USE OF ELECTROMAGNETIC STIMULATION ON AN *ENTEROCOCCUS FAECALIS* BIOFILM IN ROOT CANAL TREATED TEETH *IN VITRO*

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INTRODUCTION

In 1965, Kakehashi et al. proved that microbes are the primary etiological factor in pulpal and periapical disease.[1] Currently, it is globally accepted that disinfection of the root canal system is of utmost importance in the success or failure of root canal treatment, though complete sterility is not possible.[2] Sodium hypochlorite is the time-tested gold standard of endodontic irrigation due to its nonspecific microbial killing as well as its ability to dissolve organic tissue.[3,4] The concentration of sodium hypochlorite which achieves the ideal balance of microbial killing and tissue dissolution while providing the lowest risk for cellular toxicity remains controversial. For standard nonsurgical root canal therapy, 6.0-percent sodium hypochlorite is commonly used as it is inexpensive and readily available.[5] In the case of an immature tooth undergoing regenerative treatment (REP), gentle irrigation with 1.5-percent sodium hypochlorite followed by gentle irrigation with 17-percent EDTA improves survival of stem cells of the apical papilla.[6] Indeed, 1.5-percent sodium hypochlorite is the irrigant of choice for REP as recommended by the American Association of Endodontists but must be followed by an intracanal medicament such as double antibiotic paste to achieve an acceptable level of disinfection, which requires multiple treatment appointments.[7] In either regenerative or traditional endodontic therapy, achieving an acceptable antimicrobial effect requires fresh sodium hypochlorite remain in the canal space for an extended time which is dependent upon the concentration.[8-10] Unfortunately, sodium hypochlorite is toxic to virtually all human cell types and leaving it in the canal space during instrumentation increases the risk of apical extrusion. Apical extrusion of sodium hypochlorite can incite an intense

inflammatory reaction resulting in long lasting swelling, bruising and severe pain for the patient. Presumably a higher concentration would produce a more intense inflammatory response than a lower concentration if the same amount were extruded.[11-15] To reduce the risk of adverse reaction and also reduce treatment time, other methods of disinfecting the root canal system have been evaluated, such as chlorhexidine, diode laser, gaseous ozone, and photodynamic therapy.[16-20] The present study will evaluate the anti-biofilm effect of electromagnetic stimulation (EMS) in conjunction with 1.5-percent sodium hypochlorite or 0.9-percent saline for disinfection during nonsurgical root canal therapy.

As of today, no other solution or material has supplanted the widespread use of sodium hypochlorite as a direct method of disinfection. However, much effort has been devoted to supplementing the action of sodium hypochlorite, such as passive ultrasonic irrigation and sonically activated irrigation; although both are widely used and highly successful in achieving acceptably disinfected canals in shorter periods of time, they both present an inherent risk for apical extrusion of irrigants and debris by their very nature.[21-27]. Heating sodium hypochlorite prior to its use can also increase its antibacterial efficacy and tissue solvent action. At temperatures ranging from 37°C to 45°C, antimicrobial activity increases by as much as 100-fold when compared to use at room temperature.[28, 29]

The International Society for Electromagnetic Dentistry (Tominaga Dental Clinic, Naruto, Japan) has developed a novel method for achieving root canal disinfection by energizing lower concentrations of sodium hypochlorite with electromagnetic waves, creating a synergistic reaction via electric and thermal energy.[30] An electromagnetic wave irradiation device attached to an active electrode (a specially coated ISO size 10 hand file) will create a circuit much in the same manner as an electronic apex locating

device. Due to an insulating coating along the file (the active electrode), the electromagnetic waves are concentrated at the tip. The waves energize solutions through electric and thermal energy and has been coined electromagnetic stimulation (EMS) by its initial researchers. Unfortunately, the research on EMS's potential as an enhancing agent for root canal disinfection is very limited. So far, it has only been shown to be effective against planktonic bacteria.[30-32] Since endodontic pathogens exist as biofilms, which are up to 1000-fold more resistant to antimicrobials,[33-35] the existing literature on EMS is of little value in a clinical setting. In addition to the antimicrobial effect of EMS, there is potential for increased organic tissue dissolution, as well. Perhaps due to a thermal effect, electrically activated sodium hypochlorite has been shown to dissolve bovine muscle tissue faster than non-activated sodium hypochlorite[36, 37]; although those studies did not address EMS specifically, the principle is similar and can be tested in future studies.

EMS treatment results in a localized generation of heat. In a pilot study using mounted mandibular incisors that were instrumented to a size 40.06, EMS was activated in single second bursts a total of ten times; the intracanal temperature was recorded after each activation. Activation occurred at 1 mm, 3 mm, 5 mm and 7 mm away from the root apex. The highest measurement was nearly 80°C (or approximately 43°C above body temperature), at 1 and 3 mm away from the apex, both at the first application of EMS. Each successive activation caused a minimal increase in temperature of no more than 1-2°C. Heat transfer was greatest near the apex and lessened with greater distance from the apex, presumably due to the taper of the root canal preparation and presence of fluid. Since the diameter is wider more coronally, there should be more fluid present to

dissipate the thermal reaction. In the same study, external root surface temperatures were also measured in a similar fashion. Measurements were taken at 1 mm past the apex, at the apex, and again at 1 mm, 3 mm, 5 mm and 7 mm coronal to the root apex. Interestingly, the highest temperature change occurred at 5 mm away from the root apex with a measurement of 47°C (10 degrees above body temperature) after the first activation and a measurement of approximately 62°C (25 degrees above body temperature) after the tenth activation. As in the intracanal measurements, each successive activation raised the temperature no more than 1-2°C. These measurements were taken on mounted teeth ex vivo and variations in canal diameter, amount of irrigant present, and apical diameter size may affect these values in a clinical situation. Currently, root canal fillings are being completed using heated instruments at temperatures as high as 200°C and applied to the intracanal space for as long as 4-5 seconds to thermoplasticize gutta percha for three dimensional obturation with no ill side effects.[38] Current root filling techniques have been found to raise external root surface temperatures anywhere from 8.5°C to 50°C with at least 6 minutes of time elapsing before it returns to body temperature.[39-41] Therefore, the change in external root surface temperature with use of EMS should be of little concern with regard to the periodontal ligament since this change is for a much shorter duration. From a regenerative standpoint, further research will be needed to determine the effect of such a transient temperature change on the viability of dental pulp stem cells and stem cells of the apical papilla as well as to determine proper depth of penetration of the active electrode to optimize bacterial killing while minimizing ill side effects to stem cells.

A fastidious pathogen found in many secondary and persistent endodontic

infections,[42-44] *Enterococcus faecalis*, will serve as an excellent model on which to test the antimicrobial efficacy of EMS. It is relatively easy to obtain, grow and maintain, and will form an established biofilm in a relatively short amount of time, on the scale of a couple weeks.[45]

Although beyond the scope of the present study, the use of electromagnetic waves in routine endodontic treatment may prove to be of benefit in cases of apical periodontitis that present with a periapical radiolucency upon radiographic examination. Indeed, stimulation of osteoblasts as well as necessary growth factors for bone formation has been shown when EMS was applied to rat calvaria, resulting in increased bone healing.[46,47] With the potential for antimicrobial synergism, enhanced tissue dissolution, and more expedient bone healing, EMS has the potential to change the way current nonsurgical root canal treatment is performed. This initial study on the antimicrobial effectiveness of EMS will open a wide variety of research avenues and may eventually serve to maintain or enhance the current success rates of nonsurgical root canal therapy while being less toxic to cells.

OBJECTIVE

- The aim of the current study will be to evaluate the anti-biofilm activity of electromagnetic stimulation (EMS) on a known endodontic biofilm of *E. faecalis*.

HYPOTHESES

- Null 1: EMS in combination with 1.5-percent sodium hypochlorite will not demonstrate an anti-biofilm effect in comparison to 6.0-percent sodium hypochlorite.
- Null 2: EMS in combination with 0.9-percent saline will not have a greater

antibacterial effect than 0.9-percent saline alone.

- Alternative 1: EMS in combination with 1.5-percent sodium hypochlorite will have an anti-biofilm effect comparable to 6.0-percent sodium hypochlorite in a synergistic reaction.

- Alternative 2: EMS with 0.9-percent saline will demonstrate a significant anti-biofilm effect over 0.9-percent saline alone.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

The earliest theory on tooth pain was that a "tooth worm" bored a hole into the tooth and then wiggled around inside, causing pain.[48] Many of the earliest treatments for tooth pain were aimed at destroying the worm or stopping its movement, which would relieve the afflicted of their suffering. Such treatments included acupuncture, the application of mixed substances (olive oil, dates, onions, beans and green lead for instance) against the affected tooth, magic, and the application of a dead mouse to the tooth.[49] Though these practices continued throughout much of ancient history, modern dentistry (and endodontics) can trace its roots to 1687 when Charles Allen released the first English-language book devoted to the subject of dentistry.[50] In 1700, the father of modern microscopy, Anton von Leeuwenhoek, reported (incorrectly) to the Royal Society of London (RSL) that fly worms (fly larva) birthed in hollow teeth gnawed at the tooth pulp, causing pain. The destruction of the worms by the application of sulfuric acid was considered the remedy to the noxious stimulus.[51] This was, however, the first explanation of an external source depositing material into the tooth to cause pain.

Perhaps the most influential person in the development of endodontics was Pierre Fauchard, who released his book, *The Surgeon Dentist*, 28 years after Leeuwenhoek's letter to the RSL. In it, he described accurately the contents of the dental pulp which led to the fall of the tooth worm theory and gave rise to the so-called "Empirical Era."[52] Fauchard illustrated his technique of opening teeth to drain abscesses/pus, leave them open for 2-3 months and then fill the pulp chamber with lead foil; he also described the

use of a pin to extirpate the pulp.[53]

No more than three decades after Fauchard's explanation of pulpal treatment, Pfaff, a German dentist, first described a pulp-capping technique in which he placed the concave surface of a piece of gold or lead directly over the pulpal exposure.[54] Woofendale was the first to perform an endodontic procedure in North America, in which he cauterized the painful pulp with a hot instrument and stuffed cotton in the open canals. For nerve exposures, he used oil of cinnamon, cloves, turpentine, or any chemical oil for pain relief; he also cauterized pulpal nerves by repeated applications of crude opium and camphor.[55-57] Frederick Hirsch, a German dentist, diagnosed periapical pathosis by using percussion as a testing method – treatment was perforation of the tooth at the cervical and repeated insertion of a red-hot probe followed by a lead filling.[53]

The next era of endodontics, which lasted about 70 years, saw two opposing theories at war: the vitalistic theory and the non-vitalistic theory.[52] Koecker, whose beliefs would prevail for over 50 years, believed that a necrotic pulp caused the whole dentinal core to die. The tooth would then become a foreign body, forcing extraction to prevent inflammation, suppuration and death of the surrounding vital tissues.[58] In 1829 Fitch indoctrinated the vitalistic theory in his book, *System of Dental Surgery*. According to his theory, the crown was nourished solely by the dental pulp, whereas the root could be nourished by the pulp or the alveolar membrane. As such, death of the pulp meant only the crown lost its vitality, teeth could be retained by extirpating the pulp, decoronation and placement of a pivot crown. Non-vitalists, led by Hunter, Cuvier and Robertson, thought dentin had no sensibility or circulation and therefore no ability to repair itself.[59] In 1836 Spooner advocated the use of arsenic to devitalize the vital pulp

prior to extirpation, rendering the process painless; this, however also killed the surrounding periodontium.[57]

Endodontic files like those used today were pioneered by Edwin Maynard who developed the first broach in 1838 using a watch spring.[60] Baker extrapolated on this by recording the first published account of pulp extirpation, cleaning the canal, and filling it with gold foil.[53] The 19th century saw many other great advances in the field of endodontics, including the invention of the rubber dam by Barnum,[53, 57] the use of gutta percha as a root filling material by Bowman,[61] the extension of Lister's surgical antisepsis to pulpal treatment,[54] and the introduction of the electric pulp tester.[62]

In 1878 Rogers suggested that pathogenic organisms caused pulpal disease, which led to the demise of vitalism and the birth of the septic theory.[59] Underwood expanded on Rogers' theory by adding that pulpal suppuration and subsequent alveolar abscesses were a direct consequence of pathogen toxicity. He theorized that sterilization of the pulp space with caustic antiseptics could prevent or cure disease, regardless of pulp vitality; such caustic agents were widely used for more than 30 years.[63] Some examples include arsenic, formalin, chlorophenol, sodium dioxide, sulphuric acid, paraformaldehyde, formocresol (introduced by Buckley and still used today), and glycerol with hydrochloric acid.[56, 57, 59, 64-66]

Breuer of Vienna was the first to use electric current to sterilize root canals, a technique introduced to the United States by Rhein in 1895; this was an important step in veering the profession away from such caustic agents for root canal sterilization. Dr. Herman Prinz perfected electro-sterilization in 1917 and recommended the use of 1.0-percent sodium chloride during treatment.[66, 67] Considered one of the greatest pioneers in

endodontics, Louis Grossman described a device capable of creating a galvanic current inside the root canal, which he later used for disinfection.[68] It was a crude device, which actually shocked the patient and had to run from 5 min to 30 min per canal to be effective; Grossman claimed its use took the average number of treatment visits from five to three.[69] Another pioneer, Harry Johnston, who coined the term "endodontia" and was the first to limit his practice to endodontics, preferred to treat root canals by passing a galvanic current through an iodine solution.[70]

Several materials were advocated for obturation during the 19th century. In 1895 Bowman began using a solution of chloroform and gutta percha, termed chloropercha, to obturate root canals.[71] Rhein perfected the technique a decade later in New York.[56,57] Baker advocated the use of gold foil to fill the root canal,[53] Richmond used carbolized orangewood,[64] and Gramm used copper points.[57] In 1911 Callahan advocated the use of rosin filling material for filling root canals as a method of better penetrating dentinal tubules and gaining a hermetic seal.[72]

Several important strides were made in pulpal anesthesia in the 1800s. Briggs routinely used cocaine as a topical anesthetic in 1890.[53, 64] The same year, Funk improved on the use of cocaine as an anesthetic by inventing a plunger-like device that deposited a cocaine solution directly into the pulp.[56, 59] In 1905 Einhorn developed procaine (Novocaine), but it was a burden to use due to the tedious preparation process.[60,66,73] The first person to use infiltration for pulpal anesthesia prior to extirpation was H.S. Vaughn in New York.[61]

Perhaps the most important discovery in medicine occurred in 1895 by Konrad Wilhem von Roentgen. He discovered high energy particles that could penetrate hard

structures, aptly termed x-rays.[74-76] Shortly after his discovery, a dentist in Germany named Otto Walkhoff took the first dental x-ray.[50] Dr. Kells in Charlotte, NC was the first to use x-rays routinely in his dental practice and he gave the first clinic on their use in 1896.[63] The use of x-rays extended to endodontics in 1908, when Rhein developed a method of determining the length of the root canal and quality of obturation using wire and x-rays. G.V. Black further promoted this method to avoid overfilling, which was a common error at the time.[57,62,66,77]

During this time of advancement, root canal therapy should have enjoyed a time of progress and discovery. However, a cataclysmic series of events led to the near demise of endodontics before it was recognized as a specialty. In 1909 E.C. Rosenow introduced the theory of focal infection by showing streptococci in diseased organs capable of spreading through the bloodstream to a distant site.[66] At the same time, Mayrhofer showed that streptococci were involved in approximately 96 percent of pulpal infections.[67] This deadly combination almost led to the destruction of dentistry, and particularly endodontics, when William Hunter, an English physician, lectured the faculty at McGill University (Montreal, Quebec, Canada) on focal infection.[78] The same lecture was published a year later in *Lancet.* Hunter called the gold crown a “mausoleum of gold over a mass of sepsis.” This type of thinking resonated within the medical community and subsequently the dental community, leading to widespread full edentulism for many unfortunate patients in the name of disease prevention. This practice persisted for at least 25 years.[79-81]

Fortunately, the preservation of the pulpless tooth survived thanks to the efforts of pioneers such as Coolidge, Johnson, Rhein, Callahan, Grove, Prinz and others, who

improved their procedures through the use of aseptic techniques, bacteriological and histological methods, and diagnostic x-rays.[57,82] In 1937 three publications led to the downfall of the focal infection theory. First, Logan observed that the presence of microorganisms in tissue does not necessarily imply infection; in other words, bacteria are often found in tissues without having pathological consequences. Second, Tunnicliff and Hammond found microorganisms in the pulps of extracted teeth without evidence of inflammation or disease.[55,66,83] Finally, Burket found no benefit of surgical removal of suspected foci in over 200 cases of arthritis, leading him to conclude clinical improvement after foci removal was simply an associated relation rather than a causal one.[84] By the late 1940s or early 1950s, there was enough clinical evidence and laboratory research to prove that the devitalized tooth did not play a role in systemic disease and endodontic treatment once again began to flourish.[85]

As root canal therapy became more widespread, a group of 20 dentists, led by Harry Johnston, met in Chicago for the first organized meeting of endodontics. Their efforts set the standard for endodontic treatment and resulted in the formation of the American Association of Endodontists (AAE).[82] By 1963 more than 200 American dentists limited their practice to endodontics. The same year, the American Dental Association recognized endodontics as its own specialty and the first specialists became board certified two years later.[86] Today the AAE has over 8,000 members globally.

