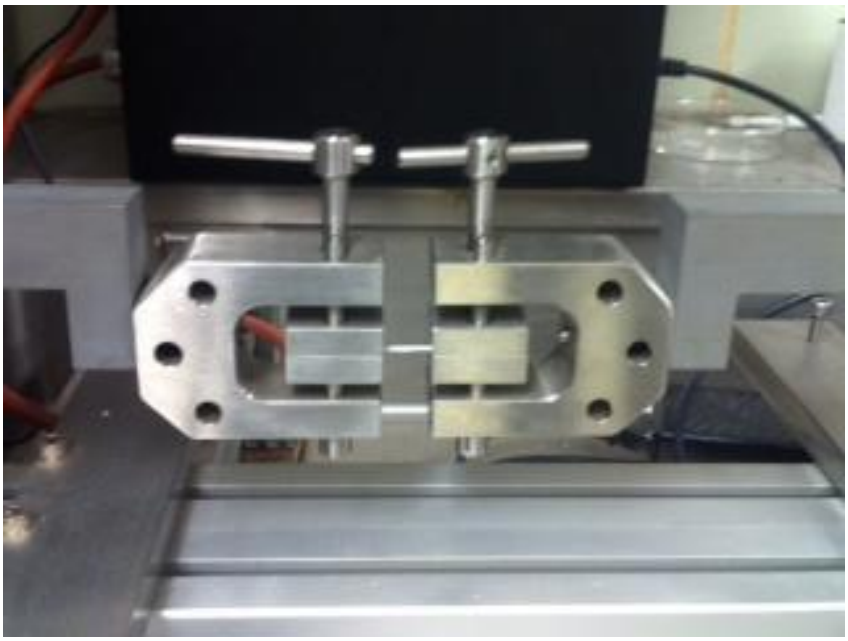

FIGURE 11. Mechanical testing of scaffolds.

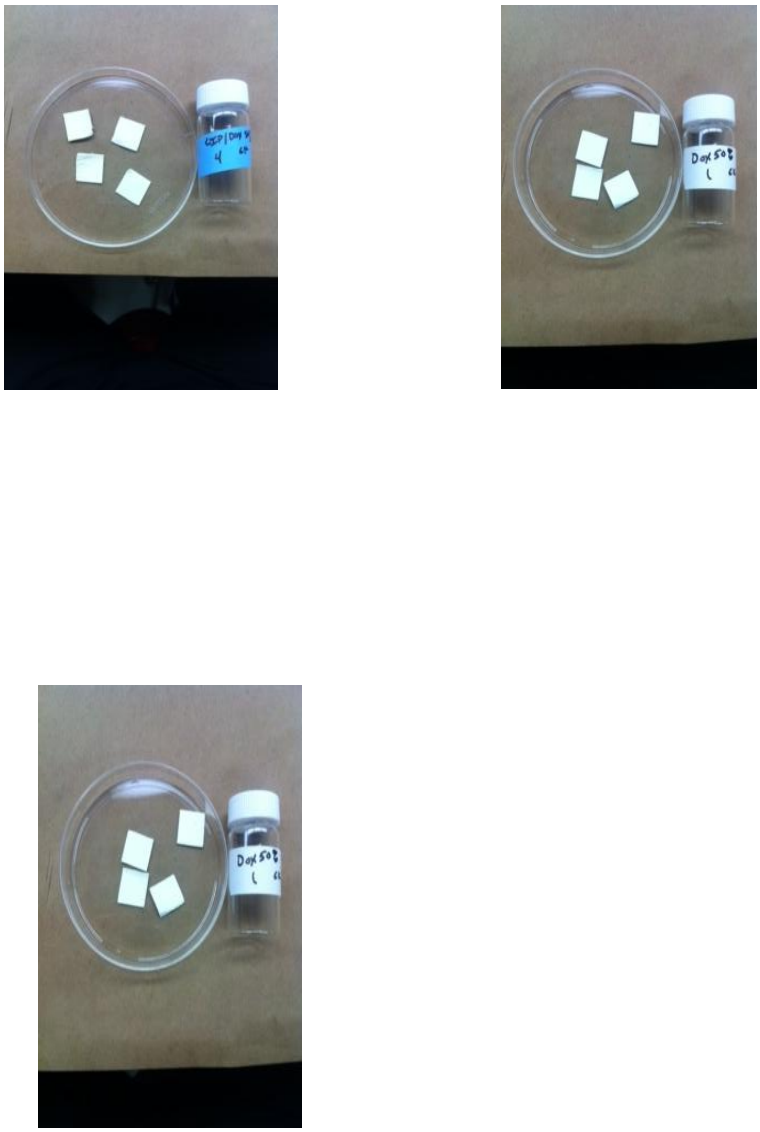

FIGURE 12. Electrospun scaffolds.

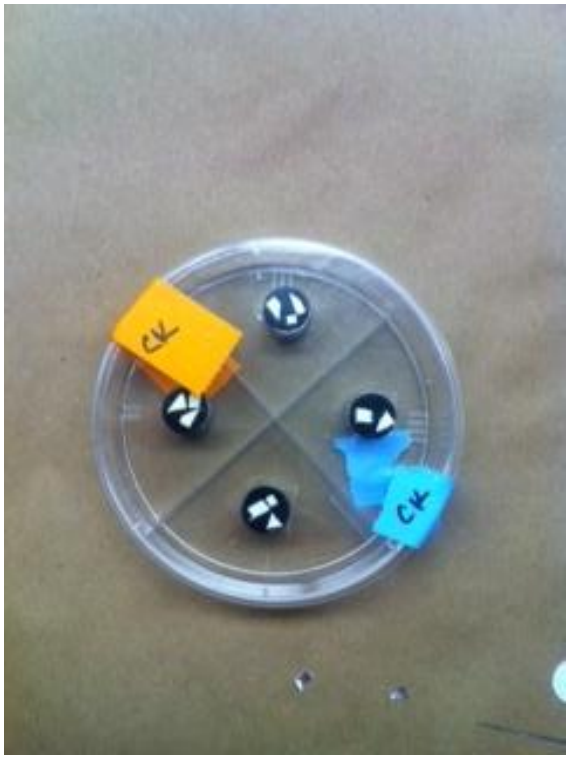

FIGURE 13a. SEM preparation and gold sputtering.

FIGURE 13b. SEM preparation and gold sputtering.

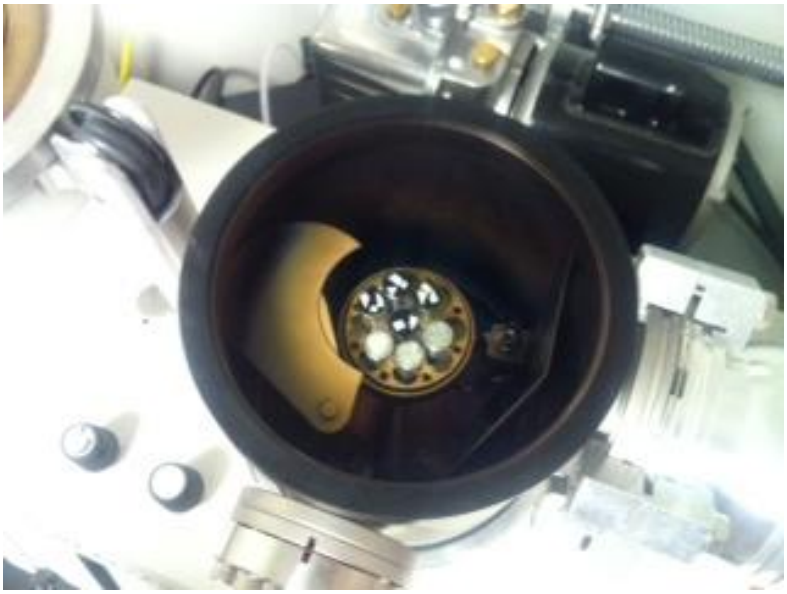

FIGURE 13c. SEM preparation and gold sputtering.

FIGURE 14a. Scanning electron microscope.