Since the foundation of the AAE, the field and science of endodontics has exploded with advancements made in visualization using the surgical operating microscope, working length determination via the introduction of the electronic apex locator, instrumentation via nickel titanium rotary instruments, adjunct irrigation

therapies like sonic/ultrasonic irrigation or the upcoming Gentle Wave system,[87] the use of CBCT in diagnosis and treatment planning of endodontic retreatment[88] and microsurgery,[89] and progressive research in regenerative endodontics.[90] The future is definitely looking bright for endodontic patients and clinicians alike.

THEORY OF ENDODONTICS

For decades it was widely accepted that the root canal system (RCS) should not be sealed until a sterile culture was obtained. Clinicians understood the connections among the presence of microorganisms in the RCS and the persistence or progression of pulpal and periapical disease. However, it wasn't until 1965 that Kakehashi, Stanley and Fitzgerald showed that microorganisms not only propagate endodontic and periapical pathosis, but they are the cause of it.[1] In what is now considered a monumental study, they exposed the pulps of germ-free (gnotobiotic) rats as well as conventional rats whose oral environments contained complex microflora. They observed the pulp exposure of the conventional rats resulting in complete pulpal necrosis with granuloma and abscess formation, whereas the gnotobiotic rats were disease free. Thanks to this study we now know that endodontic and periapical pathosis begins when normal microflora enter the RCS by way of caries, a previous restoration, or trauma.

The findings of Kakehashi, Stanley and Fitzgerald led others to investigate the association of microorganisms in pulpal and periapical disease. Inflammatory cytokines[91,92] and neuropeptides[93,94] have been found in vital pulp tissue when bacteria and their virulence factors, such as lipopolysaccharides (LPS) are present; in addition, the presence of LPS has been associated with pulpal symptoms.[95-97] If inflammation persists, micro-abscesses develop, which lead to pulpal necrosis.[98] More bacteria can enter these

areas of necrosis and subsequently into the dentinal tubules[99] and/or lateral and accessory canals.[100] The spread of bacteria into these areas allows the pathogens and. their LPS to reach the periapical tissue, resulting in apical periodontitis.[101-104] This body of knowledge allows us to identify the main goal of endodontic treatment: the eradication or reduction of pathogens and their byproducts to a level the host immune response can properly defend itself; this is achieved by mechanical debridement and chemical cleaning of the RCS.[87, 105] Given such, the success of endodontic treatment directly relies upon the reduction of pathogenic bacteria from the RCS.[106]

G.G. Stewart identified the three phases of endodontic therapy, known today as the endodontic triad: chemomechanical preparation, elimination of microbes, and obturation/sealing of the RCS.[107] Chemomechanical preparation is the key phase as it both reduces microbes and creates the shape and space needed for obturation of the apical region. Further expounding upon Stewart's triad, Louis Grossman identified 13 aspects of endodontic treatment essential to its success[108]:

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 irritating to the periapical tissues.

11. If an acute alveolar abscess is present, proper drainage must be established.

12. Injections into infectious areas should be avoided.

13. Apical surgery may be required to promote healing of the pulpless tooth.

In 1967 Schilder discussed the importance of a three-dimensional fill when obturating the root canal system. Not only was chemomechanical debridement important to lower microbial load, he argued, but an adequate three-dimensional fill was required to fully seal the apical area. His technique involved adding small increments of gutta percha, heating it with a spreader, then compacting it with a plugger to achieve a dense, homogenous obturation.[109] Schilder's technique has been credited with revolutionizing the way the RCS is filled and increasing reported success rates from 80 percent to above 95 percent.[110]

The sentiments of Schilder were echoed by Pitt Ford, who rationalized three-dimensional obturation via three concepts. First, a dense, homogenous fill would leave little to no space for bacterial colonization. Second, apical contamination could not occur if bacteria cannot colonize. Finally, movement of bacteria along the canal periphery would be prevented. According to Pitt, an aseptic endodontic technique was tantamount to prevent further RCS contamination. Rubber dam isolation, a satisfactory coronal restoration, and appropriate follow-up of endodontically treated teeth were advised.[111] All of these concepts have shaped the success of endodontic practice today. We are able to retain infected teeth utilizing the following: a reduction of bacteria through chemomechanical preparation,[107,108] three-dimensional obturation and sealing of the

RCS,[109] a coronal restoration that minimizes coronal leakage,[112-114] and appropriate long-term maintenance.[111]

MECHANICAL PREPARATION/INSTRUMENTATION

Mechanical instrumentation of the RCS is the first phase of endodontic therapy and is the foundation for proper chemical disinfection and obturation.[115] There are biological and mechanical objectives during this phase of therapy. The biological objectives include disruption of bacterial biofilms, reducing the bacterial load, neutralizing or eliminating any virulent potential of microbial components left in the canal, and the removal of all organic debris.[116] The mechanical objectives include enough taper and size to facilitate the delivery of antibacterial irrigants and/or intracanal medicaments while providing space for a dense, homogenous three-dimensional obturation.[87] It is important to maintain the original shape of the canal to prevent unnecessary weakening of tooth structure and damage to the surrounding periapical tissues.[117] Although mechanical preparation alone can reduce the microbial load by 100- to 1000-fold,[118] it does not eliminate all microbes or their byproducts from the RCS due to its anatomical complexity. The presence of anatomic intricacies such as accessory canals, lateral canals, fins, anastomoses, and apical deltas prevent our current file systems from physically reaching all areas accessible to microbes.[119] Indeed, bacteria have been shown to penetrate dentinal tubules up to 300 microns.[120] Furthermore, up to 53 percent of root canal walls will remain untouched even with adequate instrumentation.[121-123] Therefore, chemical disinfection via irrigation must also occur in order to achieve an adequately disinfected canal space.

CHEMICAL IRRIGATION

In medicine, to irrigate means to "wash out a body cavity or wound with water or a medicated fluid." In endodontics, we irrigate to disinfect the RCT.[87] The benefits of using an irrigant during root canal therapy are many and include the removal of necrotic debris, destruction of microorganisms, dissolution of organic material, removal of the smear layer, disinfection and cleaning of areas which endodontic files cannot reach, and lubrication. The ideal irrigant has a multitude of characteristics and should do the following[87]:

- Be an effective germicide and fungicide.
- Be nonirritating to the periapical tissues.
- Remain stable in solution.
- Have a prolonged antimicrobial effect.
- Be active in the presence of blood, serum, and protein derivatives of tissue.
- Have low surface tension.
- Not interfere with repair of periapical tissues.
- Not stain tooth structure.
- Be capable of inactivation in a culture medium.
- Not induce a cell-mediated immune response.
- Be able to completely remove the smear layer, and be able to disinfect the underlying dentin and its tubules.
- Be nonantigenic, nontoxic, and noncarcinogenic to tissue cells surrounding the tooth.

- Have no adverse effects on the physical properties of exposed dentin.
- Have no adverse effects on the sealing ability of filling materials.
- Have a convenient application.
- Be relatively inexpensive.

In reality, there is no such irrigant, so multiple irrigants are used to achieve all of this. The most widely used irrigant is sodium hypochlorite (NaOCl), commonly considered the gold standard in endodontics.[3] NaOCl's advantages are many: it is highly antibacterial against planktonic and biofilm arranged organisms, it dissolves organic tissue, it is inexpensive and it lubricates the canal space for instrumentation.[3] These effects, particularly organic tissue dissolution, are increased at higher concentrations or when used at higher temperatures; at lower concentrations, it loses its antibacterial and tissue solvent properties more rapidly and must be replenished much more frequently.[124,125] The antimicrobial effects of NaOCl are due to several mechanisms. At a pH of 11, it exists primarily as hypochlorous acid ($HClO^-$), which disrupts cellular activities like oxidative phosphorylation, DNA synthesis, and multiple cell membrane associated activities.[126-128] Despite its perks, there are several disadvantages to using NaOCl as an endodontic irrigant. It displays dose (concentration)-dependent cytotoxicity to periapical cells when extruded, it does not kill all microorganisms, it does not remove the smear layer, it has a foul odor/taste, it cannot inactivate endotoxins, and it lacks substantivity.[124,129-131]

To mitigate these shortcomings, chlorhexidine gluconate (CHX) has been advocated for use as an alternative or in conjunction with NaOCl. Usage modalities of CHX include caries prevention, periodontal therapy and antiseptic mouthwash.[132] CHX

has a pleasant taste and odor, less cytotoxicity, and can provide extended periods of antimicrobial activity with up to 12 weeks of substantivity.[133] It is bacteriostatic at low concentrations, bactericidal at high concentrations and disrupts cell membranes by electrostatic binding due to its cationic nature.[134,135] Recent awareness of a potentially harmful precipitate known as parachloraniline (PCA), parachlophenylrurea (PCU), or parachlorophenylguanidyl-1,6-diguanidyl-hexane (PCGH) that is formed when NaOCl and CHX interact requires the clinician to utilize great caution when alternating between the two.[136, 137] Formation of the precipitate can be prevented if the canal is flushed with saline between use of NaOCl or CHX.[137]

As neither NaOCl nor CHX can remove the inorganic smear layer, a chelating agent such as EDTA or citric acid must be used. Removal of the smear layer provides several benefits including better penetration of NaOCl into dentinal tubules[138] and improved sealing ability of obturation materials.[139] Irrigation of a canal for 1 minute with 17-percent EDTA adequately removes the smear layer[140] and subsequently removes canal debris more effectively when used with NaOCl.[141]

OBTURATION

The third objective of endodontic therapy is to provide a “hermetic” seal that adequately and densely fills the RCS.[142] Filling materials should be biocompatible and nontoxic to tissues if extruded beyond the apex.[108] However, the highest rates of success are seen when the material ends 0-1 mm from the radiographic apex.[143] The major cause of pulpal and periapical disease are microorganisms; however, their complete elimination is often not possible (or required) for successful treatment. Thankfully, a wide variety of instruments, irrigants, and obturation materials exist that the prudent clinician can utilize

to provide long-lasting endodontic treatment.

IMMATURE TEETH WITH PULPAL NECROSIS

In the case of mature teeth with closed apices, follow up studies have found conventional root canal therapy to have success rates as high as 97 percent.[114] In the case of immature teeth with pulpal necrosis, conventional root canal therapy is not as predictable. Challenges specific to immature teeth with pulpal necrosis include a wide-open apex through which endodontic materials may be extruded into the apical tissue,[144] difficulty in chemomechanical disinfection due to wide/divergent walls, and thin root walls which lead to future fractures and restorative failures.[145] Such challenges have forced the development of different management strategies of these teeth.

APEXOGENESIS

Maintenance of the vital pulp is the preferred method of treatment for immature teeth with irreversible pulpitis. The rationale for this method, known as apexogenesis, is to allow continued root development and apical closure.[146] Clinically, apexogenesis entails partial or full pulpotomy followed by a biocompatible pulpal dressing and a definitive restoration. Factors that dictate the amount of tissue removal necessary include the size of the exposure, amount of time passed before treatment and adequate hemorrhage control.[7, 147] Historically, calcium hydroxide has been used as the pulp dressing of choice for apexogenesis due to its ability to disinfect and stimulate hard tissue formation. Despite these advantages, dentin formation induced by calcium hydroxide is usually incomplete and its high pH results in pulpal and tissue inflammation.

The advent of MTA as a pulp capping material has led to superior dentin bridge

formation without inducing pulpal inflammation.[148] Its use has resulted in more predictable outcomes with greater long-term success. Nevertheless, MTA has several disadvantages including high cost, difficulty in handling, long setting time and tooth discoloration.[149] Other bioceramic materials such as Biodentine have been developed to overcome some of MTA's drawbacks. They are used in a manner similar to calcium hydroxide; the inflamed pulp is partially or fully removed from the chamber under aseptic conditions. After controlling hemorrhage, the bioceramic derivative is placed directly over the remaining pulp tissue followed by a direct restoration, either resin or amalgam. Symptoms and further root development are monitored through occasional follow-up visits with radiographs and pulpal/periapical testing. This allows the clinician to assess continued root development with maintenance of a symptom free vital pulp. Apexogenesis is the preferred treatment in immature teeth with vital pulps, but for immature teeth with necrotic pulps, treatment modalities include apexification and regenerative endodontic procedures (REPs).

APEXIFICATION

Apexification was the original method of inducing root end closure of an immature tooth with pulpal necrosis. The objective is to induce a hard tissue barrier in against which endodontic filling materials can be compacted. Traditionally this has been done by gentle chemomechanical disinfection of the root canal system just shy of the blunderbuss apex followed by long-term placement of a disinfecting intracanal medicament such as calcium hydroxide.[150,151] Due to its high pH, calcium hydroxide denatures microbial proteins and induces apical barrier formation by causing a low-grade inflammation in the canal space. This treatment modality requires excellent patient

compliance due to the need for multiple visits over 9-24 months.[152] The amount of time and number of visits necessary to complete apical barrier formation are reliant upon the stage of root development. Once a barrier is visible radiographically, the root canal is filled with MTA and/or gutta percha with sealer followed by a direct restoration for the coronal seal.[146]

The main drawbacks of traditional apexification include the type of hard tissue barrier formed, patient compliance, and the side effects of long-term calcium hydroxide application such as increasing dentin susceptibility to fracture. The hard tissue formation is typically made of cementoid and osteoid material that is porous with small remnant communication with the apical tissues.[152, 153] Its formation does not induce increased root width or length development. Due to the longevity of treatment, the patient is required to return for multiple visits over a long period of time, require excellent patient compliance with a disciplined follow-up regimen. In addition, long-term calcium hydroxide has been shown to decrease the fracture resistance of dentin, heightening the chance of future root fracture.[154-157]

An alternative treatment to traditional apexification is the bioceramic apical barrier technique. This can be done in 1[158] or 2 visits with short term application of calcium hydroxide. Once the canal has been adequately disinfected and the patient's symptoms have resolved, an artificial apical barrier is made using a 4-mm to 5-mm layer of bioceramic material, which allows immediate obturation of the RCS with traditional techniques.[159] The advantages of bioceramic apexification are the biocompatibility of bioceramic materials, reduced need for patient compliance, and the capability of bioceramics to form an adequate seal in the presence of moisture.[160,161] Success rates of

bioceramic apexification range from 81 percent to 95.3 percent.[158,162,163] In spite of such high success, bioceramic apexification does not induce further root maturation, placing the root at risk for future fracture. These drawbacks have necessitated the development of other treatments, including REPs.

REGENERATIVE ENDODONTIC PROCEDURES (REP)

The origin of REPs can be traced back to the 1960s through the work of Nygaard-Østby in which he described tissue healing and repair in the presence of a blood clot in the root canal space.[164] In the last 15-20 years, research on REPs has exploded, including the discovery of the four elements required for successful REPs: stem cells, scaffolds, growth factors, and adequate disinfection.[165] Current AAE guidelines recommend REP procedures over two visits.[166] The first visit begins the disinfection process by irrigation with 1.5-percent NaOCl and 17-percent EDTA followed by application of an intracanal medicament such as triple antibiotic paste,[167] double antibiotic paste,[168] or calcium hydroxide. After 1 to 2 weeks, anesthesia is administered without vasoconstrictor and the RCS is again rinsed with 17-percent EDTA. The EDTA rinse removes the smear layer by chelating inorganic material and demineralizing the superficial dentin layer. This results in the exposure of dentinal collagen fibers and the release of growth factors.[169] Bleeding is induced by lacerating the apical papilla with a large hand file, thereby providing stem cells and the scaffold necessary for tissue formation. The blood is allowed to fill the tooth to the CEJ and a collagen barrier is then placed. A coronal seal is obtained by placement of a bioceramic base followed by a direct restoration. Varying types of soft and hard connective tissue will form in the canal space including cementum, bone, and reparative dentin.[170] The main goals for REPs are to 1) alleviate patient symptoms, 2)

continued root length and width development with apical closure, and 3) obtain pulp vitality/sensibility.[90,166,171-173]

MICROORGANISMS

Classification of endodontic infections depend upon whether the RCS has been previously treated; infections can be primary (never treated) or secondary (a recurrent or refractory infection). Either type of infection is associated with particular groups or types of bacteria, which are typically arranged in a biofilm. The four types of endodontic biofilms are intracanal, extraradicular, periapical and biomaterial-centered.[174] Biofilm organized bacteria have certain mechanisms which increase resistance to environmental stress and thereby enhance their odds of survival.[175]

Biofilms can be up to 1,000-fold more resistant than their planktonic counterparts – they are arranged in an extracellular matrix (ECM) of exopolysaccharides, which impedes antibiotic diffusion and uptake.[34] Furthermore, extracelluar enzymes that inactivate antibiotics, such as beta-lactamase, are abundant and highly concentrated in the ECM. Quorum sensing encourages growth of species beneficial to biofilm structure and growth. Gene expression can be altered to protect certain subpopulations. The deeper microorganisms are protected from medicaments that only act on peripheral cells. In a biofilm state, individual microorganisms grow more slowly with less metabolism than planktonic cells, allowing them to evade antimicrobials. When nutrients are depleted or waste products over accumulate, growth halts, preventing uptake of antibiotics. The altered pH and low oxygen levels in biofilms may also alter antibiotic efficacy.[174]

The majority of microorganisms in primary endodontic infections are gram-positive anaerobic rods and cocci.[176] Some examples include *Actinomyces naeslundii,*

Fusobacterium nucleatum, and *Porphyromonas gingivalis.*[175,177] *A. naeslundii* activates the host innate immune system, kickstarting cytokine production and the inflammatory process.[178-181] A critical component of microbial biofilms, *F. nucleatum* is a relatively large gram-negative rod capable of allowing numerous gram-positive and -negative organisms to attach. It invades host tissue cells to elicit an immune response.[177] The main virulence factors for *P. gingivalis*, found in nearly 50 percent of primary endodontic infections, include LPS, lipoproteins, capsule, and fimbriae.[175, 177, 182] It is a gram-negative obligate anaerobe that is unable to withstand exposure to NaOCl.[183]

Gram-positive facultative cocci, rods and filaments comprise the majority of microorganisms in secondary endodontic infections.[184,185] Though the flora of secondary infections is less well understood, predominant species include *Actinomyces israelii, Enterococcus faecalis,* and *Propionibacterium spp.*[186-188] *E. faecalis* is a gram-positive facultative cocci that is found in primary and secondary infections and its importance in endodontics is well documented.[177,184,189-193] It possesses multiple virulence factors aimed at inciting the host inflammatory response including the ability to adhere to dentin, invasion of dentinal tubules, the ability to suppress lymphocytic action, the ability to use serum for nutrition, and the possession of a proton pump which lowers internal pH and increases resistance to calcium hydroxide. It also possesses lytic enzymes, cytolysin, lipoteichoic acid, aggregation substance and pheromones.[191] *E. faecalis* has a high propensity to form and thrives in biofilms, augmenting its virulence and resistance factors.[192] In fact, the contact time to eradicate a mature monospecies biofilms comprised of *E. faecalis* with 5.25-percent NaOCl is at least 40 minutes, a representation of its ability to survive in the harshest conditions.[193]

USE OF ELECTRIC CURRENT FOR DISINFECTION

In endodontics, electromagnetic fields have the capability provide disinfection or drug delivery. In the late 19th/early 20th centuries, “electro-sterilization” had gained massive popularity and was used to disinfect root canals throughout Europe and the United States.[67-69] Sturridge wrote a comprehensive guide on the use of electric current in dentistry in 1918. He described its applications in root canal sterilization, periodontal disease, neuralgia, bleaching, and anesthesia. His proposed mechanism of action for the antibacterial effect of electric current was the movement of ions in solution, particularly zinc and silver.[194]

Despite its early popularity and versatility, electrical field usage root canal therapy died off and did not rekindle until the late 1990s, when the Endox Endodontic System was developed in Germany and later studied in Italy, where it is still used by many today.[195] Endox emits high-frequency electric impulses through proprietary electrodes of varying diameters that last for approximately 0.14 seconds at a frequency of 312.5 kHz and potential of 1100 V. The electrodes also have an electronic apex locating feature, but their use requires preliminary shaping to a certain size and taper. Pulsing a root canal with Endox has three main effects: a local rise in temperature, increased ozone due to medium ionization and production of UV rays.[195] Presumably these local changes imbue Endox with its proposed antimicrobial activity, though findings are mixed. One *in-vitro* study demonstrated an enhanced antibacterial effect compared to saline,[196] while two others exhibited a diminished antibacterial effect compared to 2.5-percent NaOCl/MTAD, 2.5-percent NaOCl/EDTA,[197] HealZone, and MTAD.[198] Pulsation with Endox also dissolves organic tissue to a degree, but the effect is minimal without

mechanical preparation – in in fact, to rely on Endox solely for tissue dissolution would be tedious and time consuming as it would likely take several hundred pulses to fully dissolve organic debris.[199]

Another *in-vitro* study described a prototype device in Turkey that is used differently than Endox. Rather than single high-frequency pulses, it emits a continuous low-frequency current in combination with sonic agitation for a time period chosen by the operator. In the study, the prototype did not eradicate a mature *E. faecalis* biofilm.[200]

Perhaps the most intriguing electrical device for root canal disinfection today is the J. MORITA prototype, the result of a partnership between researchers at the International Society for Electromagnetic Dentistry (ISEM) and J. MORITA. Spearheaded by Dr. Toshihiko Tominaga, early studies on the device show bactericidal activity against planktonic microorganisms (*S. mutans, E. faecalis, P. gingivalis,* and *S. intermedius*), downregulation of inflammatory cytokine production by THP-1 lymphocytes, destruction of *P. gingivalis* gingipains,[30] increased osteoblast proliferation and differentiation, and upregulation of bone growth factors by activating the ERK1/2 and p38 MAPK pathways.[46,47] J. MORITA theorizes that the antimicrobial capability of its prototype device is due to synergism between the electromagnetic pulses and any antimicrobial solution in the canal, which they have coined electromagnetic stimulation (EMS).

Unlike the Endox system and the Turkish prototype, the J. MORITA prototype has years of clinical use at the ISEM, and the results are promising. With over 300 cases treated, and a success rate between 95 percent to 99 percent,[31,32,201] the future is bright for the J. MORITA prototype. In the most innovating clinical study by the ISEM, individual

patients were used as their own controls. Bi-rooted teeth with periapical rarefactions associated with each root were treated with the prototype device and conventional root canal therapy. A preoperative periapical radiograph and CBCT were taken of each tooth. The diameter of each lesion was measured on the PA, and the volumes were measured on the CBCT. One root was treated conventionally in two visits with an interappointment medicament and obturation at the 2nd visit. The other root was shaped to size, the canal filled with saline at 4°C, and immediately treated with the prototype device by 1 pulse directly into the lesion and 1 pulse at the root apex. The irradiated root was obturated at the first visit. The teeth were then followed and reimaged at 1, 3 and 6 months. Irradiated teeth were divided into rapid healing and slow healing groups, the data quantified and compared to the control group. In the control group, monthly diameter reduction averaged 0.38 mm/month and volume reduction 8.12 percent per month. In the slow healing experimental group, diameter reduction averaged 1.12 mm per month and volume reduction 17.26 percent per month. In the rapid healing experimental group, diameter reduction averaged 2.97 mm per month and volume reduction 33.33 percent per month. The average time to complete healing of the periapical lesions in the rapid healing group was three (3) months.[31] Proven clinical success is paramount to operator acceptance of any new technique or device and this is the main advantage the J. MORITA prototype has over Endox and the Turkish prototype.

Treatment with the J. MORITA device is through high-frequency electromagnetic impulses emitted through an insulated K-file, with current concentrated at the tip of the file. The number of pulses required to reach the desired antimicrobial effect has yet to be determined, and its efficacy on a biofilm has not been tested. The settings vary from 500

kHz to 1000 kHz, 70 percent to 80 percent duty cycle, and 50-150 mA. The frequency and duty cycle are preset on the individual device, with only the mA varying depending on the clinical situation. Thickness of dentin, canal diameter, and amount of solution all affect the current output the device is able to produce.[30]

The current incarnation of the J. MORITA prototype looks exactly like a Root ZX electronic apex locator (FIGURE 1), has an apex locating function in addition to EMS capability, and is clinically in a manner similar to an EAL. That is, a circuit is completed by attaching a clip to the patient's lip (counter electrode) and an endodontic K-file is inserted into the tooth (active electrode) to utilize both functions. Electromagnetic impulses are activated by pressing a rheostat.

ELECTROMAGNETIC FIELD MECHANISM OF ACTION

The exact mechanism by which electromagnetic fields kill bacteria is not well understood, but it is believed to work by disrupting the biofilm extracellular matrix or individual cell wall organization.[202] Biofilm disruption by electric current is known as the bioelectric effect. Several theories abound that explain how biofilms may be disrupted by the application of electromagnetic waves. First, electric impulses may disrupt charges in the ECM, resulting in better penetration of antimicrobial agents. Second, individual cell walls may be affected through a process called electroporation, which destroys barriers that prevent antimicrobials from diffusing across the membrane. Third, the electrolytic generation of oxygen or oxygen radicals may increase metabolic activity and growth rate of biofilm bacteria, which would make them more susceptible to antibiotic uptake and lysis. Finally, the electrochemical generation of potentiating oxidants or ions may also be responsible for the bioelectric effect, though studies on this theory deliver

mixed results.[203] Several authors show no antibiofilm effects when applying an electric current without antimicrobials,[204-206] whereas others have found direct application of an electric current to a biofilm has some antibacterial effect.[207,208]

MATERIALS AND METHODS

HUMAN TOOTH SELECTION

An overview of the entire experimental methodology is provided in Figure 2. Thirty-seven single rooted maxillary and mandibular human permanent teeth were collected and stored in a mixture of glycerine with 6.0-percent NaOCl. Only teeth with completely formed roots, free of decay, and at least 4 mm midroot diameter buccolingually or mesiodistally were included. Teeth exhibiting hypocalcification, restorations, decay, hypoplasia, fractures or cracks, incomplete radicular formation, fluorosis, and dentinogenesis or amelogenesis imperfecta were excluded. To determine whether teeth fit into these criteria, they were visually inspected.

ROOT SPECIMEN PREPARATION

A diamond saw with water irrigation (Li'l Trimmer, Lapcraft; Powell, OH) was used to cut off the crowns of the teeth (Figure 3). Root samples were prepared to a standard length of 12 mm. The canal spaces of the prepared root specimens were first negotiated with a #10 endodontic hand file, then a #15 endodontic hand file the full length of the root or 12 mm from the orifice to the root apex (Dentsply Sirona; York, PA). This was followed by preparation with a size 25.07 Wave One Gold file (Dentsply Sirona; York, PA) using a reciprocating motion in a Promark endodontic motor (Dentsply Sirona; York, PA) to a length of 13 mm from the orifice, to standardize the apical foramen size at the D1 level of the reciprocating file. A second Wave One Gold reciprocating file, size 45.05, was used in the same manner for final preparation. During treatment, specimens were irrigated with 6.0-percent NaOCl. Following preparation, the specimens were irrigated with 6.0-percent NaOCl and 17-percent EDTA for 3 minutes to eliminate the smear layer as described in the literature.[209] Teeth were stored in 6.0-

percent NaOCl and glycerine at a ratio of 2:1 until ready for use, at which time they were autoclaved for 20 minutes at 121°C.

INOCULATION AND BIOFILM FORMATION

A standard strain of *E. faecalis* (ATCC 29212) was used to inoculate the prepared specimens in the following manner: a solution of brain-heart infusion broth (BHI) was inoculated with a single colony and incubated for 24 hours at 37°C at 5.0-percent CO_2 to form the stock culture (Figure 4). The root specimens were coated with clarified and filter-sterilized pooled human whole saliva and prepared via 1 hour 37°C incubation.[210] Saliva was collected anonymously at the Oral Health Research Institute and was frozen until day of use. To thaw, the saliva was placed in an incubator at 37°C for one-hour and centrifuged for 10 minutes at 5000 rpm (Eppendorf 5804 R, Eppendorf; Hauppauge, NY). The supernatant was discarded and the remaining liquid was sterilized by filtration through a 0.22 µm PES membrane filter (Genesee Scientifics; San Diego, CA) (Figure 5). The saliva-coated roots were placed in 24-well culture plates (1 sample per well) filled with 1.8 mL of sterile BHI and 0.2 mL of fresh 24-hour stock inoculum and incubated at 37°C and 5.0-percent CO_2 for 14 days.[211] BHI solution was replaced every 24 hours without the addition of new inoculum in order to prevent nutrient depletion (Figure 6).

EXPERIMENTAL GROUPS

After removal from the 24 well-plates, specimens were divided randomly into three experimental groups and two control groups with 6 specimens each (8 specimens in 0.9-percent saline with EMS), depending on the disinfection protocol used (n = 6; n = p = 8 in 0.9-percent saline with EMS). Samples were prepared for treatment by mounting in

a sterilized sample cap 5A-1 (SKY-I, Japan) in fast-set alginate. The alginate was supplied in individual use packets that were packaged in an aseptic environment. The experimental and control groups were as follows:

Group 1: Treatment using 5 mL of 6.0-percent NaOCl under gentle irrigation (positive control)

Group 2: Treatment using 5 mL of 1.5-percent NaOCl under gentle irrigation

Group 3: Treatment with 5 mL of 1.5-percent NaOCl under gentle irrigation followed by EMS

Group 4: Treatment with 5 mL of sterile saline under gentle irrigation followed by EMS

Group 5: Treatment with 5 mL of sterile saline under gentle irrigation (negative control)

ELECTROMAGNETIC STIMULATION EXPERIMENTS

The EMS device (J. Morita; Japan) requires a complete circuit in order to function. In a clinical scenario, a counter electrode is placed over the patient's lip in the form of a shepherd hook. The active electrode is attached to an endodontic hand file via a clip. In the experimental scenario, the counter electrode is a periodontal probe mounted within the sample 5A cap containing the tooth specimen, while the active electrode is clipped to an endodontic hand file in the same manner as the clinical situation. Teeth were mounted as previously described; for circuit completion, a hole was bored in the inferior portion of the cap to allow the counter electrode, a periodontal probe (YDM Corporation; Japan), to be inserted. This area was sealed using Revolution flowable composite (Kerr; Orange, CA) and light cured for 20 seconds. The specimen was

mounted in alginate, leaving approximately 2 mm of tooth structure above the alginate. The active electrode was created by connecting an insulated #10 endodontic hand file (Dentsply Sirona, York, PA) to the device. The parylene coating serves as an insulator, so the electromagnetic burst only activates at the last 2 mm to 3 mm of the hand file. The active electrode was inserted to a working length of 12 mm and activated for a 1-second burst at the manufacturer's recommended setting of 500 kHz, 80 mA, 70-percent duty. A total of 7 one-second bursts were administered in the following manner: 3 bursts at the working length of 12 mm, 3 bursts at a working length of 9 mm, and 1 burst at a working length of 6 mm, as recommended by the manufacturer. The lengths were demarcated on the endodontic hand file with a marker to allow for expedient movement of the file during treatment. The one-second bursts were controlled by a rheostat, which is pressed each time a burst is desired. In the combination group, the canals were gently irrigated with either 1.5-percent NaOCl or saline with a 27-gauge needle up to 1 mm short of working length followed by immediate use of the EMS device.

CONTROL GROUPS

In the 1.5-percent NaOCl group, as well as the positive and negative control groups, the canals were gently irrigated with 5 mL volumes as described above.

ASSESSMENT OF ANTIMICROBIAL ACTIVITY

After treatment, coronal samples were immediately taken using a spiral utility brush (Versa Brush, Vista Dental; USA) in a slow speed hand piece at 250 rpm for 1 minute at a depth of 6 mm. Apical samples were taken by inserting a sterile size 30.04 paper point to a working length of 12 mm for 1 minute. The same procedure was used

for all groups.

The spiral brush and paper point were transferred to 15 mL Falcon tubes (Fisher Scientific; USA) containing 5 mL of sterile saline. Biofilms were detached by sonication for 30 seconds then vortexing for 30 seconds. A ten-fold serial dilution was completed, followed by plating onto blood agar plates. After anaerobic incubation for 48 hours in 5.0-percent CO_2 at 37°C, colonies were counted and CFUs/mL determined for statistical analysis (Figure 7).

CONFOCAL IMAGING

In addition to the specimens in each of the 5 groups, one specimen was prepared and completed as described above in the 2 EMS experimental groups, as well as the two NaOCl and 0.9-percent saline groups, for a total of 5 teeth. Prior to sterilization, the teeth were scored longitudinally as described in a previous study[212] using a straight handpiece with a diamond saw (Figure 8). This allowed separation of the specimen with a scalpel after treatment, exposing the root canal space for imaging (Figure 9). The canal space was stained with Live/Dead® Bacterial Viability Kit (Baclight Bacterial Viability kit L7012; Molecular Probes, Inc.). Three 0.5 mm stacks were taken starting from the apex and moving coronally for visualization of the treated biofilms at this portion of the tooth. A fourth 0.5 mm stack was taken individually at 6 mm from the apex to visualize a snapshot of the middle third of the tooth root.

STATISTICAL ANALYSIS

Due to a nonparametric distribution of data, CFUs were converted to $\log_{10}$. The effect of treatment group on $\log_{10}$ bacteria counts was made using Wilcoxon Rank Sum tests. A 5.0-percent significance level was used.

SAMPLE SIZE

The coefficient of variation was estimated to be 1.0. With a sample size of 6 in the 6.0-percent NaOCl, 1.5-percent NaOCl, 1.5-percent NaOCl with EMS and 0.9-percent saline and 8 in the 0.9-percent saline with EMS, the power to detect a 3x difference was 99 percent between the NaOCl groups and 0.9-percent saline, 99 percent between the two 0.9-percent saline groups and 89 percent between the NaOCl groups and 0.9 percent saline with EMS.