FIGURE 14b. Scanning electron microscope.

FIGURE 15a.

FIGURE 15b.

Vials with PBS and scaffolds at days 1,4,7,14.

FIGURE 15c.

FIGURE 15d.

Vials with PBS and scaffolds at days 1,4,7,14.

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FIGURE 16. Fourier transform infrared spectroscopy apparatus.

FIGURE 17. Plating of bacteria for agar diffusion testing.

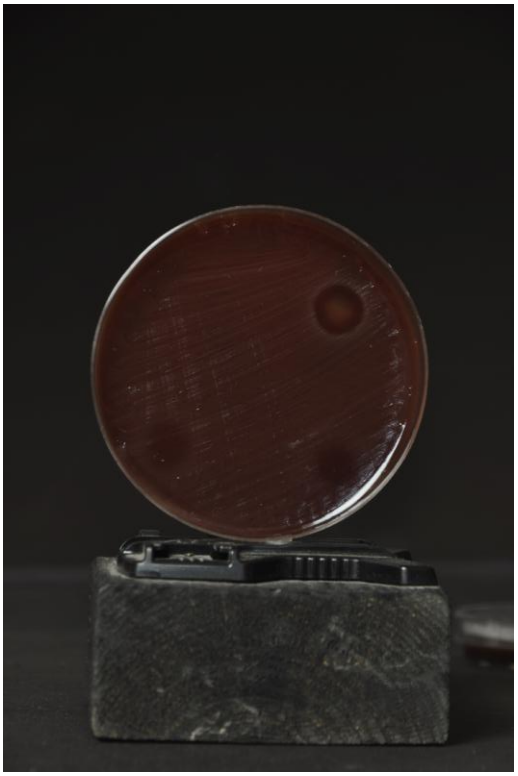

FIGURE 18. Zones of inhibition Ef CIP/DOX50% (bottom left day 4, bottom right day 7, top left pure PDS, top right 0.12% CHX).

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FIGURE 19. Zones of inhibition Ef CIP/DOX50% (bottom left day 1, bottom right day 14, top left pure PDS, top right 0.12% CHX).

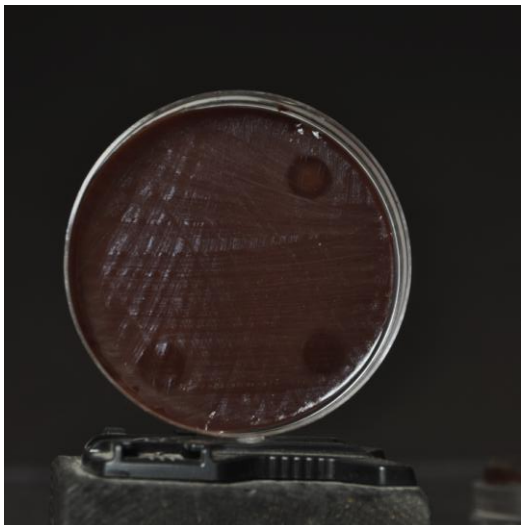

FIGURE 20. Zones of inhibition Ef CIP50% (bottom left day 1, bottom right day 14, top left pure PDS, top right 0.12% CHX).

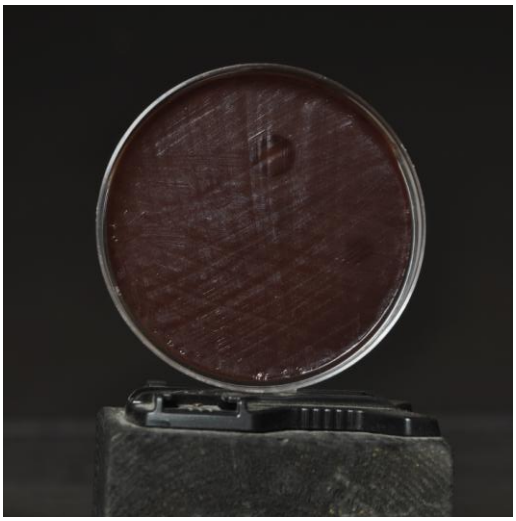

FIGURE 21. Zones of inhibition Ef DOX50% (bottom left day 1, bottom right day 14, top left pure PDS, top right 0.12% CHX).

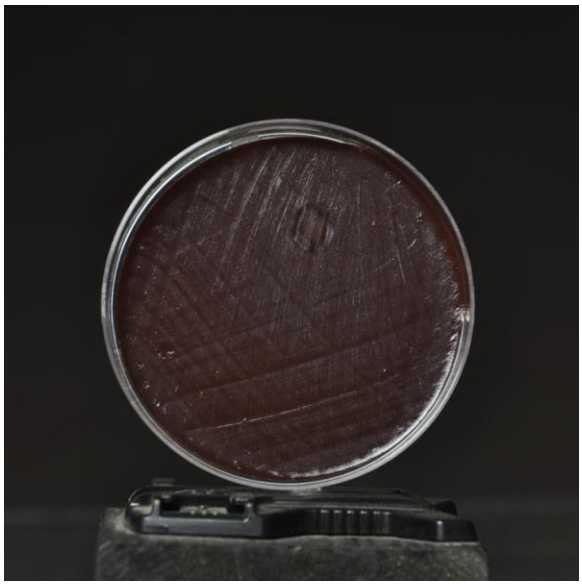

FIGURE 22. Zones of inhibition Ef DOX50% (bottom left day 4, bottom right day 7, top left pure PDS, top right 0.12% CHX).

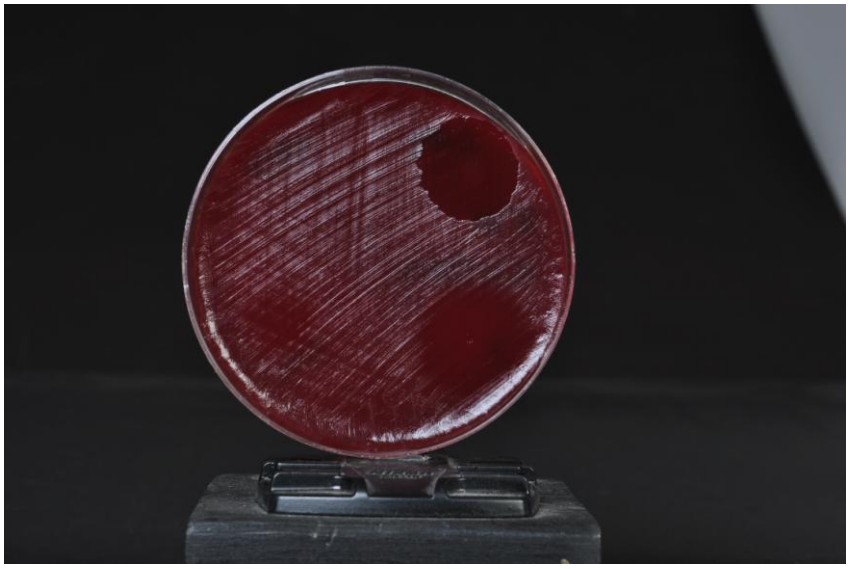

FIGURE 23. Zones of inhibition Fn CIP/DOX50% (bottom left day 1, bottom right day 14, top left pure PDS, top right 0.12% CHX).

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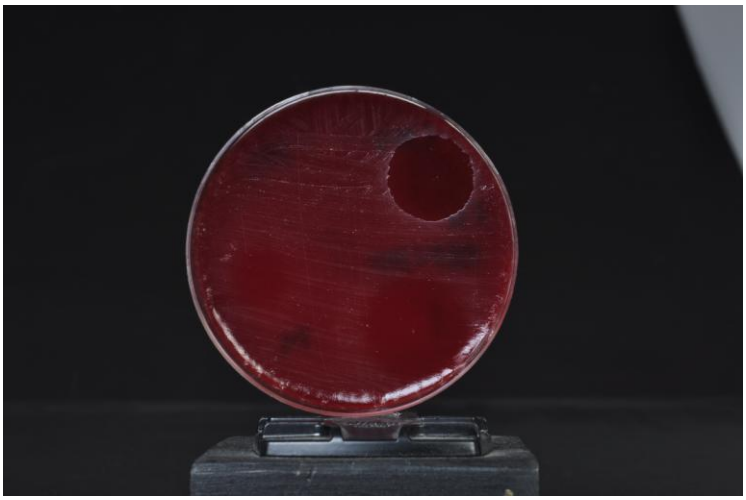

FIGURE 24. Zones of inhibition Fn CIP/DOX50% (bottom left day 4, bottom right day 7, top left pure PDS, top right 0.12% CHX).

FIGURE 25. Zones of inhibition Fn CIP50% (bottom left day 1, bottom right day 14, top left pure PDS, top right 0.12% CHX).

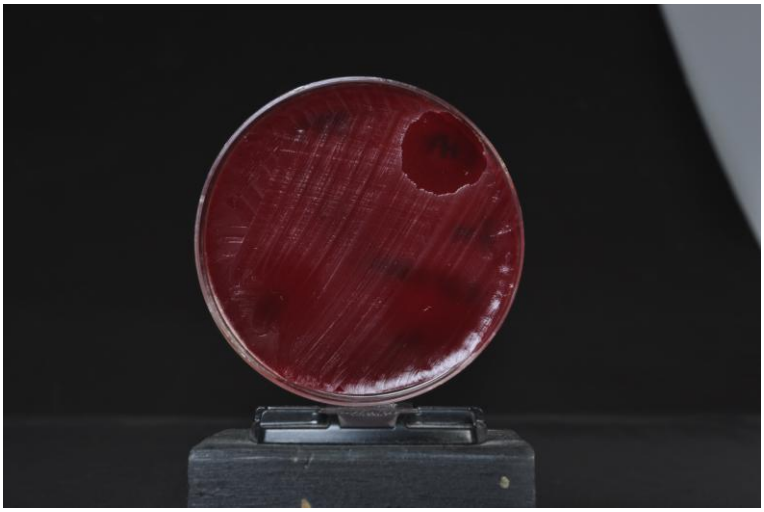

FIGURE 26. Zones of inhibition Fn CIP50% (bottom left day 4, bottom right day 7, top left pure PDS, top right 0.12% CHX).

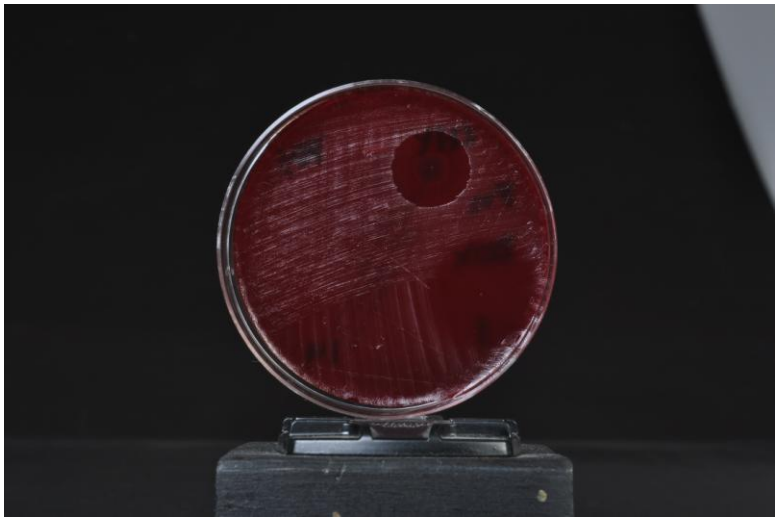

FIGURE 27. Zones of inhibition Fn DOX50% (bottom left day 1, bottom right day 14, top left pure PDS, top right 0.12% CHX).

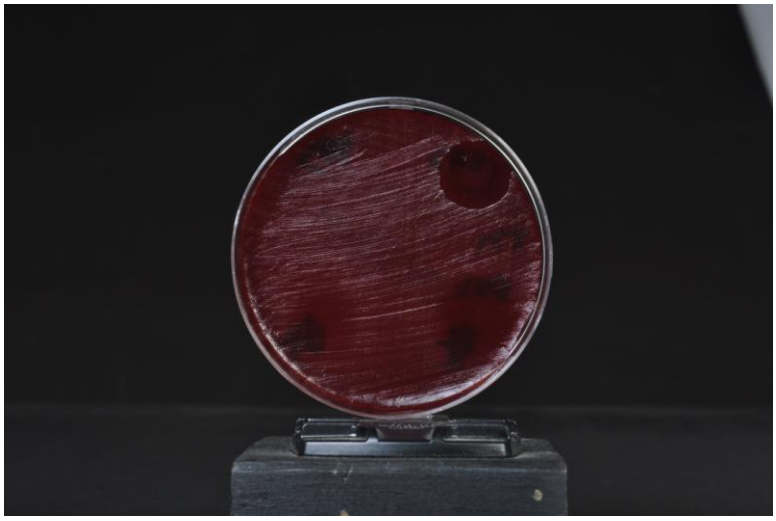

FIGURE 28. Zones of inhibition Fn DOX50% (bottom left day 4, bottom right day 7, top left pure PDS, top right 0.12% CHX).

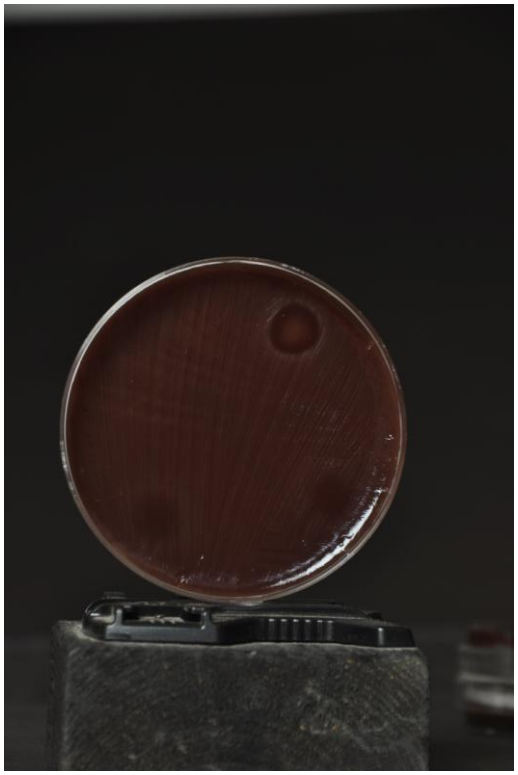

FIGURE 29. Zones of inhibition Sg DOX50% (bottom left day 4, bottom right day 7, top left pure PDS, top right 0.12% CHX).

FIGURE 30. Zones of inhibition Sg DOX50% (bottom left day 1, bottom right day 14, top left pure PDS, top right 0.12% CHX).

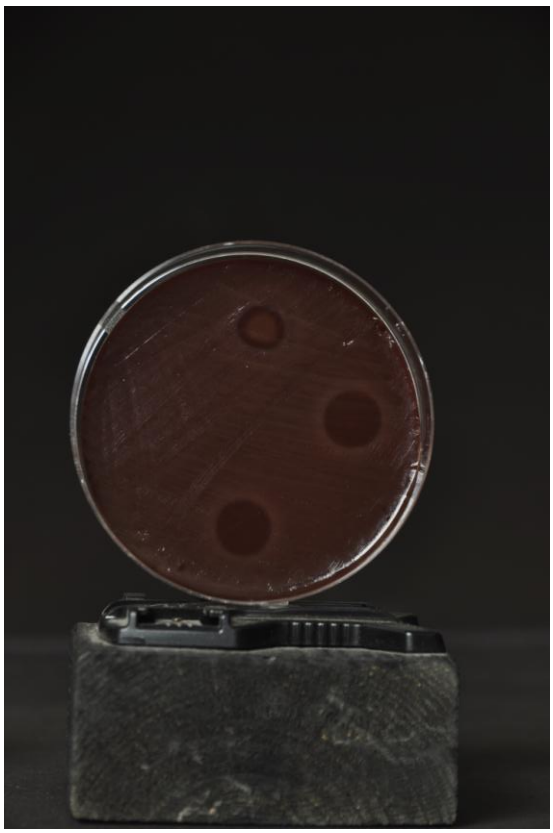

FIGURE 31. Zones of inhibition Sg CIP50% (bottom left day 1, bottom right day 14, top left pure PDS, top right 0.12% CHX).