RESULTS

CFUs were converted to $\log_{10}$ and compared for differences using Wilcoxon Rank Sum Tests due to a nonparametric distribution of the data. In all cases of disinfection with NaOCl, no colonies formed after treatment. CFUs were counted in both the 0.9-percent saline and 0.9-percent saline with EMS groups (Table I). There was a significant effect with the use of NaOCl with or without EMS versus 0.9-percent saline with or without EMS ($p = 0.012$ and 0.003, respectively). EMS appeared to have an anti-biofilm effect, however, as there were fewer CFUs formed when using 0.9-percent saline and EMS versus 0.9-percent saline alone ($p =.002$, Table II, Figure 10).

For confocal imaging, groups are laid out by section. Confocal imaging provides a three-dimensional image of a biofilm. Live cells fluoresce a bright green color whereas damaged cells fluoresce a bright red color, which is a product of the molecules used for staining and imaging. The cells are stained with SYOT9 and propidium iodide (PI), both of which have high affinity for nucleic acid.[213,214] SYOT9 is the molecule responsible for cells that fluoresce green is small with minimal charge; this allows it to enter the membrane of any cell, whether live or dead. PI, which fluoresces red, is a large molecule with an intense positive charge, which prevents it from entering intact cells; it therefore can only enter if the outer membrane has been damaged. However, PI has a higher affinity for nucleic acid than SYOT9, so it is capable of displacement when used as the secondary stain.[213,214] During preparation, the samples are placed in several alternating washes of saline and stain solutions. This procedure can cause some dead cells to wash away, leaving black space. The apical 0.5 mm is presented in Figure 11, the apical 0.5 to

1.0 mm is presented in Figure 12, and the apical 1.0 to 1.5 mm is presented in Figure 13. Figure 14 represents a 0.5 mm stack taken 6 mm coronal to the apex, for a snapshot into the middle third of the root canal space. In all instances in which NaOCl was used as an irrigant, confocal imaging shows complete eradication of the biofilm at the apical 1.5 mm, regardless of whether EMS was also used. When saline was used without EMS, the apical 1.5 mm contained a full thickness biofilm and nearly all cells were green, indicating no anti-biofilm effect. When saline was used with EMS, there was a mixture of red cells, black space, and green cells, indicating some anti-biofilm effect in the apical 1.5 mm.

FIGURES AND TABLES

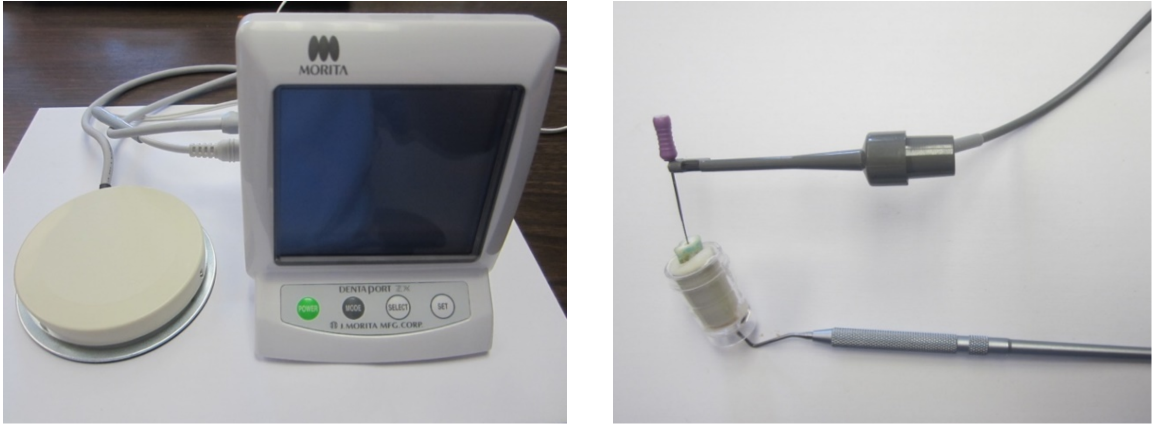


FIGURE 1. J. MORITA prototype.

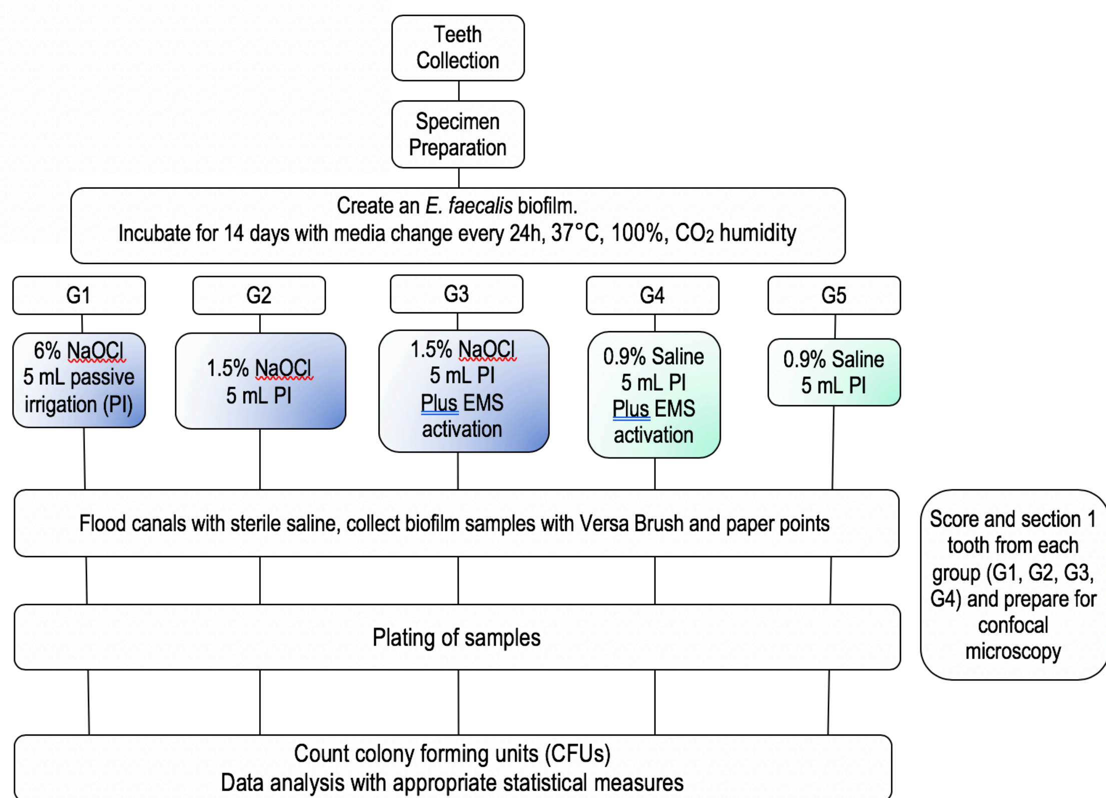


FIGURE 2. Experimental methodology.

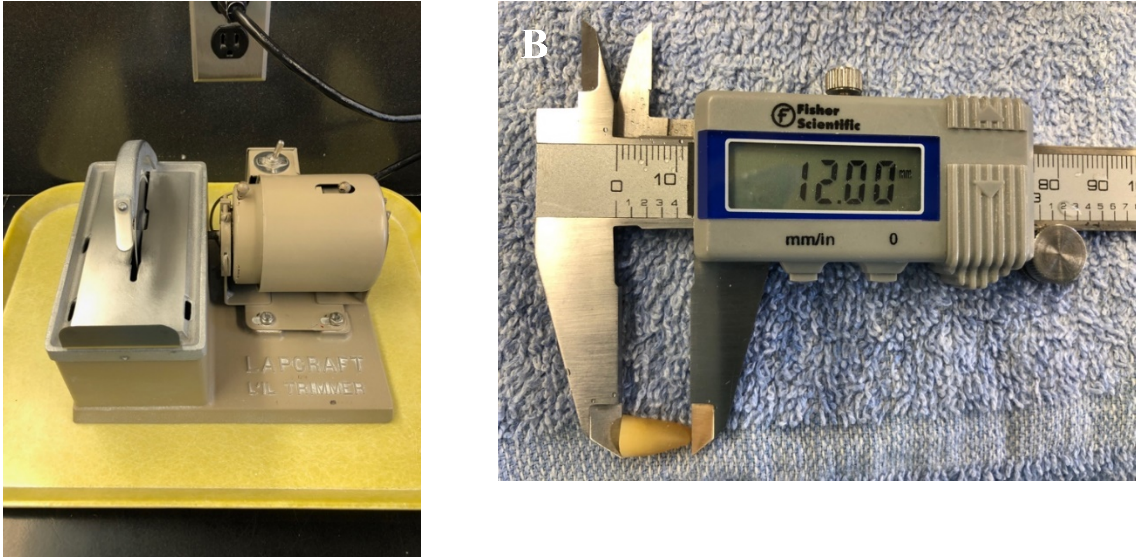


FIGURE 3. Diamond saw (A) used to standardize roots to 12 mm (B).

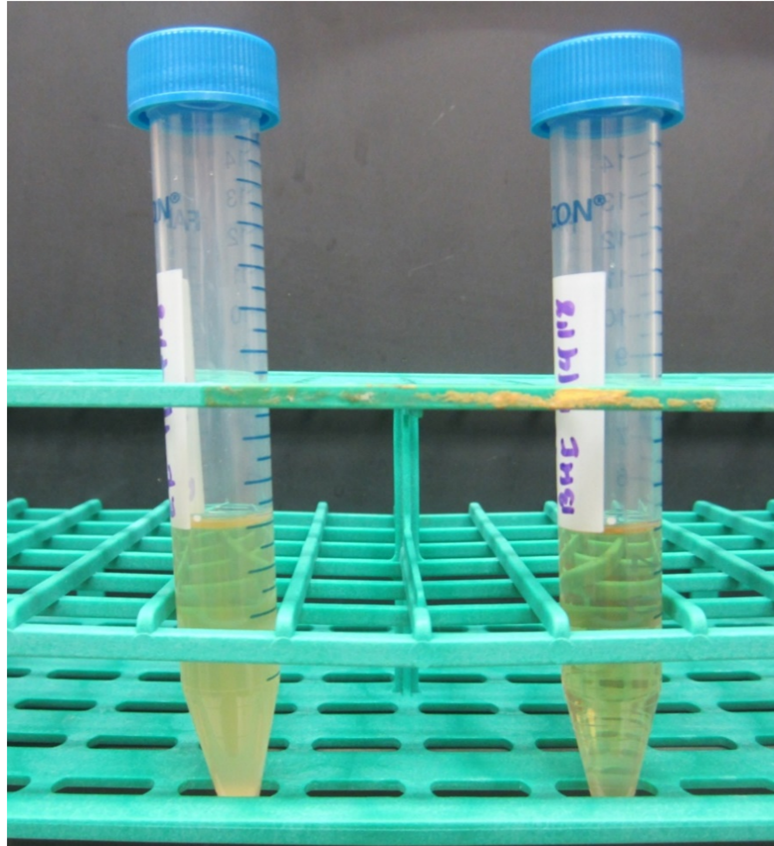


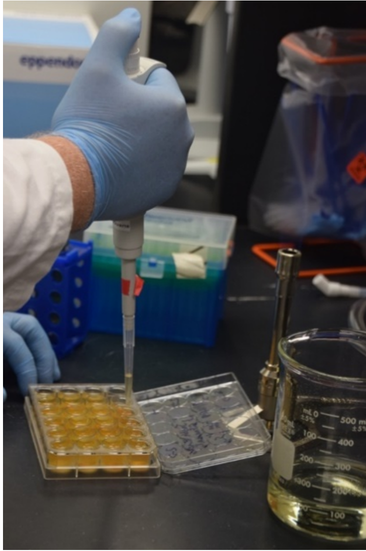
FIGURE 4. Stock inoculum (left) is cloudy compared with the sterile control (right).

A.

C.

B.

FIGURE 5. Centrifuge to spin saliva (A), vacuum filtration system (B), saliva coated roots (C).



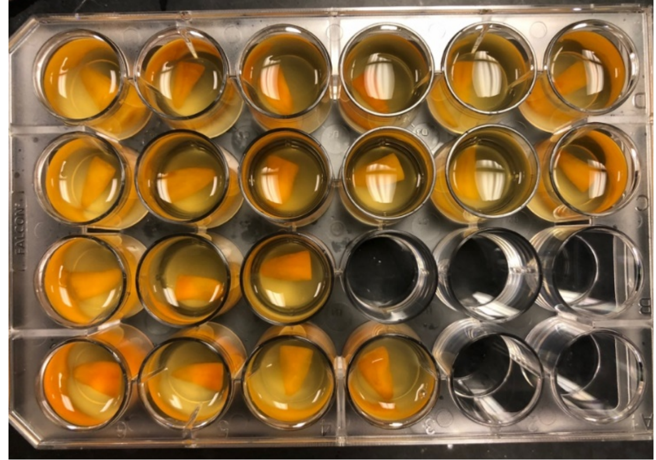


FIGURE 5. Changing BHI media (A), inoculated specimens (B).

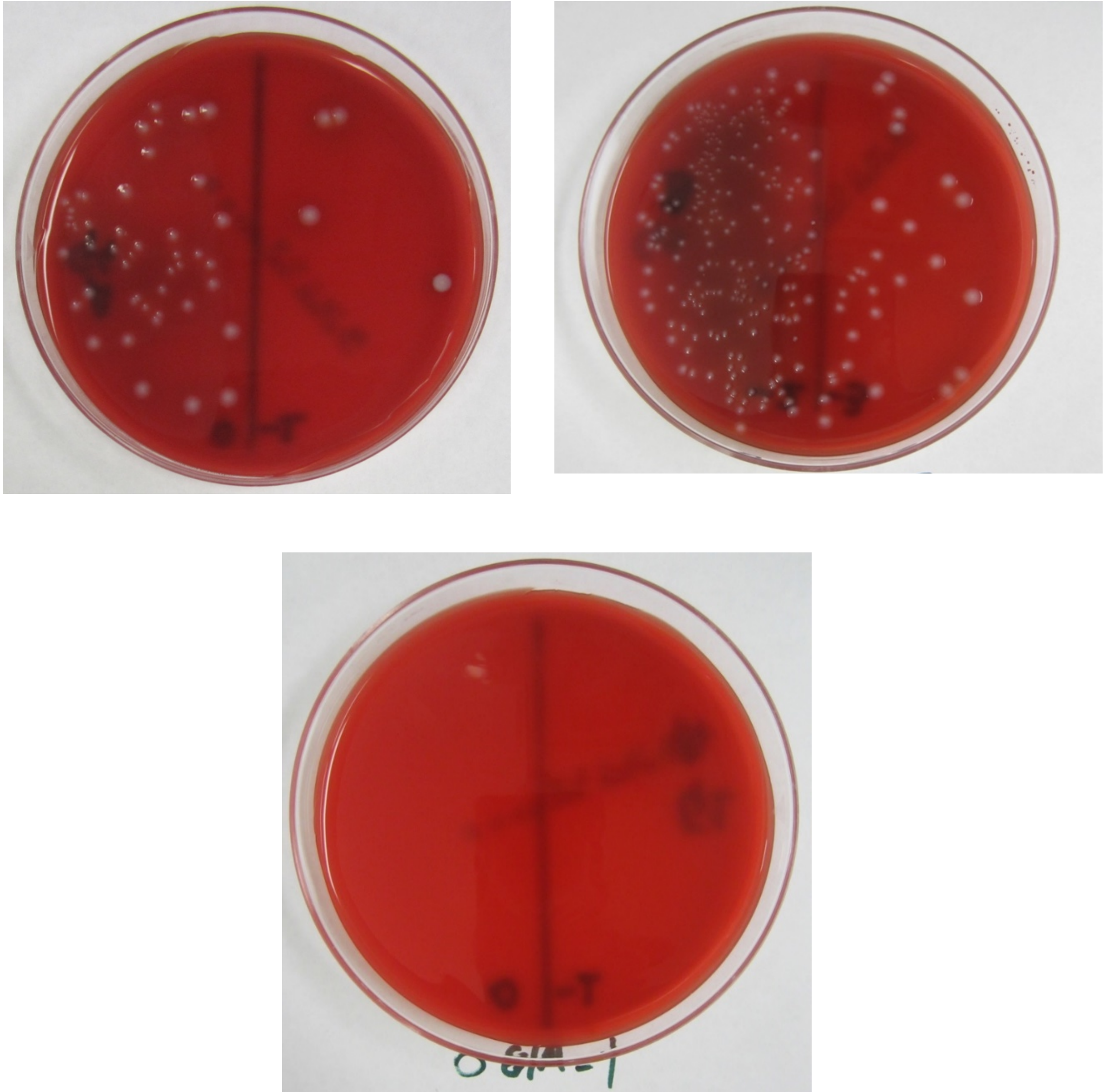
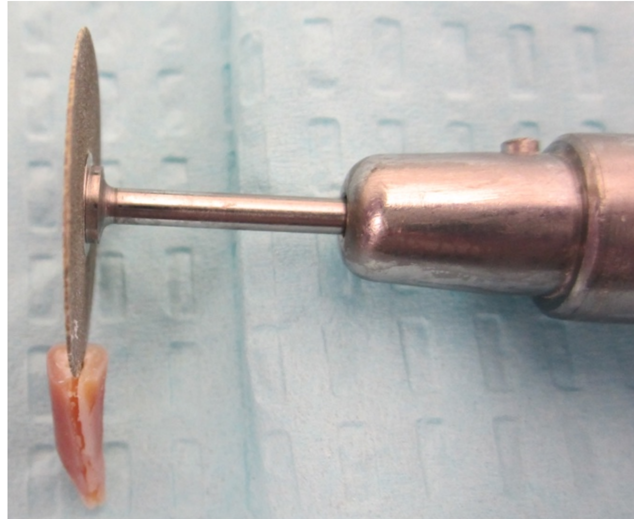


FIGURE 6. Representative growth plates. ***Clockwise:*** 0 G4A -1; -2 G5B -3; 0 G1A -1.