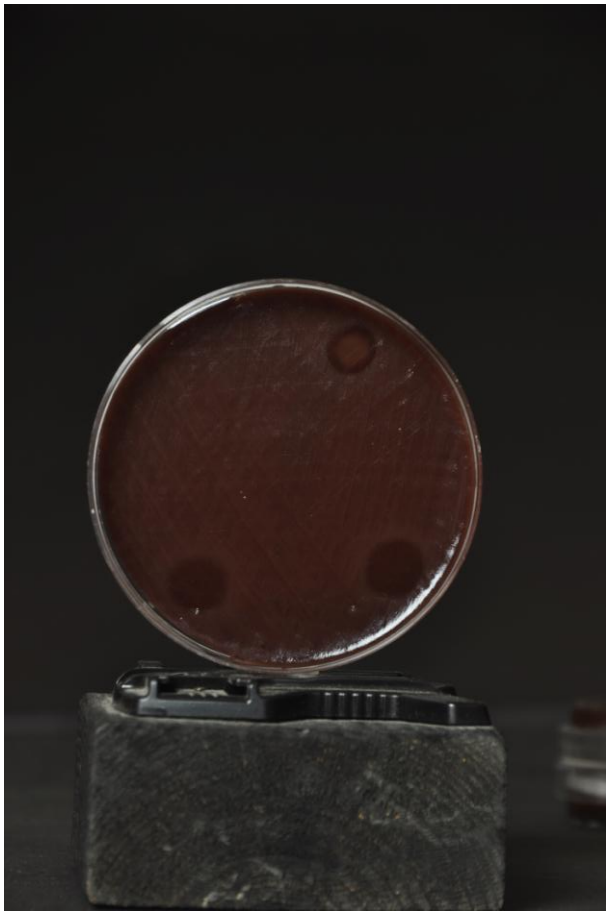

FIGURE 32. Zones of inhibition Sg CIP50% (bottom left day 4, bottom right day 7, top left pure PDS, top right 0.12% CHX).

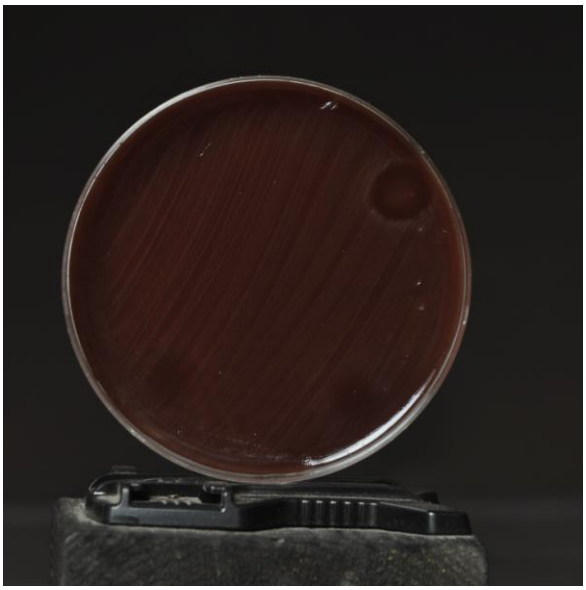

FIGURE 33. Zones of inhibition Sg CIP/DOX50% (bottom left day 4, bottom right day 7, top left pure PDS, top right 0.12% CHX).

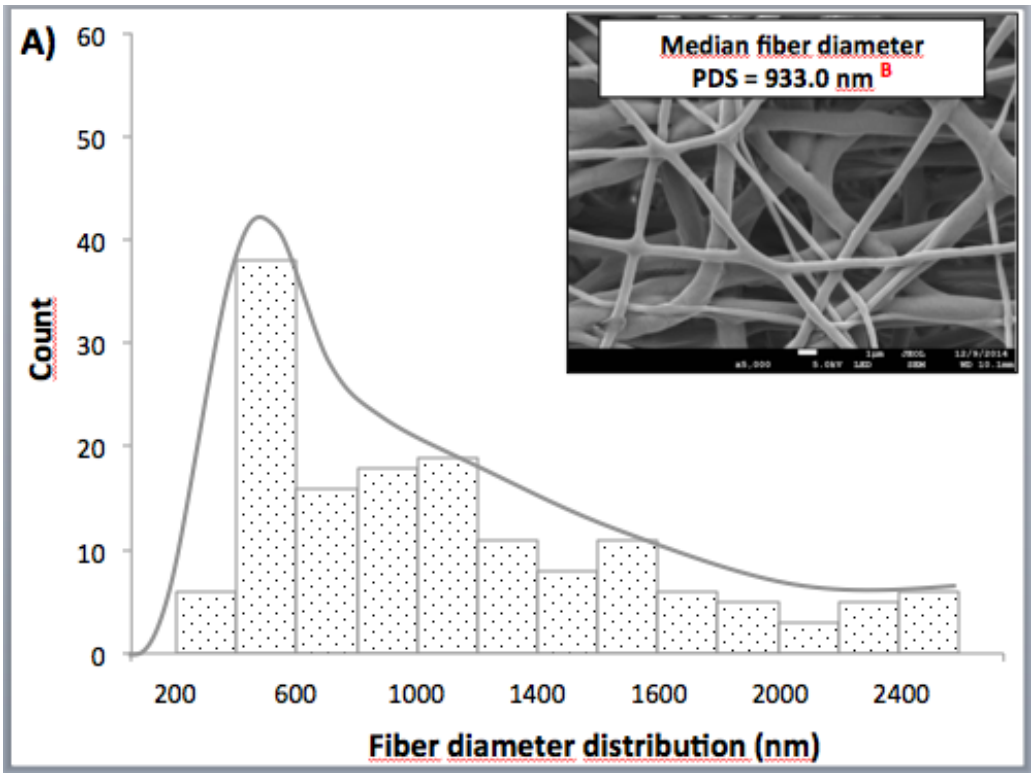

FIGURE 34a. Demonstrates the distribution of fiber diameter of pure PDS scaffolds with an SEM image giving a visual representation of the fibers.

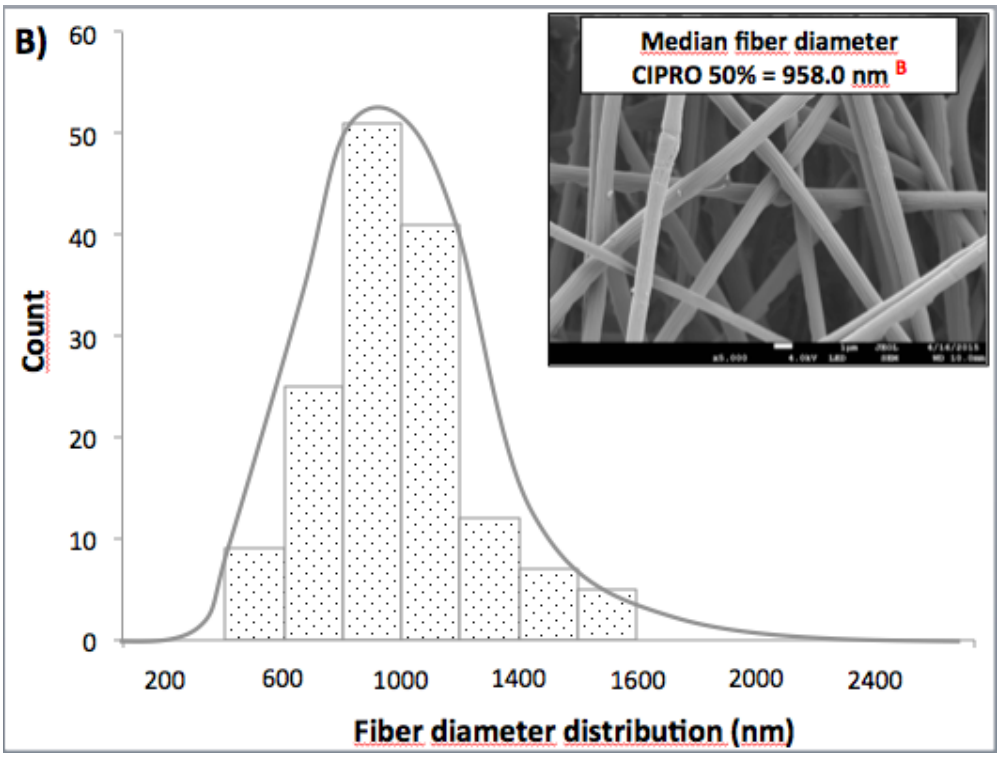

FIGURE 34b. Demonstrates the distribution of fiber diameter of pure PDS scaffolds with an SEM image giving a visual representation of the fibers.

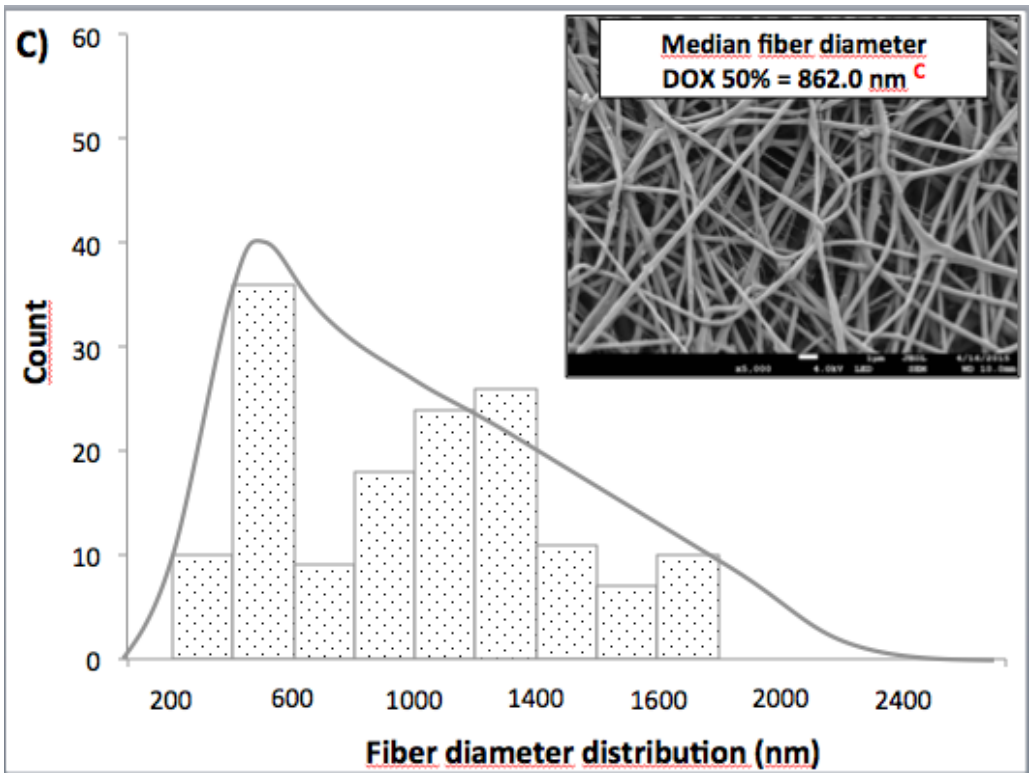

FIGURE 34c. Demonstrates the distribution of fiber diameter of DOX50% scaffolds with an SEM image giving a visual representation of the fibers.

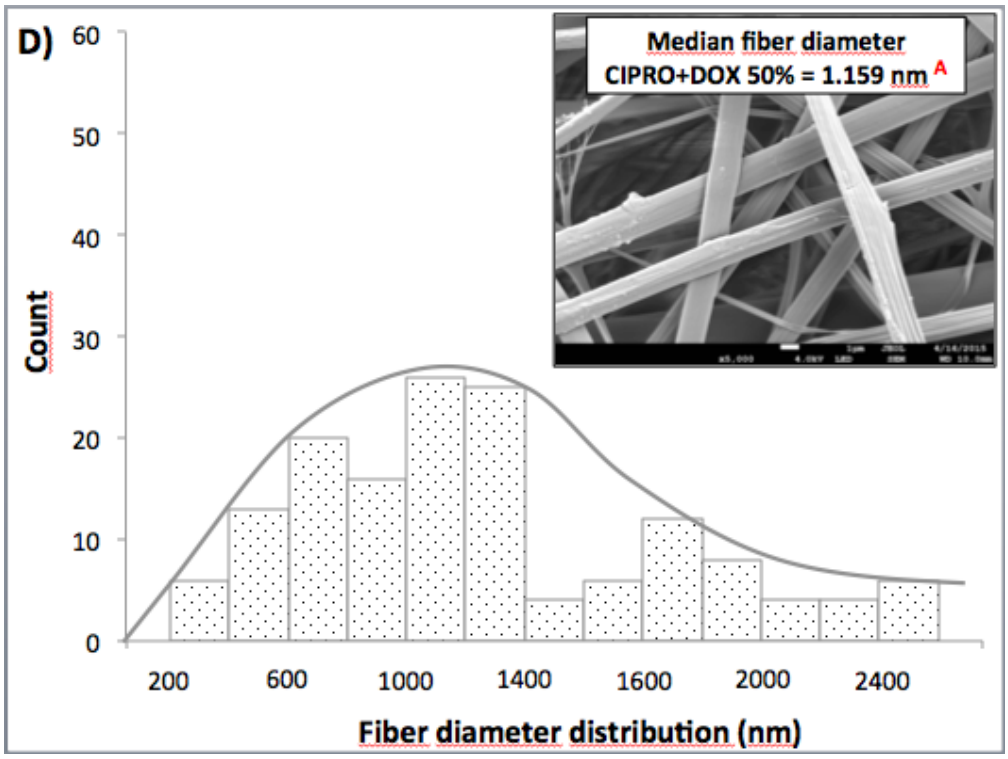

FIGURE 34d. Demonstrates the distribution of fiber diameter of CIP-DOX50% scaffolds with a SEM image giving a visual representation of the fibers.

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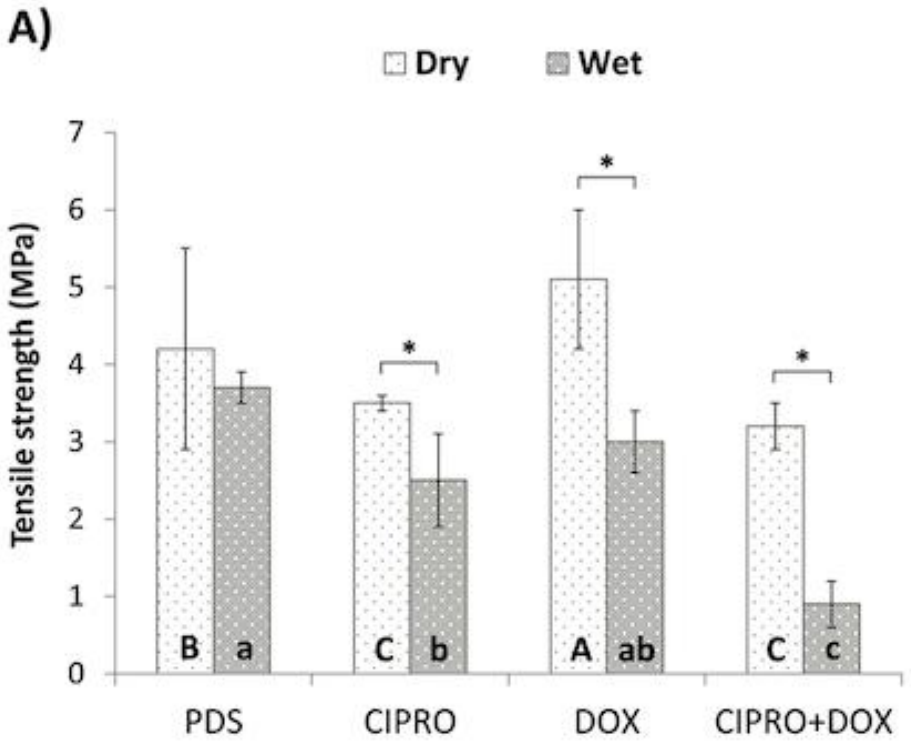

FIGURE 35. Shows the tensile strength (MPa) of each scaffold tested. Capital letters (i.e., A,B) correspond to dry scaffolds and are related to their significance ($p < 0.05$) between the dry scaffolds. Lower case letters (i.e. a,b,c) correspond to the wet scaffolds and are related to their significance between the wet scaffolds.