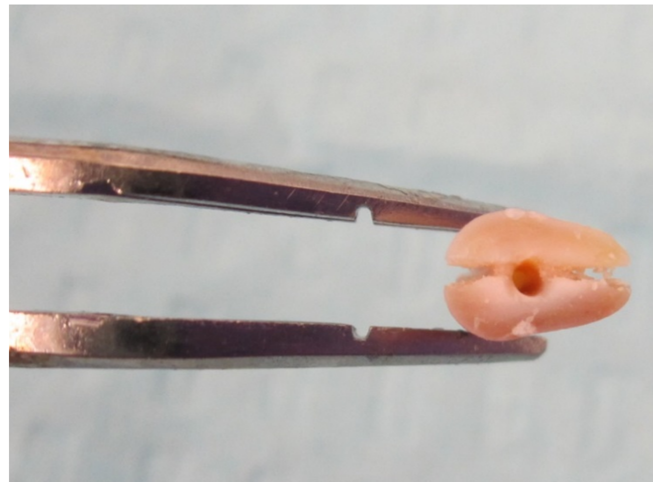
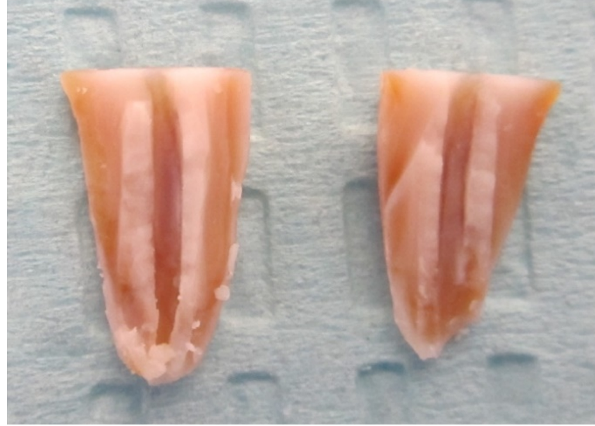


FIGURE 7. Scoring a tooth for confocal imaging.



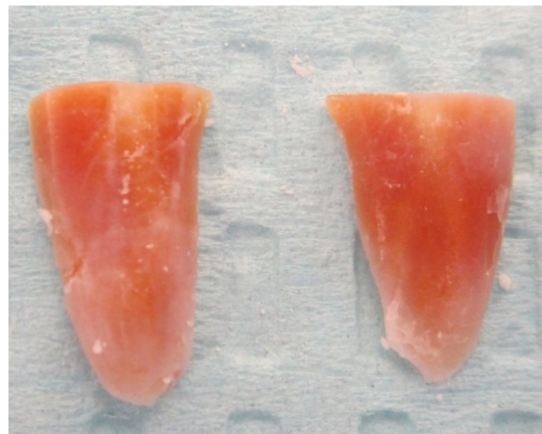


FIGURE 8. A scored and split tooth.

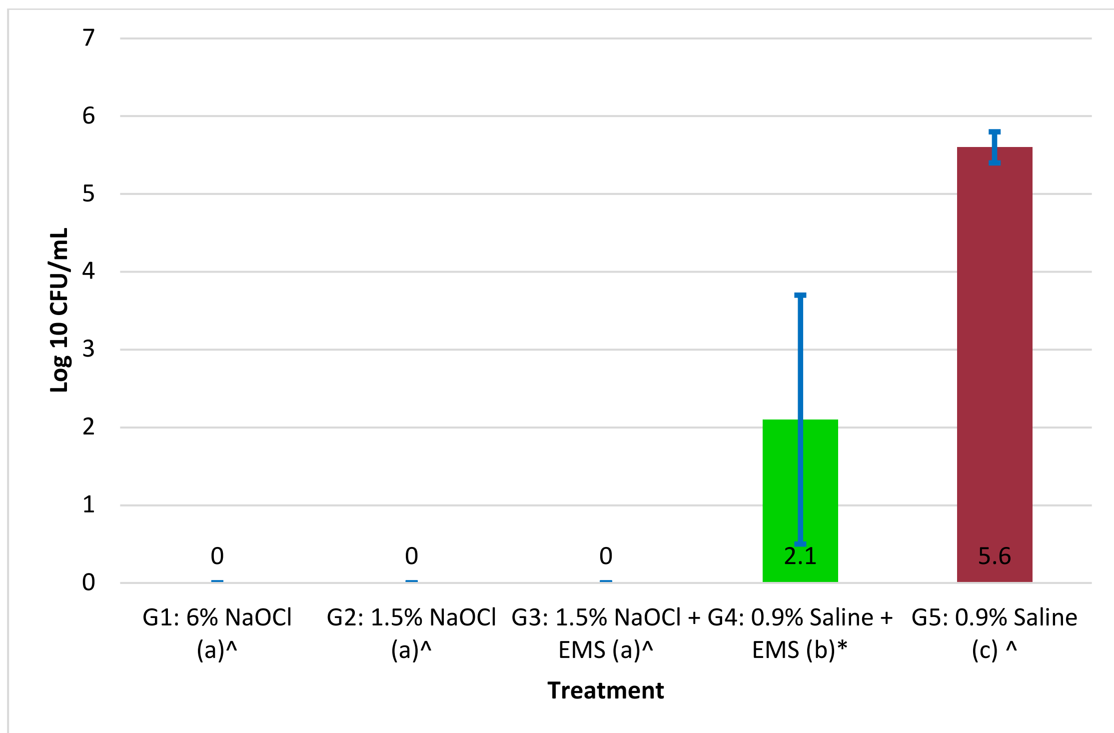


FIGURE 9. Average $\log_{10}$ CFU/mL count per group (^ indicates n = 6; * indicates n = 8; a different letter indicates that group was statistically significant from the other groups).

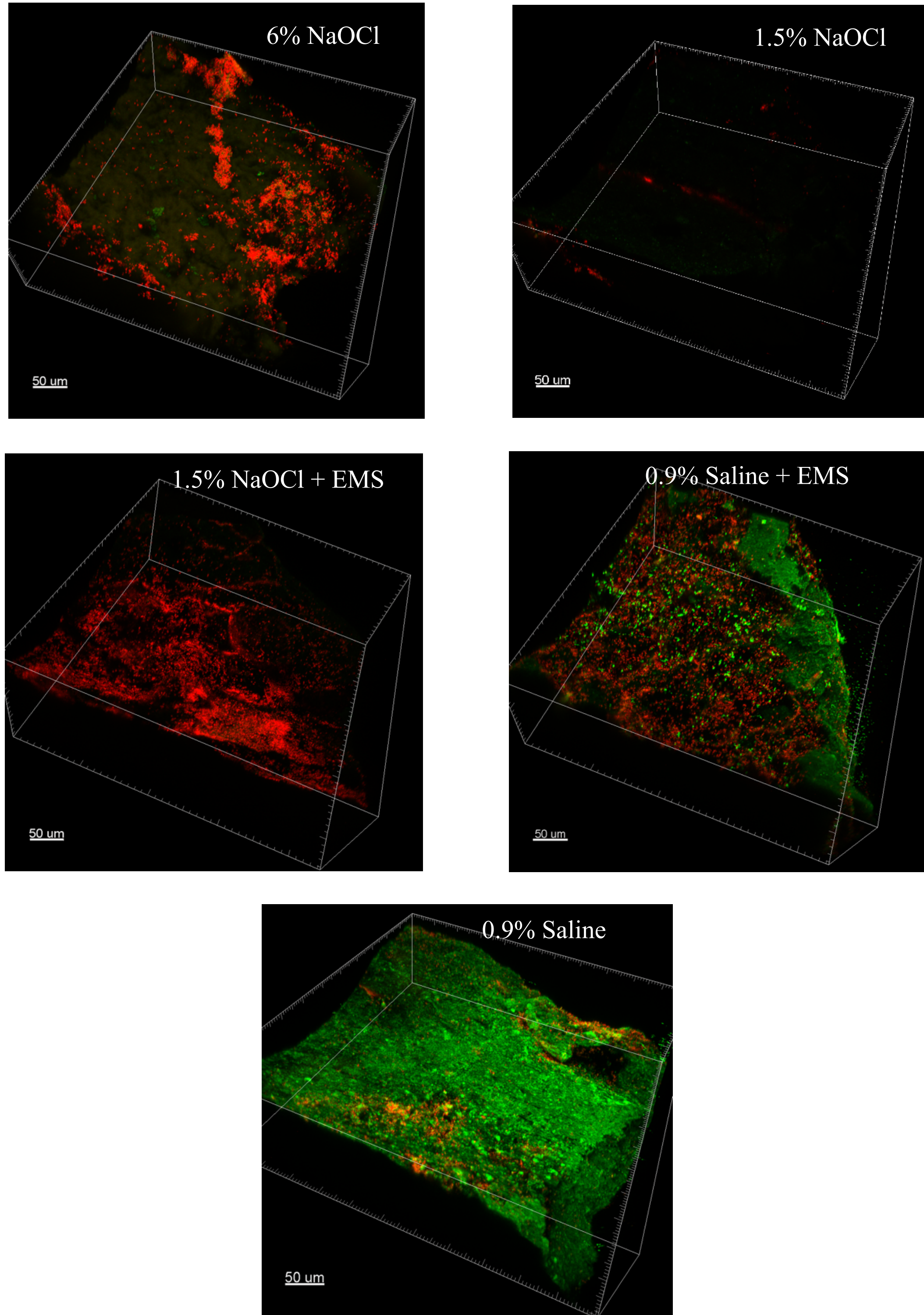
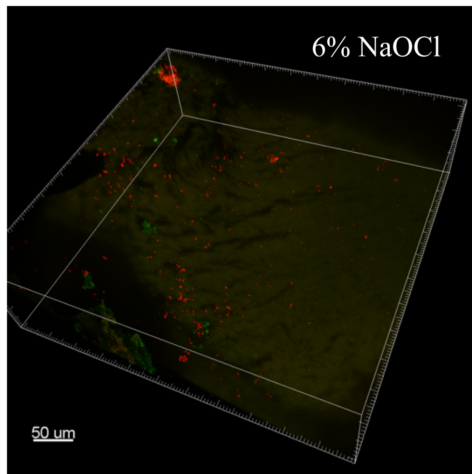
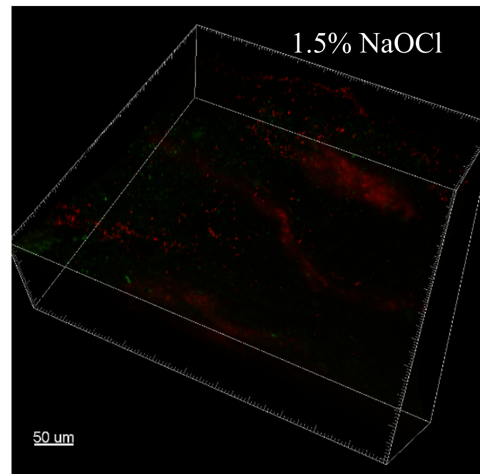
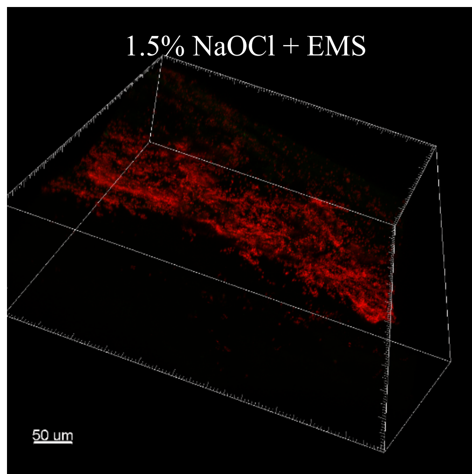


FIGURE 10. Apical 0-mm to 0.5-mm confocal images.







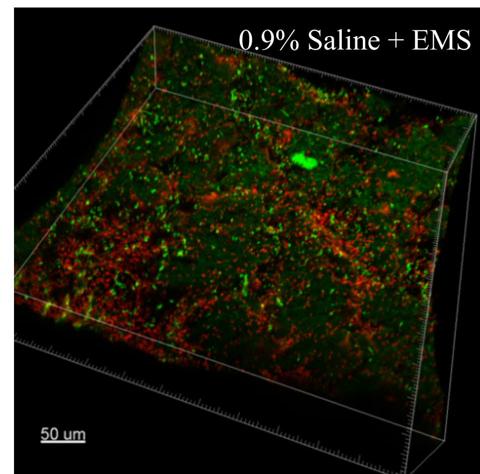
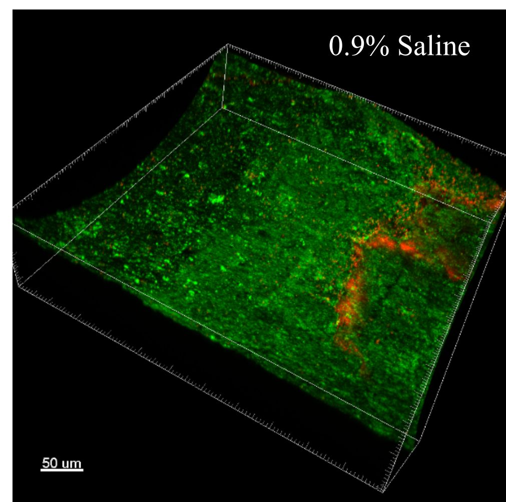


FIGURE 11. Apical 0.5-1.0 mm confocal images.



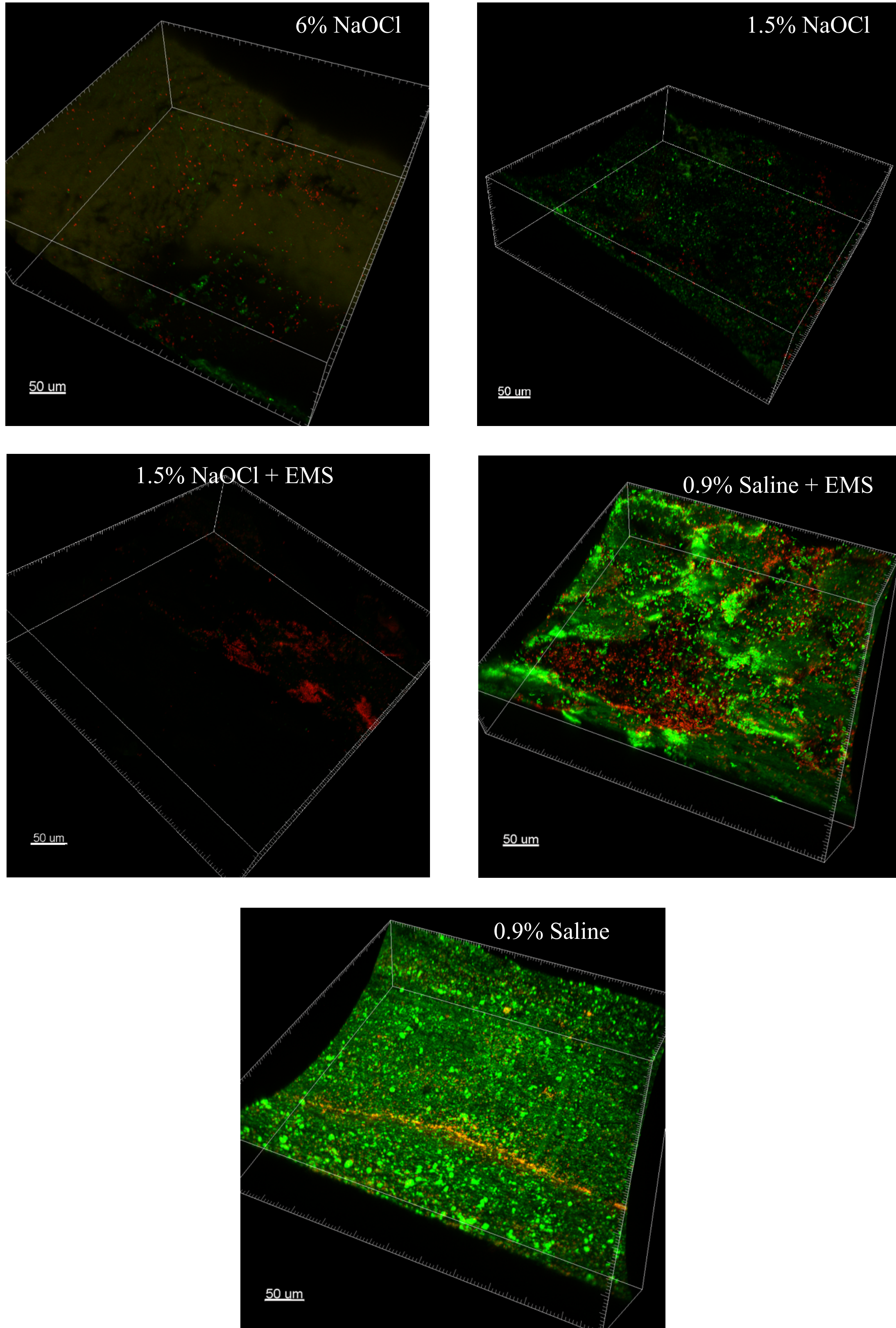


FIGURE 12. Apical 1.0-mm to 1.5-mm confocal images.