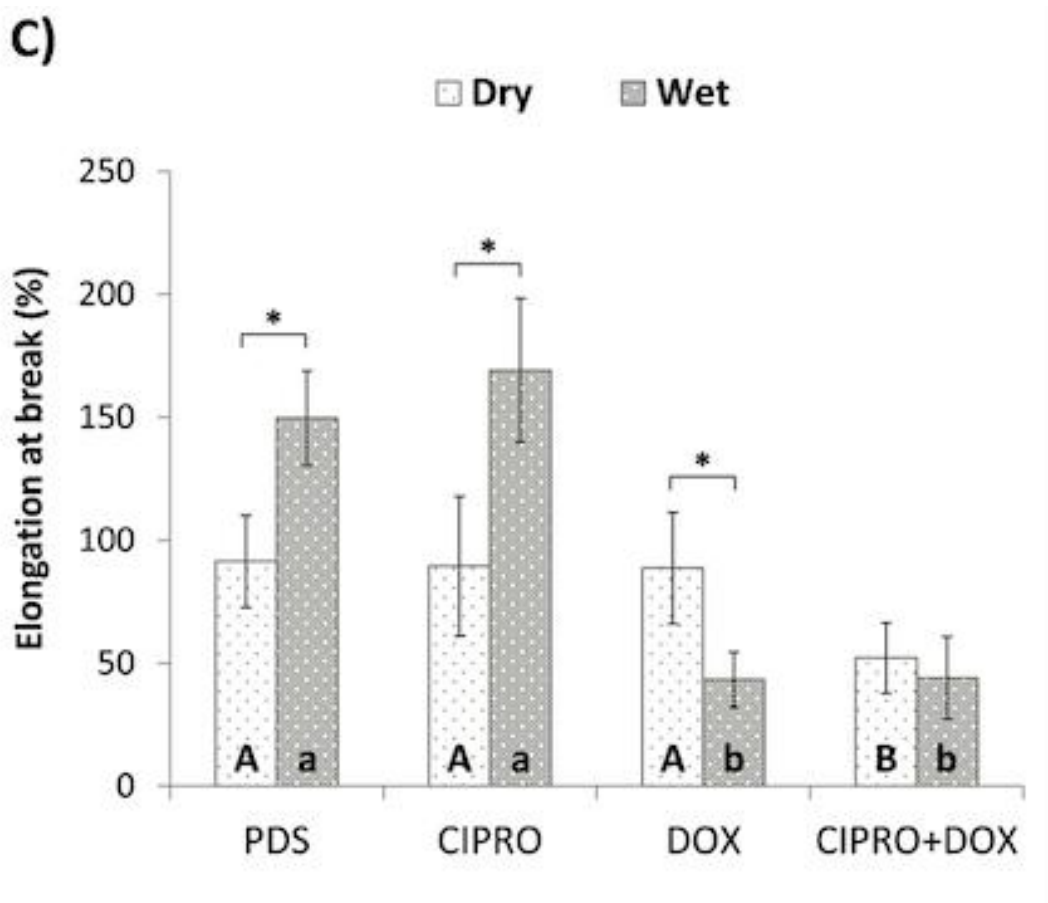

FIGURE 36. This figure illustrates the Elongation at break (%) of each scaffold tested. Capital letters (i.e., A,B) correspond to dry scaffolds and are related to their significance between the dry scaffolds. Lower case letters (i.e. a,b,c) correspond to the wet scaffolds and are related to their significance between the wet scaffolds.

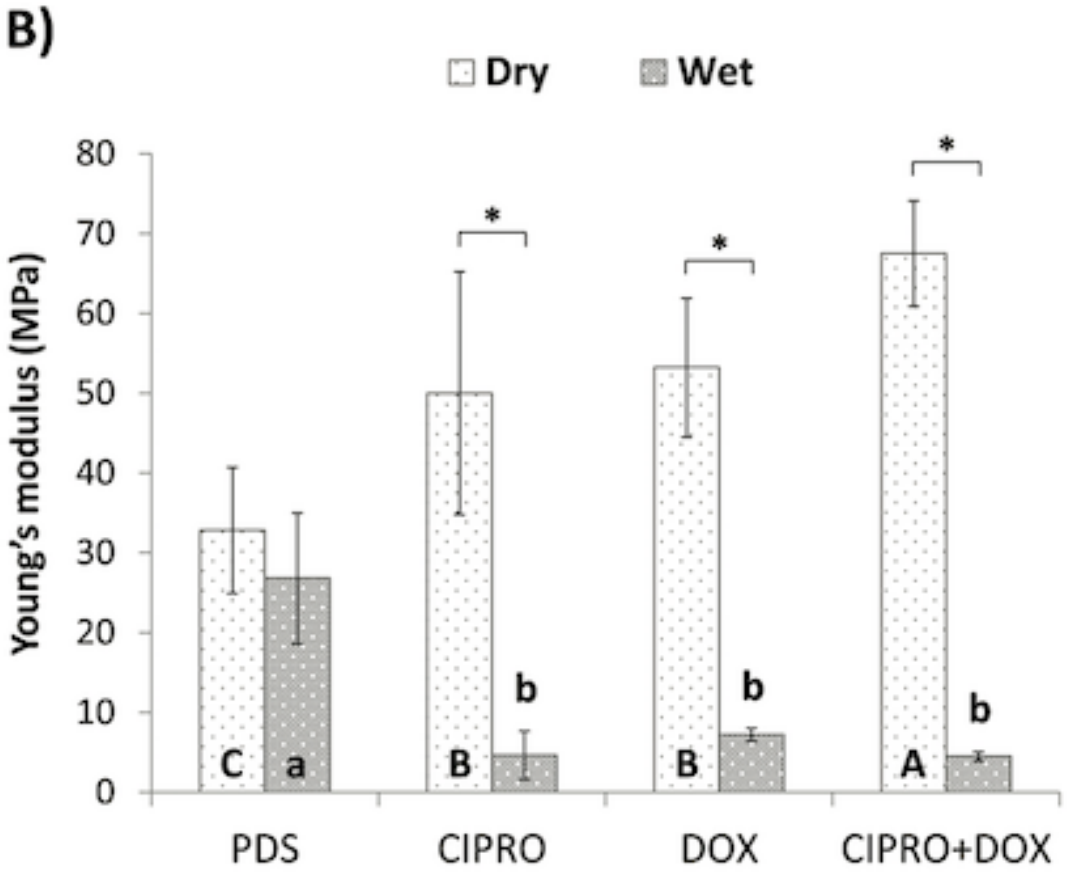

FIGURE 37. This figure illustrates the Young's modulus (MPa) of each scaffold tested. Capital letters (i.e., A,B,C) correspond to dry scaffolds and are related to their significance between the dry scaffolds. Lower case letters (i.e. a,b) correspond to the wet scaffolds and are related to their significance between the wet scaffolds.