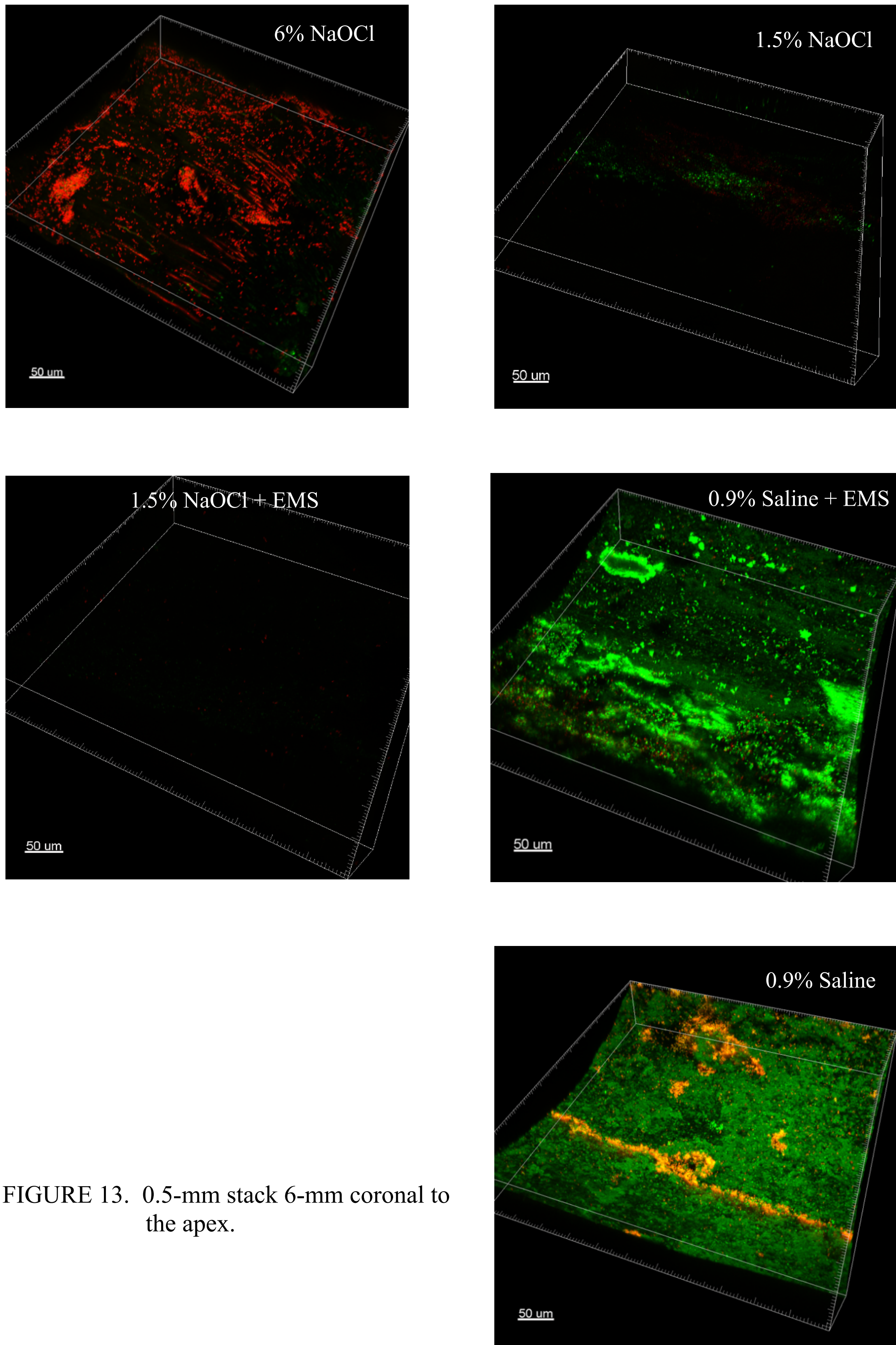


FIGURE 13. 0.5-mm stack 6-mm coronal to the apex.

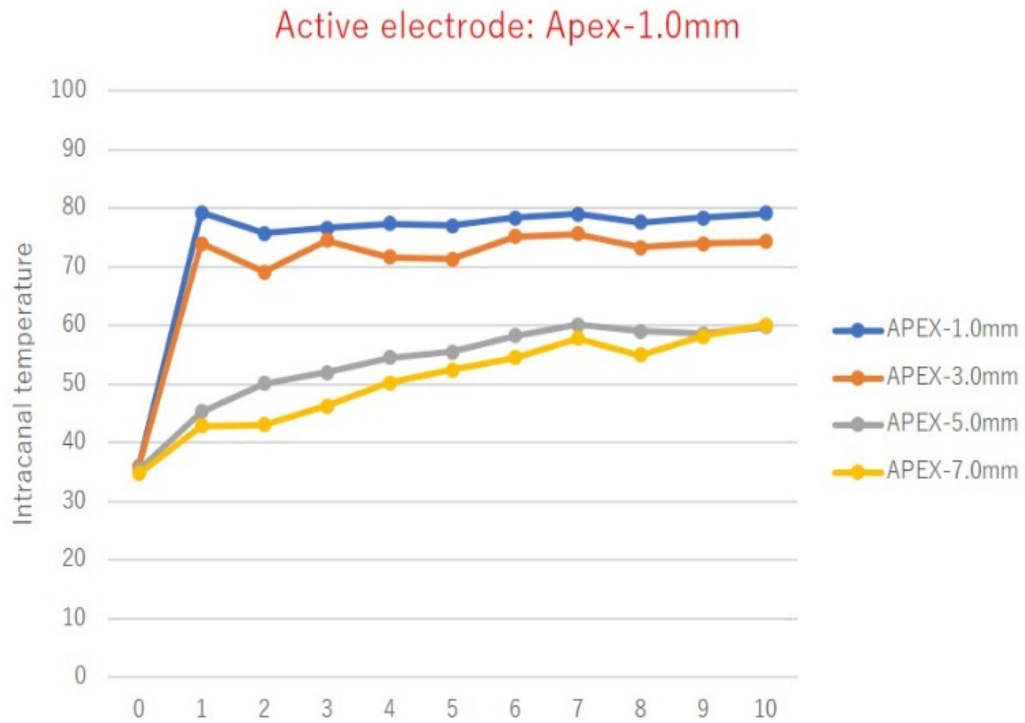


FIGURE 15. Intracanal temperature rises of mandibular incisors after 1 second EMS activation at various depths from the apex NOTE: y-axis unit is degrees Celsius; x-axis unit is the number of times EMS activated for 1 second (Courtesy of Dr. Tominaga, International Society for Electromagnetic Dentistry).

TABLE I

Log_{10} CFU/mL counts as a function of treatment rendered

Group	*N*	*Mean*	*SD*	*SE*	*Median*	*IQR*		*Min*	*Max*
6% NaOCl	6	0	0	0	0	0	0	0	0
1.5% NaOCl	6	0	0	0	0	0	0	0	0
1.5% NaOCl + EMS	6	0	0	0	0	0	0	0	0
0.9% Saline + EMS	8	2.14	1.63	0.58	2.45	0.75	2.99	0.00	4.79
0.9% Saline	6	5.60	0.17	0.07	5.63	5.52	5.67	5.31	5.83

SD= Standard Deviation

SE= Standard Error

IQR=Interquartile Rating

TABLE II

Statistical significance of differences seen in $\log_{10}$ CFUs/mL

p-values:	1.5% NaOCl	1.5% NaOCl + EMS	0.9% Saline + EMS	0.9% Saline
6% NaOCl	1	1	0.012*	0.003*
1.5% NaOCl		1	0.012*	0.003*
1.5% NaOCl + EMS			0.012*	0.003*
0.9% Saline + EMS				0.002*

NOTE: Statistical significance was set at $p < 0.05$

DISCUSSION

Based on our results, the J. MORITA prototype device is capable of elucidating an anti-biofilm effect against a 2-week-old biofilm of *E. faecalis.* This is evident as the CFU/mL counts in the saline with EMS group were less than half of what they were in the saline only group. This finding was corroborated with confocal imaging, where there were many more dead or missing cells in the saline with EMS group, whilst the saline only group showed a healthy, intact biofilm. Previous studies measuring the antibacterial effect of the J. MORITA device used planktonic microorganisms[30]; to our knowledge this is the first study that used a biofilm model.

A synergistic reaction between the prototype device and NaOCl could not be determined. This was due to the fact that no colonies grew when root canals were irrigated with 1.5-percent or 6.0-percent NaOCl. Previous studies have shown eradication of an *E. faecalis* biofilm with as low as 0.000625-percent NaOCl in one minute.[215,216] Other studies have found 2.5-percent NaOCl incapable of eradicating *E. faecalis* biofilms with as much as 40 minutes of contact time.[216] These differences are likely explained by study methodologies; it stands to reason, that with the high potential for shear forces in such straight and wide canals, as well as much higher concentrations of NaOCl being used, even a 2-week-old biofilm would be eradicated. Future studies should consider utilizing lower concentrations of NaOCl, or perhaps using contact time rather than passive irrigation as the method for measuring solution delivery. For instance, rather than gentle irrigation with a set volume of irrigant, which will create shear forces, the canal could be filled with selected irrigant and allowed to sit for a predetermined

amount of time before EMS activation takes place. This type of comparison would make a synergistic or additive effect more readily determinable.

The purpose of the confocal images was to provide a visual confirmation of what the CFU counts would tell about the antibiofilm effects of the various treatment modalities. In all images in which NaOCl was used as the irrigant, there was either a mass of red cells, indicating cell death, or a large area or areas of black space, indicating removal of dead cells during the staining phase or during treatment with NaOCl. In addition to the effects seen with saline alone, we can see that the remaining cells in the 1.5-percent NaOCl + EMS samples fluoresced a very intense red, indicating a high PI to nucleic acid ratio. The cells in the 1.5-percent NaOCl group, however, do not appear and the ones that are fluoresced display a lower intensity of red. If we assume the 1.5-percent NaOCl samples washed out during treatment and not staining, this could indicate an enhancement of NaOCl (or one of its byproducts) uptake by outer membrane damage from the EMS treatment. However, this is difficult to confirm since both groups were irrigated with the same amount of NaOCl for the same amount of time prior to EMS treatment. One explanation could be that the author used a more forceful irrigation pattern in the 1.5-percent NaOCl only group as compared to the 1.5-percent NaOCl + EMS group, which is plausible, but unlikely. An alternative explanation is that NaOCl was highly effective in all scenarios and some damaged/dead cells were washed away during staining of certain samples. This could be explained by a simple bump on the table by a researcher/bystander or a jerky movement of the imaging table under the microscope. Confirmation of EMS's enhancement of NaOCL could have been more easily attained had more cells remained viable in the 1.5-percent NaOCl group.

This study used a monospecies biofilm of *E. faecalis* which grew for 2 weeks. Endodontic infections are polymicrobial in nature.[215] Owing to greater genetic diversity, polymicrobial biofilms are more resistant to environmental stresses. As oral biofilms mature, they can better establish themselves; in the case of a polymicrobial biofilm community, anaerobic bacteria can reside deeper, making them more difficult to eradicate.[217] Follow up studies should assess the efficacy of EMS on a mature, polymicrobial biofilm.

As previously stated, the mechanism by which electric current exerts its effects on biofilms is currently unknown, but most theories involve increased uptake of antimicrobials by biofilm cells.[203] Since EMS exhibits an antibiofilm effect in 0.9-percent saline, perhaps its effect is due to a local generation of ions or oxygen, which disrupts or kills biofilm cells. Such an explanation is purely speculation, however. Alternatively, a localized generation of heat could have been responsible for the effects seen. In the *in-vitro* study on planktonic bacteria, the solution's temperature increased by 4-5°C with each activation.[30] However, in unpublished data by the International Society for Electromagnetic Dentistry, intracanal temperature rose as much as 45°C when EMS was used at 1mm from the apex (Figure 15); the rise was less dramatic farther back from working length likely due to an increase in canal diameter and the amount of solution present. The presence of more fluid would allow heat to dissipate, resulting in a smaller increase in heat. Standardized strains of *E. faecalis* have been shown to be susceptible to temperatures from 65-80°C for 1 min to 10 min, but clinical strains have also been shown to be resistant to 80°C for as long as 3 minutes.[218] Although another potential explanation for the effects seen in the present study, susceptibility of the *E. faecalis*

biofilm to rises in temperature appears to be related to the particular strain being studied, with lab strains being more susceptible than wild types; therefore, if heat is the responsible factor, clinical applications of EMS for bactericidal purposes without the addition of an antimicrobial may be limited.

Not much is known about the effects that local electromagnetic current and its associated heat increase will have on host cells, such as osteoblasts, dental pulp stem cells, cells of the apical papilla, fibroblasts, periodontal ligament, etc. In rat calvaria, upregulation of osteoblast proliferation as well as an increase in growth factors necessary for bone mineralization were noted.[47] Clinically, periapical lesions in bone were found to heal up to 4 times faster when treated with the prototype device as compared to control.[32] Follow-up studies should therefore examine these effects on other cell lines such as stem cells and cells of the periodontal ligament.

In a study examining the effects of tobramycin on a *Pseudomonas aeruginosa* biofilm, the authors applied a 2-mA current to the biofilm in the presence of tobramycin and found a significant increase in bacterial killing over biofilms injected with oxygen and tobramycin or a control in which tobramycin was used alone. The bioelectric effect could not be explained away by a change in pH, temperature increase, or disruption of the biofilm extracellular matrix by the addition of gas.[207] In regenerative endodontic procedures (REPs), the most commonly used antibiotics are TAP or DAP, which contain ciprofloxacin, metronidazole, and/or clindamycin or minocycline, depending on the clinical use or clinician's preference.[219] Given the increase in bactericidal activity seen in the previously mentioned study on *P. aeruginosa*, follow up studies with EMS should test the effects when used with TAP or DAP to determine if there are potential uses in REPs

or if these antibacterials are sufficient for use in non-surgical root canal therapy.

Based on the confocal images obtained, the present insulation design may result in a limited zone of effect with EMS. At the apex and at 6 mm back from the apex, two locations directly affected by activation, more bacterial killing is visualized in the form of red cells or black space than at 1 mm or 1.5 mm coronal to the apex. Follow-up studies should modify file insulation design to see if the EMS effect can be spread more evenly throughout the canal. For instance, horizontal slits could be placed in the insulation material every 1 mm to 2 mm to increase the area affected. This may also result in less need for multiple activations.

The main limitations of the study include the inability to determine what, if any synergistic effect is seen with EMS and NaOCl, the small sample size, the small stack sizes for confocal imaging, a lack of statistical analysis of the confocal images, and the inability to directly measure its effects *in vivo*. The reasons for inability to detect any synergism have already been explored. As for sample size, although small, the power to detect the observed differences were very high – ranging from 89 percent to 99 percent depending on which groups were being compared. The confocal stack sizes were small out of necessity. When a pilot study was initiated without a biofilm to determine how large the stack sizes should be and how long it would take to image, we calculated it would take 23 hours to image all 12 mm of one root half. This time would certainly increase if a biofilm had been present. When enough data points are taken, statistical analyses of biofilms can be taken from confocal images; this includes biofilm thickness, width, and volume as well as number of cells and the ratio of live to dead/damaged cells. This requires several points in a given sample, and they must be repeated. In addition,

there are relatively few programs available for this type of analysis. Again, time was certainly a factor in deciding whether to take enough images for statistical analysis, but future studies would benefit from numerically analyzable confocal data. Finally, certain *in vivo* characteristics may affect the current flow of EMS, such as dentin thickness, canal diameter, and amount of solution present. All of these variables must remain standardized to determine the actual effects of EMS, so clinical results may differ from what is found during a laboratory experiment.

SUMMARY AND CONCLUSIONS

The findings of this study suggest that the use of EMS with saline has an antibiofilm effect against *E. faecalis* when compared with irrigation with saline alone. This effect was not as great as irrigation with 6.0-percent NaOCl. Furthermore, since there was no bacterial growth in all groups in which NaOCl was used, a synergistic effect cannot be determined. Therefore, the null hypothesis that EMS used with 1.5-percent NaOCl would have an antibiofilm effect similar to irrigation with 6.0-percent NaOCl alone cannot be rejected. However, we accept the alternative hypothesis that 0.9-percent saline used with EMS would have a greater antibiofilm effect than 0.9-percent saline alone. Follow-up studies should focus on utilizing lower concentrations of NaOCl, consider other disinfectants such as CHX, TAP or DAP, modify file insulation designs, or examine the effects on stem cells, osteoblasts or cells of the periodontal ligament. At present, the most applicable clinical use for EMS may be its ability to expedite bone healing as already proven via clinical studies.

REFERENCES

1. Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg Oral Med Oral Pathol 1965;20:340-349.

2. Lin LM, Pascon EA, Skribner J, Gangler P, Langeland K. Clinical, radiographic, and histologic study of endodontic treatment failures. Oral Surg Oral Med Oral Pathol 1991;71(5):603-611.

3. Zehnder M. Root canal irrigants. J Endod 2006;32(5):389-398.

4. Kandaswamy D, Venkateshbabu N. Root canal irrigants. J Conserv Dent 2010;13(4):256-264.

5. Haapasalo M, Shen Y, Qian W, Gao Y. Irrigation in endodontics. Dent Clin North Am 2010;54(2):291-312.

6. Martin DE, De Almeida JF, Henry MA, Khaing ZZ, Schmidt CE, Teixeira FB, et al. Concentration-dependent effect of sodium hypochlorite on stem cells of apical papilla survival and differentiation. J Endod 2014;40(1):51-55.

7. Diogenes AR, Ruparel NB, Teixeira FB, Hargreaves KM. Translational science in disinfection for regenerative endodontics. J Endod 2014;40(4):S52-S57.

8. Siqueira JF, Jr., Rocas IN, Favieri A, Lima KC. Chemomechanical reduction of the bacterial population in the root canal after instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite. J Endod 2000;26(6):331-334.

9. Harrison JW, Hand RE. The effect of dilution and organic matter on the antibacterial property of 5.25% sodium hypochlorite. J Endod 1981;7(3):128-132.

10. Yesilsoy C, Whitaker E, Cleveland D, Phillips E, Trope M. Antimicrobial and toxic effects of established and potential root canal irrigants. J Endod 1995;21(10):513-515.

11. Bosch-Aranda ML, Canalda-Sahli C, Figueiredo R, Gay-Escoda C. Complications following an accidental sodium hypochlorite extrusion. J Clin Exp Dent 2012;4(3):e194-198.

12. Caliskan MK, Turkun M, Alper S. Allergy to sodium hypochlorite during root canal therapy: a case report. Int Endod J 1994;27(3):163-167.

13. Kaufman AY, Keila S. Hypersensitivity to sodium hypochlorite. J Endod 1989;15(5):224-226.

14. Hulsmann M, Hahn W. Complications during root canal irrigation--literature review and case reports. Int Endod J 2000;33(3):186-193.

15. Guivarc'h M, Ordioni U, Ahmed HM, Cohen S, Catherine JH, Bukiet F. Sodium hypochlorite accident: A systematic review. J Endod 2017;43(1):16-24.

16. Bitter K, Vlassakidis A, Niepel M, Hoedke D, Schulze J, Neumann K, et al. Effects of diode laser, gaseous ozone, and medical dressings on Enterococcus faecalis biofilms in the root canal ex vivo. Biomed Res Int 2017;2017:1-9.

17. Poggio C, Arciola CR, Dagna A, Florindi F, Chiesa M, Saino E, et al. Photoactivated disinfection (PAD) in endodontics: an in vitro microbiological evaluation. Int J Artif Organs 2011;34(9):889-897.

18. Vaziri S, Kangarlou A, Shahbazi R, Nazari Nasab A, Naseri M. Comparison of the bactericidal efficacy of photodynamic therapy, 2.5% sodium hypochlorite, and 2% chlorhexidine against Enterococcous faecalis in root canals; an in vitro study. Dent Res J (Isfahan) 2012;9(5):613-618.

19. Xu Y, Young MJ, Battaglino RA, Morse LR, Fontana CR, Pagonis TC, et al. Endodontic antimicrobial photodynamic therapy: Safety assessment in mammalian cell cultures. J Endod 2009;35(11):1567-1572.

20. Menezes MM, Valera MC, Jorge AO, Koga-Ito CY, Camargo CH, Mancini MN. In vitro evaluation of the effectiveness of irrigants and intracanal medicaments on microorganisms within root canals. Int Endod J 2004;37(5):311-319.

21. Genc Sen O, Kaya M. Comparative safety of needle, EndoActivator, and laser-activated irrigation in overinstrumented root canals. Photomed Laser Surg 2017.

22. Karatas E, Ozsu D, Arslan H, Erdogan AS. Comparison of the effect of nonactivated Self-Adjusting File system, Vibringe, EndoVac, ultrasonic and needle irrigation on apical extrusion of debris. Int Endod J 2015;48(4):317-322.

23. Iriboz E, Bayraktar K, Turkaydin D, Tarcin B. Comparison of apical extrusion of sodium hypochlorite using 4 different root canal irrigation techniques. J Endod 2015;41(3):380-384.

24. Rodriguez-Figueroa C, McClanahan SB, Bowles WR. Spectrophotometric determination of irrigant extrusion using passive ultrasonic irrigation, EndoActivator, or

syringe irrigation. J Endod 2014;40(10):1622-1626.

25. Mitchell RP, Baumgartner JC, Sedgley CM. Apical extrusion of sodium hypochlorite using different root canal irrigation systems. J Endod 2011;37(12):1677-1681.

26. Mitchell RP, Yang SE, Baumgartner JC. Comparison of apical extrusion of NaOCl using the EndoVac or needle irrigation of root canals. J Endod 2010;36(2):338-341.

27. Desai P, Himel V. Comparative safety of various intracanal irrigation systems. J Endod 2009;35(4):545-549.

28. Cunningham WT, Joseph SW. Effect of temperature on the bactericidal action of sodium hypochlorite endodontic irrigant. Oral Surg Oral Med Oral Pathol 1980;50(6):569-571.

29. Sirtes G, Waltimo T, Schaetzle M, Zehnder M. The effects of temperature on sodium hypochlorite short-term stability, pulp dissolution capacity, and antimicrobial efficacy. J Endod 2005;31(9):669-671.

30. Yumoto H, Tominaga T, Hirao K, Kimura T, Takahashi K, Sumitomo T, et al. Bactericidal activity and oral pathogen inactivation by electromagnetic wave irradiation. J Appl Microbiol 2012;113(1):181-191.

31. Tominaga T, Yumoto H, Matsuo T. Clinical application of electromagnetic wave irradation for infected root canal treatment—EMAT (electromagnetic apical treatment). J Endod 2012;38(3):e56, PR93.

32. Tominaga T, Yumoto H, Hirao K, Bando N, Takahira K, Matsuo T. EMAT (electromagnetic apical treatment): Clinical application of pulsed electric current energization for root canal treatment. J Endod 2015;40(3):e39, PR75.

33. Neelakantan P, Romero M, Vera J, Daood U, Khan AU, Yan A, et al. Biofilms in endodontics: Current status and future directions. Int J Mol Sci 2017;18(8).

34. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999;284(5418):1318-1322.

35. Mohammadi Z, Dummer PM. Properties and applications of calcium hydroxide in endodontics and dental traumatology. Int Endod J 2011;44(8):697-730.

36. Ertugrul IF, Maden M, Orhan EO, Ozkorucuklu SP. The effect of micro-electric

current and other activation techniques on dissolution abilities of sodium hypochlorite in bovine tissues. BMC Oral Health 2015;15:161.

37. Ertugrul IF, Maden M, Orhan EO, Ozkorucuklu SP, Aglarca AV. Rapid tissue dissolution efficiency of electrically-activated sodium hypochlorite on bovine muscle. Eur J Dent 2014;8(4):464-468.

38. Cumbo E, Russo R, Gallina G. System B, Endo-Twinn and E-Fill. True temperatures inside the canal. J Conserv Dent 2012;15(4):342-345.

39. Lipski M. Root surface temperature rises in vitro during root canal obturation using hybrid and microseal techniques. J Endod 2005;31(4):297-300.

40. Lipski M. In vitro infrared thermographic assessment of root surface temperatures generated by high-temperature thermoplasticized injectable gutta-percha obturation technique. J Endod 2006;32(5):438-441.

41. Viapiana R, Guerreiro-Tanomaru JM, Tanomaru-Filho M, Camilleri J. Investigation of the effect of sealer use on the heat generated at the external root surface during root canal obturation using warm vertical compaction technique with System B heat source. J Endod 2014;40(4):555-561.

42. Rocas IN, Jung IY, Lee CY, Siqueira JF, Jr. Polymerase chain reaction identification of microorganisms in previously root-filled teeth in a South Korean population. J Endod 2004;30(7):504-508.

43. Haapasalo M, Udnæs T, Endal U. Persistent, recurrent, and acquired infection of the root canal system post-treatment. Endodontic Topics 2003;6(1):29-56.

44. Peciuliene V, Balciuniene I, Eriksen HM, Haapasalo M. Isolation of Enterococcus faecalis in previously root-filled canals in a Lithuanian population. J Endod 2000;26(10):593-595.

45. Estrela C, Sydney GB, Figueiredo JAP, Estrela CRA. A model system to study antimicrobial strategies in endodontic biofilms. J Appl Oral Sci 2009;17(2):87-91.

46. Sato T, Sugaya T, Kudo M, Nakatani M, Maeda N, Kawanami M, et al. Effects of high-frequency electromagnetic wave stimulation on bone repair in rat calvaria defects. J Oral Tissue Engineering 2016;14(1):59-64.

47. Yumoto H, Hirao K, Tominaga T, Bando N, Takahashi K, Matsuo T. Electromagnetic wave irradiation promotes osteoblastic cell proliferation and up-regulates growth factors via activation of the ERK1/2 and p38 MAPK pathways. Cell

Physiol Biochem 2015;35(2):601-615.

48. Gerabek WE. The tooth-worm: historical aspects of a popular medical belief. Clinical Oral Investigations 1999;3(1):1-6.

49. Forrai J. The beginnings of dental caries and its treatments. Rev Clin Pesq Odontol 2009;5(2):187-192.

50. Castellucci A. A brief history of endodontics. In: Castellucci A, editor. Endodontics. 1 ed. Prato, Italy: Il Tridente Edizioni Odontoiatriche; 2004. p. 2-5.

51. Ring ME. Anton van Leeuwenhoek and the tooth-worm. J Am Dent Assoc 1971;83(5):999-1001.

52. Cruse WP, Bellizzi R. A historic review of endodontics, 1689-1963, part 1. J Endod 1980;6(3):495-499.

53. Curson I. History and endodontics. Dent Pract Dent Rec 1965;15:435-439.

54. Francke OC. Capping of the living pulp: from Philip Pfaff to John Wessler. Bull Hist Dent 1971;19(2):17-23.

55. Farley JR. Brief history of endodontics. Tex Dent J 1974;92(2):9.

56. Koch C. History of dental surgery. Chicago: National Arts Publishing Company; 1909.

57. Anthony LP, Grossman LI. A brief history of root-canal therapy in the United States. J Am Dent Assoc 1945;32(1):43-50.

58. Monica W. Outline of dental history. Hackensack, NJ: Fairleigh Dickinson University Dental School; 1972.

59. Denton G, Zachariou C. The history of vitalism in pulp treatment. Dent Cosmos 1931;73:267-273.

60. Ostrander FD. The practice of endodontics: past, present, and future. J Dent Ed 1967;31(3):386-388.

61. Lightfoot Jr. V. A brief history of root canal therapy. Dent Student 1955;33(9):11-17, 42.

62. Tagger M. Endodontics: a review of the past and is present status. Alpha Omegan 1967;60(2):107-118.

63. Cruse WP, Bellizzi R. A historic review of endodontics, 1689-1963, part 2. J Endod 1980;6(4):532-535.

64. Cohn C. A brief history of root canal therapy. Dent Outlook 1938;25:20-27.

65. Ottolengui R. A review of the life work of J.R. Callahan. Pacific Dent Gazette 1920;28:260-276.

66. Coolidge ED. Past and present concepts in endodontics. J Am Dent Assoc 1960;61:676-688.

67. Prinz H. Electro-sterilization of root-canals. Dent Cosmos 1917;59(4):373-394.

68. Grossman L. An electrolyzer for use in electro-sterilization. Dent Cosmos 1929;71(7):708-710.

69. Appleton J, Grossman L. A study in experimental and applied electro-sterilization. J Dent Res 1932;12(2):311-319.

70. Grossman LI. Pioneers in endodontics. J Endod 1987;13(8):409-415.

71. Grossman LI. A brief history of endodontics. J Endod 1982;8:S36-S40.

72. Callahan J. Rosin solution in root filling. J Allied Dent Soc 1914;9:53-63.

73. Grossman LI. Endodontics: A peep into the past and the future. Oral Surg Oral Med Oral Pathol 1974;37(4):599-608.

74. Jacobsohn PH, Fedran RJ. Making darkness visible: the discovery of X-ray and its introduction to dentistry. J Am Dent Assoc 1995;126(10):1359-1367.

75. JD M. Dental and oral radiography. St. Louis: The C.V. Mosby Company; 1923.

76. Mazzola PV. Early reports of X-ray dangers. Bull Hist Dent 1974:31-34.

77. Weine F. Endodontic Therapy. St. Louis: C.V. Mosby Co.; 1972.

78. Francke OC. William Hunter's "Oral Sepsis" and American odontology. Bull Hist Dent 1973;21(2):73-79.

79. Bellizzi R, Cruse WP. A historic review of endodontics, 1689-1963, part 3. J Endod 1980;6(5):576-580.

80. Duke W. Oral sepsis in its relationship to systemic disease. St. Louis: The C.V. Mosby Company; 1918.

81. EM H. Resume of root-canal problems and a practical aseptic technique. J Am Dent Assoc 1922;9:939-945.

82. Ingle J. Endodontics. Philadelphia: Lea & Febiger; 1965.

83. Sommer R, et al. Clinical endodontics. Philadelphia: W.B. Saunders Co.; 1966.

84. Wolfsohn BL. Endodontia and tooth conservation. J Calif State Dent Assoc 1949;25(3):105-108.

85. Grossman LI. A personal history of dental practice: 1920-1980. J Am Dent Assoc 1981;102(3):360-365.

86. Milas V. A history of the American Association of Endodontists 1943-1968.

87. Peters O, Peters C, Basrani B. Cleaning and shaping the root canal system. In: Hargreaves K, Berman L, editors. Cohen's pathways of the pulp. 11th ed. St. Louis: CV Mosby; 2016. p. 209-279.

88. Cotton TP, Geisler TM, Holden DT, Schwartz SA, Schindler WG. Endodontic applications of cone-beam volumetric tomography. J Endod 2007;33(9):1121-1132.

89. Fayad M, Azevedo B, Rubinstein R. Cone beam computed tomography in treatment planning of periapical surgery. In: Torabinejad M, Rubinstein R, editors. The art and science of contemporary surgical endodontics. Hanover Park: Quintessence Publishing Company Incorporated; 2017. p. 97-112.

90. Diogenes A, Simon S, Law A. Regenerative endodontics. In: Hargreaves K, Berman L, editors. Cohen's Pathways of the pulp. 11th ed. St. Louis: C.V. Mosby; 2016. p. 447-473.

91. Hosoya S, Matsushima K. Stimulation of interleukin-1 beta production of human dental pulp cells by Porphyromonas endodontalis lipopolysaccharide. J Endod 1997;23(1):39-42.

92. Nakanishi T, Matsuo T, Ebisu S. Quantitative analysis of immunoglobulins and inflammatory factors in human pulpal blood from exposed pulps. J ndod1995;21(3):131-136.

93. Caviedes-Bucheli J, Arenas N, Guiza O, Moncada NA, Moreno GC, Diaz E, et al. Calcitonin gene-related peptide receptor expression in healthy and inflamed human pulp tissue. Int Endod J 2005;38(10):712-717.

94. Caviedes-Bucheli J, Muñoz HR, Azuero-Holguín MM, Ulate E. Neuropeptides in dental pulp: The silent protagonists. J Endod 2008;34(7):773-788.

95. Dahlen G, Bergenholtz G. Endotoxic activity in teeth with necrotic pulps. J Dent Res 1980;59(6):1033-1040.

96. Horiba N, Maekawa Y, Abe Y, Ito M, Matsumoto T, Nakamura H. Correlations between endotoxin and clinical symptoms or radiolucent areas in infected root canals. Oral Surg Oral Med Oral Pathol 1991;71(4):492-495.