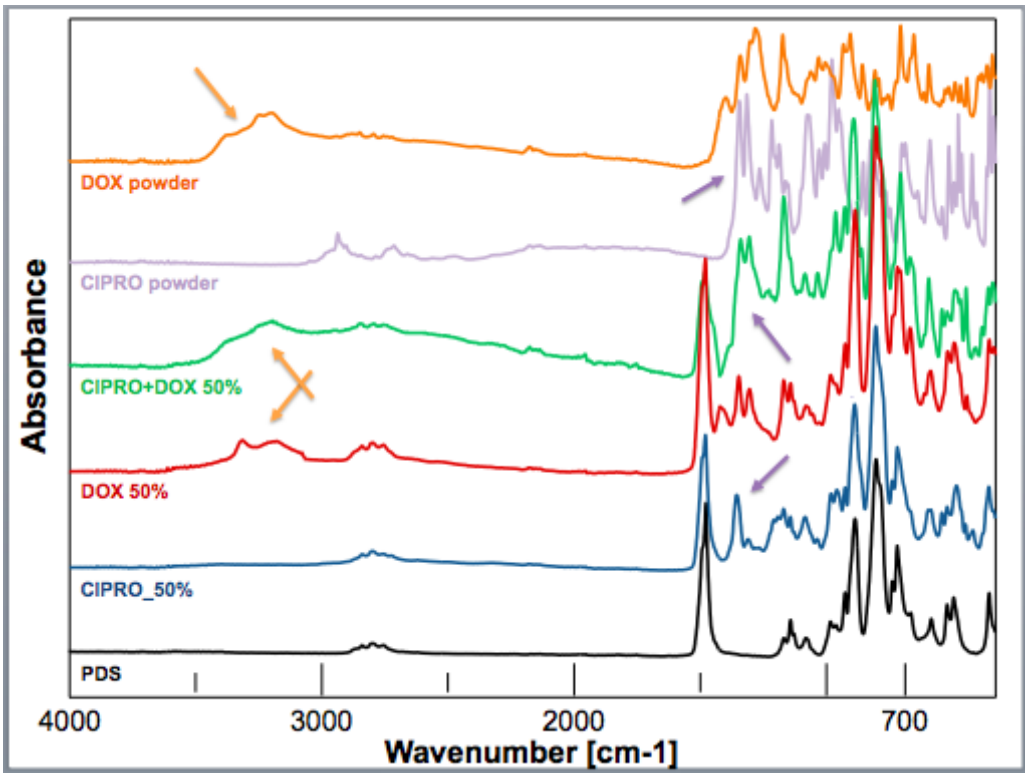

FIGURE 38. Fourier transform infrared spectroscopy of each fibrous scaffold and antibiotic powder. Orange arrows highlight DOX incorporation into the scaffolds. Purple arrows highlight CIP incorporation into the scaffolds.

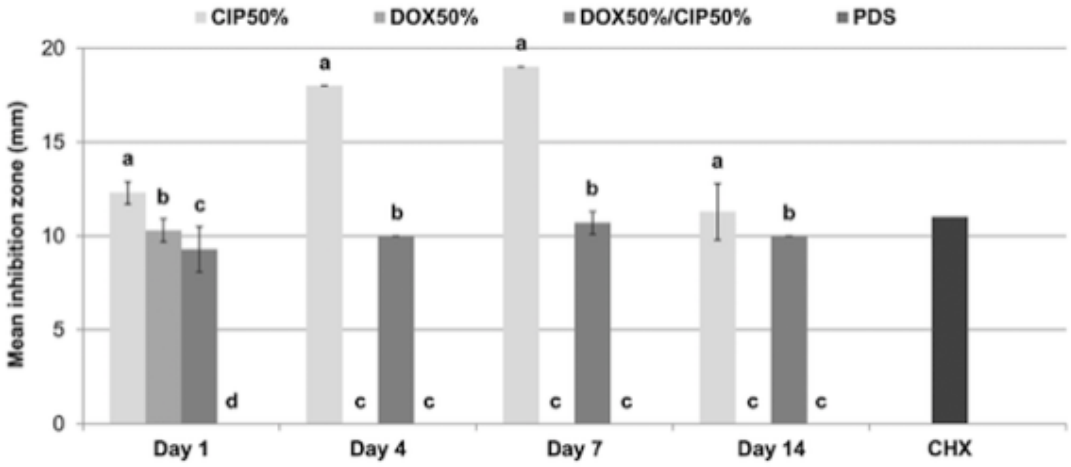

FIGURE 39. Agar diffusion, mean inhibition zones (in mm), against E.f, Same letters indicate no significant difference compared with the results of the same day of elution aliquot at time intervals of 1, 4, 7 and 14 days. Positive (0.12% CHX) and negative controls (pure PDS) were used.

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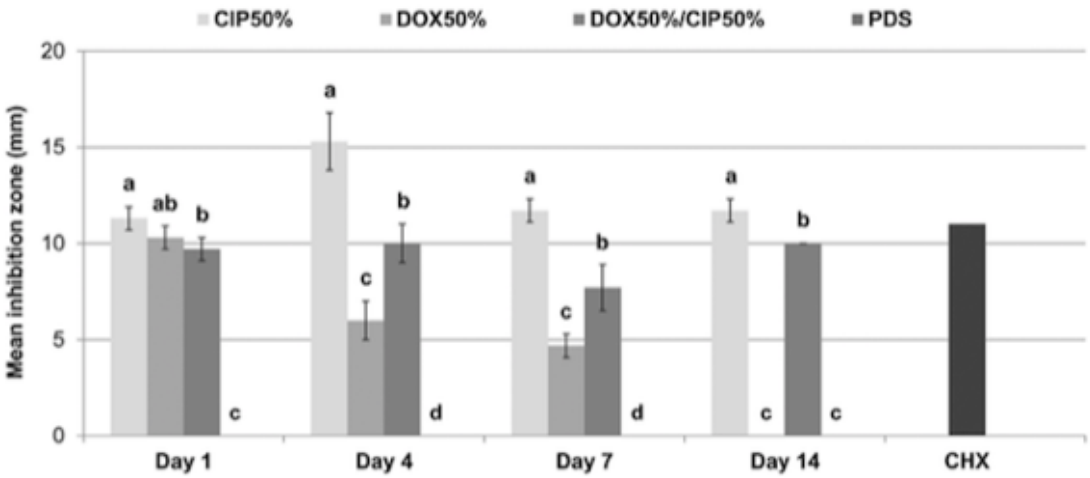

FIGURE 40. Agar diffusion, inhibition zones, against S.g. Same letters indicate no significant difference compared with the results of the same day of eluted aliquot at time intervals of 1, 4, 7, and 14 days. Positive (0.12% CHX) and negative control (pure PDS) were used.

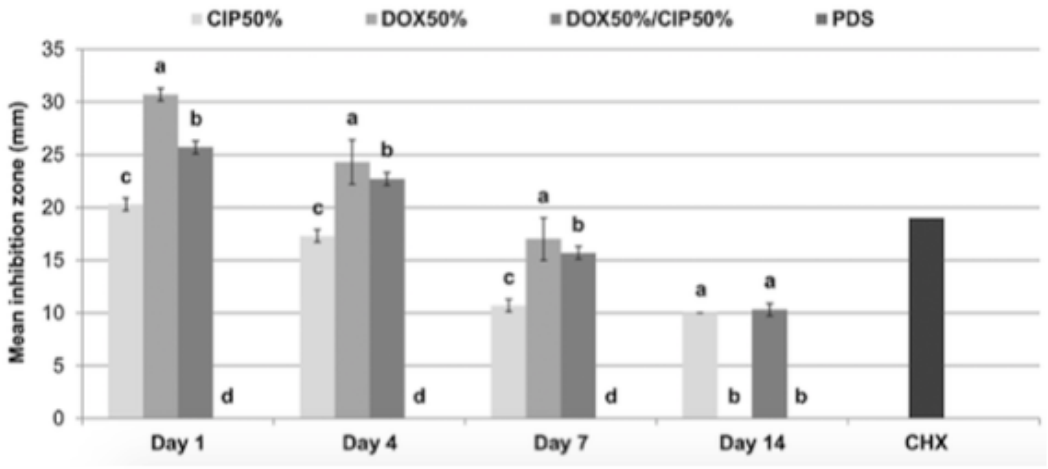

FIGURE 41. Agar diffusion, inhibition zones, against F.n. Same letters indicate no significant difference compared with the results of the same day of eluted aliquots at time intervals of 1, 4, 7, and 14 days. Positive control (0.12% CHX) and negative control (pure PDS) were used.

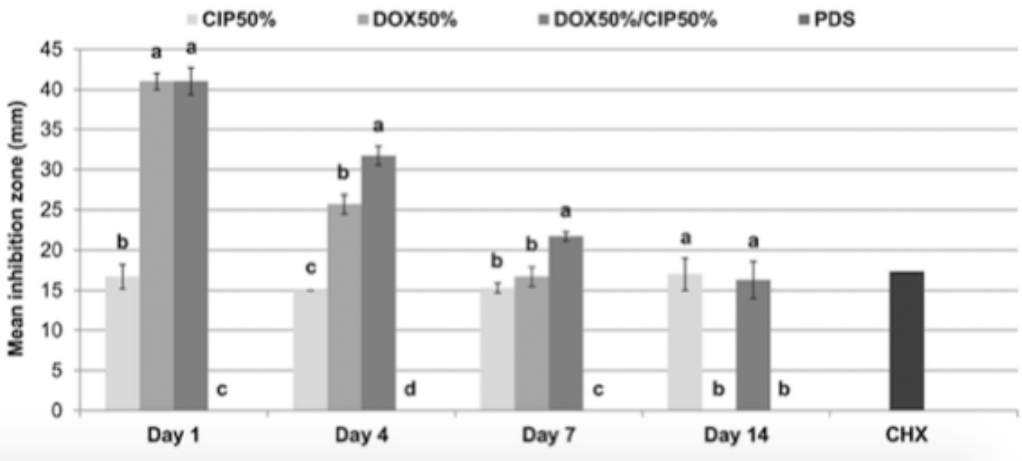

FIGURE 42. Agar diffusion, inhibition zones, against F.n. Same letters indicate no significant difference compared with the results of the same day of eluted aliquots at time intervals of 1, 4, 7, and 14 days. Positive control (0.12% CHX) and negative control (pure PDS) were used.

DISCUSSION

Disinfection of the root canal system is paramount in necrotic teeth with immature apices, mature apices or previously-treated root canals containing persistent infections.[2,55,56,66,128] Through meticulous research and proven case studies it has been shown that antibiotic combinations of double antibiotic and triple antibiotic pastes can promote disinfection of the root canal system.[21,27,60,122]

In this study, CIP/DOX containing electrospun scaffolds showed significant antimicrobial efficacy against common and persistent endodontic bacteria (E.f, S.g, F.n, and P.g). CIP, a DNA inhibitor, did not inhibit gram-negative (F.n, P.g) bacteria as effectively when compared to gram-positive bacteria (E.f, S.g). A recent publication has reinforced that a CIP-containing electrospun scaffold has significant antimicrobial effects and that at lower concentrations (i.e., 1 and 2.5 wt%) significantly reduced the negative impact on hDPSC viability/proliferation.[129,131] At high drug concentrations there is cytotoxicity to the stem cells of the apical papilla and at low concentrations the effects are significantly decreased.[129] Doxycycline (DOX), a RNA inhibitor, demonstrated less inhibition against gram-positive (E.f, S.g) bacteria then gram-negative bacteria (F.n, P.g). This study further confirms CIP/DOX's antibacterial significance against common endodontic bacteria when used in combination. The FTIR augmented the agar diffusion findings by confirming the presence of the drugs in each scaffold (Fig. 38). Further studies need to be conducted on these scaffolds at different concentrations to evaluate toxic effects on stem cells.

The results demonstrate the scaffold may provide a greater antibacterial effect in comparison to CHX. CHX has been shown to be detrimental to SCAP's and is currently not recommended in the disinfection protocol.[16]

This research provides a promising advancement in disinfection related to root canal therapy. Alongside regenerative endodontics these antibiotic-containing scaffolds could provide great benefit in treatment of primary and/or persistent endodontic infections.

E. faecalis is the most frequently isolated microbe in persistent endodontic infections due to its ability to invade dentinal tubules and resist the high pH of calcium hydroxide.[40,55,61,64,65] *E. faecalis* is a master of survival in the root canal system and is able to form a biofilm that helps it survive by becoming 1000 times more resistant to phagocytosis, antibodies, and antimicrobials than non-biofilm organisms.[62,67] Mature teeth with persistent infections would greatly benefit from a uniform controlled local application of antibiotics. Stronger drug dosages may be used to provide the optimal therapeutic levels.

Continuous research on the best balance of antibiotic combination, delivery and concentration is a highly desirable area in modern day regenerative research.[16,119]

Electrospun scaffolds containing antibiotics may someday turn regenerative treatment into a single visit protocol. Our group continues to fully evaluate the potential of electrospun polymer-based antibiotic containing scaffolds to find the best practices in disinfection of the root canal system.[23,130]

SUMMARY AND CONCLUSION

This study addressed two areas of clinical significance in endodontics by continuing efforts to optimize regenerative endodontic disinfection through an electrospun scaffold and provide baseline research for the potential use of this scaffold as a root canal disinfectant against primary or persistent root canal bacteria. In conclusion, the findings from this research demonstrated a double antibiotic scaffold of ciprofloxacin and doxycycline reduced bacteria growth against common endodontic pathogens.

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ABSTRACT

DEVELOPMENT OF A DOUBLE ANTIBIOTIC ELECTROSPUN SCAFFOLD FOR ROOT CANAL DISINFECTION

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Introduction: Regenerative endodontics requires proper disinfection of the root canal system (RCS). The development of pulp-like tissue and continued root formation are considered a successful outcome. A biocompatible scaffold that could also help in disinfecting the RCS seems to be an exciting alternative.

Objective: This study synthesized electrospun polymer-based scaffolds containing ciprofloxacin (CIP) and doxycycline (DOX), as a scaffold mimic of double antibiotic

paste (DAP) and determined, *in vitro*, its mechanical properties, chemical composition, and antimicrobial effectiveness against multiple endodontic bacterium, such as *Enterococcus faecalis* (*E. faecalis*), *Streptococcus gordonii* (*S. gordina*), *Porphyromonas gingivalis* (*P. gingivalis*) and *Fusobacterium nucleatum* (*F. nucleatum*).

Materials and Methods: Polydioxanone sutures (PDS) were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP), mixed with CIP/DOX (i.e., 50%), and electrospun under optimized conditions into fibrous scaffolds. Fiber morphology was evaluated by scanning electron microscopy and image J software (NIH). Tensile testing was used to evaluate the mechanical properties. Fourier transform infrared spectroscopy (FTIR) was used to confirm the successful incorporation of the antibiotics. Antimicrobial efficacy was determined over time using aliquots collected at 1, 4, 7, 14 day and agar diffusion assays.

Results: Tensile strength (MPa) of the CIP/DOX scaffold did not show significant difference from the control (pure PDS). Elongation at break (%) did show a significant difference between CIP/DOX scaffolds and the control group. Young's modulus of elasticity (MPa) showed to have a significant difference between CIP/DOX scaffolds and the control. FTIR confirmed the presence of the antibiotics within the scaffolds. CIP-containing scaffolds did not inhibit Gram-negative (*F. nucleatum* and *P. gingivalis*) bacteria as effectively when compared to Gram-positive bacteria (*E. faecalis* and *S. gordina*). DOX-containing scaffolds showed less inhibition against Gram-positive (*E. faecalis* and *S. gordina*) bacteria then Gram-negative bacteria (*F. nucleatum* and *P. gingivalis*). In combination, CIP/DOX scaffolds showed significant inhibition against G(-) and G(+) bacteria.

Conclusion: Electrospun double antibiotic scaffold demonstrated increased antimicrobial efficacy proving the potential for future clinical use to disinfect the RCS in permanent immature necrotic teeth to aid in regenerative treatment and or in persistent infections.

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