97. Schein B, Schilder H. Endotoxin content in endodontically involved teeth. J Endod 1975;1(1):19-21.

98. Langeland K. Tissue response to dental caries. Endod Dent Traumatol 1987;3(4):149-171.

99. Lin L, Langeland K. Light and electron microscopic study of teeth with carious pulp exposures. Oral Surg Oral Med Oral Pathol 1981;51(3):292-316.

100. Seltzer S, Bender IB, Nazimov H, Sinai I. Pulpitis-induced interradicular periodontal changes in experimental animals. J Periodontol 1967;38(2):124-129.

101. Dwyer TG, Torabinejad M. Radiographic and histologic evaluation of the effect of endotoxin on the periapical tissues of the cat. J Endod 1981;7(1):31-35.

102. Marton IJ, Kiss C. Overlapping protective and destructive regulatory pathways in apical periodontitis. J Endod 2014;40(2):155-163.

103. Schonfeld SE, Greening AB, Glick DH, Frank AL, Simon JH, Herles SM. Endotoxic activity in periapical lesions. Oral Surg Oral Med Oral Pathol 1982;53(1):82-87.

104. Hulsmann M, Rummelin C, Schafers F. Root canal cleanliness after preparation with different endodontic handpieces and hand instruments: a comparative SEM investigation. J Endod 1997;23(5):301-306.

105. Johnson W, Noblett W. Cleaning and shaping. In: Torabinejad M, Walton R, editors. Endodontics: principles and practice. St. Louis: Saunders; 2009. p. 258-286.

106. Abbott PV. The periapical space--a dynamic interface. Aust Endod J 2002;28(3):96-107.

107. Stewart GG. The importance of chemomechanical preparation of the root canal. Oral Surg Oral Med Oral Pathol 1955;8(9):993-997.

108. Grossman LI. Rationale of endodontic treatment. Dent Clin North Am 1967:483-490.

109. Schilder H. Filling root canals in three dimensions. Dent Clin North Am 1967:723-744.

110. Goodis HE. Commentary on: Filling root canals in three dimensions. J Endod 2006;32(4):279-280.

111. Ford TRP. Relation between seal of root fillings and tissue response. Oral Surg Oral Med Oral Pathol 1983;55(3):291-294.

112. Aquilino SA, Caplan DJ. Relationship between crown placement and the survival of endodontically treated teeth. J Prosthet Dent 2002;87(3):256-263.

113. Ng YL, Mann V, Gulabivala K. A prospective study of the factors affecting outcomes of non-surgical root canal treatment: part 2: tooth survival. Int Endod J 2011;44(7):610-625.

114. Salehrabi R, Rotstein I. Endodontic treatment outcomes in a large patient population in the USA: an epidemiological study. J Endod 2004;30(12):846-850.

115. Khademi A, Yazdizadeh M, Feizianfard M. Determination of the minimum instrumentation size for penetration of irrigants to the apical third of root canal systems. J Endod 2006;32(5):417-420.

116. Haapasalo M, Endal U, Zandi H, Coil JM. Eradication of endodontic infection by instrumentation and irrigation solutions. 2005;10(1):77-102.

117. Pettiette MT, Delano EO, Trope M. Evaluation of success rate of endodontic treatment performed by students with stainless-steel K-files and nickel-titanium hand files. J Endod 2001;27(2):124-127.

118. Bystrom A, Sundqvist G. Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res 1981;89(4):321-328.

119. Kavanagh D, Lumley PJ. An in vitro evaluation of canal preparation using Profile .04 and .06 taper instruments. Endod Dent Traumatol 1998;14(1):16-20.

120. Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med 2002;13(2):171-183.

121. Peters OA, Laib A, Gohring TN, Barbakow F. Changes in root canal geometry after preparation assessed by high-resolution computed tomography. J Endod 2001;27(1):1-6.

122. Peters OA, Schonenberger K, Laib A. Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. Int Endod J 2001;34(3):221-230.

123. Hubscher W, Barbakow F, Peters OA. Root-canal preparation with FlexMaster: canal shapes analysed by micro-computed tomography. Int Endod J 2003;36(11):740-747.

124. McKenna SM, Davies KJ. The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. Biochem J 1988;254(3):685-692.

125. Moorer WR, Wesselink PR. Factors promoting the tissue dissolving capability of sodium hypochlorite. Int Endod J 1982;15(4):187-196.

126. Barrette WC, Jr., Hannum DM, Wheeler WD, Hurst JK. General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. Biochemistry 1989;28(23):9172-9178.

127. Estrela C, Estrela CR, Barbin EL, Spano JC, Marchesan MA, Pecora JD. Mechanism of action of sodium hypochlorite. Braz Dent J 2002;13(2):113-117.

128. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 1999;12(1):147-179.

129. Baca P, Mendoza-Llamas ML, Arias-Moliz MT, Gonzalez-Rodriguez MP, Ferrer-Luque CM. Residual effectiveness of final irrigation regimens on Enteroccus faecalis-infected root canals. J Endod 2011;37(8):1121-1123.

130. Martinho FC, Gomes BP. Quantification of endotoxins and cultivable bacteria in root canal infection before and after chemomechanical preparation with 2.5% sodium hypochlorite. J Endod 2008;34(3):268-272.

131. Weber CD, McClanahan SB, Miller GA, Diener-West M, Johnson JD. The effect of passive ultrasonic activation of 2% chlorhexidine or 5.25% sodium hypochlorite irrigant on residual antimicrobial activity in root canals. J Endod 2003;29(9):562-564.

132. Southard SR, Drisko CL, Killoy WJ, Cobb CM, Tira DE. The effect of 2% chlorhexidine digluconate irrigation on clinical parameters and the level of Bacteroides gingivalis in periodontal pockets. 1989;60(6):302-309.

133. Estrela C, Silva JA, de Alencar AH, Leles CR, Decurcio DA. Efficacy of sodium hypochlorite and chlorhexidine against Enterococcus faecalis--a systematic review. J Appl Oral Sci 2008;16(6):364-368.

134. Hennessey TS. Some antibacterial properties of chlorhexidine. J Periodontal Res Suppl 1973;12:61-67.

135. Hugo WB, Longworth AR. The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of Escherichia coli and Staphylococcus aureus. J Pharm Pharmacol 1966;18(9):569-578.

136. Basrani BR, Manek S, Mathers D, Fillery E, Sodhi RN. Determination of 4-chloroaniline and its derivatives formed in the interaction of sodium hypochlorite and chlorhexidine by using gas chromatography. J Endod 2010;36(2):312-314.

137. Nowicki JB, Sem DS. An in vitro spectroscopic analysis to determine the chemical composition of the precipitate formed by mixing sodium hypochlorite and chlorhexidine. J Endod 2011;37(7):983-988.

138. Torabinejad M, Handysides R, Khademi AA, Bakland LK. Clinical implications of the smear layer in endodontics: a review. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2002;94(6):658-666.

139. Shahravan A, Haghdoost AA, Adl A, Rahimi H, Shadifar F. Effect of smear layer on sealing ability of canal obturation: a systematic review and meta-analysis. J Endod 2007;33(2):96-105.

140. Calt S, Serper A. Time-dependent effects of EDTA on dentin structures. J Endod 2002;28(1):17-19.

141. Niu W, Yoshioka T, Kobayashi C, Suda H. A scanning electron microscopic

study of dentinal erosion by final irrigation with EDTA and NaOCl solutions. Int Endod J 2002;35(11):934-939.

142. Chugal NM, Clive JM, Spangberg LS. Endodontic infection: some biologic and treatment factors associated with outcome. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003;96(1):81-90.

143. Schaeffer MA, White RR, Walton RE. Determining the optimal obturation length: a meta-analysis of literature. J Endod 2005;31(4):271-274.

144. Trope M. Treatment of the immature tooth with a non–vital pulp and apical periodontitis. Dent Clin 2010;54(2):313-324.

145. Hargreaves KM, Diogenes A, Teixeira FB. Treatment options: biological basis of regenerative endodontic procedures. Pediatr Dent 2013;35(2):129-140.

146. Waterhouse P, Whitworth J. Pediatric endodontics: Endodontic treatment for the primary and young permanent dentition. In: Hargreaves K, Berman L, editors. Cohen's Pathways of the Pulp. 11th ed. St. Louis: Mosby; 2016.

147. Cvek M. A clinical report on partial pulpotomy and capping with calcium hydroxide in permanent incisors with complicated crown fracture. J Endod 1978;4(8):232-237.

148. Asgary S, Eghbal MJ, Parirokh M, Ghanavati F, Rahimi H. A comparative study of histologic response to different pulp capping materials and a novel endodontic cement. Oral Surg Oral Med Oral Pathol 2008;106(4):609-614.

149. Lenherr P, Allgayer N, Weiger R, Filippi A, Attin T, Krastl G. Tooth discoloration induced by endodontic materials: a laboratory study. Int Endod J 2012;45(10):942-949.

150. Frank AL. Therapy for the divergent pulpless tooth by continued apical formation. J Am Dent Assoc 1966;72(1):87-93.

151. Steiner JC, Van Hassel HJ. Experimental root apexification in primates. Oral Surg Oral Med Oral Pathol 1971;31(3):409-415.

152. Steiner J. Inducing root end closure of non-vital permanent teeth. J Dent child 1968;35:47-54.

153. Ham JW, Patterson SS, Mitchell DF. Induced apical closure of immature pulpless teeth in monkeys. Oral Surg Oral Med Oral Pathol 1972;33(3):438-449.

154. Andreasen JO, Farik B, Munksgaard EC. Long-term calcium hydroxide as a root canal dressing may increase risk of root fracture. Dent Traumatol 2002;18(3):134-137.

155. Doyon GE, Dumsha T, von Fraunhofer JA. Fracture resistance of human root dentin exposed to intracanal calcium hydroxide. J Endod 2005;31(12):895-897.

156. Rosenberg B, Murray PE, Namerow K. The effect of calcium hydroxide root filling on dentin fracture strength. Dent Traumatol 2007;23(1):26-29.

157. Yassen GH, Platt JA. The effect of nonsetting calcium hydroxide on root fracture and mechanical properties of radicular dentine: A systematic review. Int Endod J 2013;46(2):112-118.

158. Simon S, Rilliard F, Berdal A, Machtou P. The use of mineral trioxide aggregate in one-visit apexification treatment: a prospective study. Int Endod J 2007;40(3):186-197.

159. Valois CR, Costa ED, Jr. Influence of the thickness of mineral trioxide aggregate on sealing ability of root-end fillings in vitro. Oral Surg Oral Med Oral Pathol 2004;97(1):108-111.

160. Torabinejad M, Hong CU, McDonald F, Pitt Ford TR. Physical and chemical properties of a new root-end filling material. J Endod 1995;21(7):349-353.

161. Shabahang S, Torabinejad M, Boyne PP, Abedi H, McMillan P. A comparative study of root-end induction using osteogenic protein-1, calcium hydroxide, and mineral trioxide aggregate in dogs. J Endod 1999;25(1):1-5.

162. Witherspoon DE, Ham K. One-visit apexification: technique for inducing root-end barrier formation in apical closures. Pract Proced Aesthet Dent 2001;13(6):455-460.

163. Holden DT, Schwartz SA, Kirkpatrick TC, Schindler WG. Clinical outcomes of artificial root-end barriers with mineral trioxide aggregate in teeth with immature apices. J Endod 2008;34(7):812-817.

164. Ostby BN. The role of the blood clot in endodontic therapy: An experimental histologic study. Acta Odontol Scand 1961;19:324-353.

165. Langer R, Vacanti JP, Vacanti CA, Atala A, Freed LE, Vunjak-Novakovic G. Tissue engineering: biomedical applications. Tissue Eng 1995;1(2):151-161.

166. AAE. Considerations for regenerative procedures. 2013 [cited 4/5/2019]; Available from: http://www.aae.org/uploadedFiles/Clinical_Resources/Regenerative_Endodontics/considerationsregendo7-31-13.pdf

167. Iwaya SI, Ikawa M, Kubota M. Revascularization of an immature permanent tooth with apical periodontitis and sinus tract. Dent Traumatol 2001;17(4):185-187.

168. Banchs F, Trope M. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? J Endod 2004;30(4):196-200.

169. Galler KM, D'Souza RN, Federlin M, Cavender AC, Hartgerink JD, Hecker S, et al. Dentin conditioning codetermines cell fate in regenerative endodontics. J Endod 2011;37(11):1536-1541.

170. Nevins AJ, Finkelstein F, Borden BG, Laporta R. Revitalization of pulpless open apex teeth in rhesus monkeys, using collagen-calcium phosphate gel. J Endod 1976;2(6):159-165.

171. Bose R, Nummikoski P, Hargreaves K. A retrospective evaluation of radiographic outcomes in immature teeth with necrotic root canal systems treated with regenerative endodontic procedures. J Endod 2009;35(10):1343-1349.

172. Jeeruphan T, Jantarat J, Yanpiset K, Suwannapan L, Khewsawai P, Hargreaves KM. Mahidol study 1: comparison of radiographic and survival outcomes of immature teeth treated with either regenerative endodontic or apexification methods: a retrospective study. J Endod 2012;38(10):1330-1336.

173. Kahler B, Mistry S, Moule A, Ringsmuth AK, Case P, Thomson A, et al. Revascularization outcomes: a prospective analysis of 16 consecutive cases. J Endod 2014;40(3):333-338.

174. Yoo YJ, Perinpanayagam H, Oh S, Kim AR, Han SH, Kum KY. Endodontic biofilms: contemporary and future treatment options. Restor Dent Endod 2019;44(1):e7.

175. Siqueira J, Rocas I. Microbiology of endodontic infections. In: Hargreaves K, Berman L, editors. Cohen’s Pathways of the pulp. 11th ed. St. Louis: Mosby; 2016. p. 599-629.

176. Fabricius L, Dahlen G, Ohman AE, Moller AJ. Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure. Scand J Dent Res 1982;90(2):134-144.

177. Siqueira JF, Jr. Endodontic infections: concepts, paradigms, and perspectives. Oral Surg Oral Med Oral Pathol 2002;94(3):281-293.

178. Hotokezaka H, Sakai E, Ohara N, Hotokezaka Y, Gonzales C, Matsuo K, et al. Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF-alpha, lipopolysaccharide, or peptidoglycan. J Cell Biochem 2007;101(1):122-134.

179. Kumar A, Zhang J, Yu FS. Innate immune response of corneal epithelial cells to Staphylococcus aureus infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion. Invest Ophthalmol Vis Sci 2004;45(10):3513-3522.

180. Reed MJ. Chemical and antigenic properties of the cell wall of Actinomyces viscosus (Strain T6). J Dent Res 1972;51(5):1193-1202.

181. Wilson M, Reddi K, Henderson B. Cytokine-inducing components of periodontopathogenic bacteria. J Periodontal Res 1996;31(6):393-407.

182. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of Porphyromonas gingivalis. Periodontol 1999;20:168-238.

183. Siqueira J. Treatment of endodontic infections. 1st ed; 2011.

184. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med 2004;15(6):348-381.

185. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol 1998;85(1):86-93.

186. Barnard D, Davies J, Figdor D. Susceptibility of Actinomyces israelii to antibiotics, sodium hypochlorite and calcium hydroxide. Int Endod J 1996;29(5):320-326.

187. Rocas IN, Siqueira JF, Jr., Santos KR. Association of Enterococcus faecalis with different forms of periradicular diseases. J Endod 2004;30(5):315-320.

188. Sedgley CM, Lennan SL, Clewell DB. Prevalence, phenotype and genotype of oral enterococci. Oral Microbiol Immunol 2004;19(2):95-101.

189. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J 2002;35(3):221-228.

190. Frough-Reyhani M, Ghasemi N, Soroush-Barhaghi M, Amini M, Gholizadeh Y. Antimicrobial efficacy of different concentration of sodium hypochlorite on the biofilm of Enterococcus faecalis at different stages of development. J Clin Exp Dent 2016;8(5):e480-e484.

191. Hegde V. Enterococcus faecalis: Clinical significance and treatment considerations. Endodontology 2009;21(2):48-52.

192. Liu H, Wei X, Ling J, Wang W, Huang X. Biofilm formation capability of Enterococcus faecalis cells in starvation phase and its susceptibility to sodium hypochlorite. J Endod 2010;36(4):630-635.

193. Retamozo B, Shabahang S, Johnson N, Aprecio RM, Torabinejad M. Minimum contact time and concentration of sodium hypochlorite required to eliminate Enterococcus faecalis. J Endod 2010;36(3):520-523.

194. Sturridge E. Dental electro-therapeutics. Lea & Febiger; 1918.

195. Haffner C, Benz C, Hickel R. Endox-Endodontie-system: Weitere Laborergbnisse und erste Klinische Resultate. ZWR 1999;108(11):670-674.

196. Karale R, Thakore A, Shetty V. An evaluation of antibacterial efficacy of 3% sodium hypochlorite, high-frequency alternating current and 2% chlorhexidine on Enterococcus faecalis: An in vitro study. J Conserv Dent 2011;14(1):2-5.

197. Aranda-Garcia AR, Guerreiro-Tanomaru JM, Faria-Junior NB, Chavez-Andrade GM, Leonardo RT, Tanomaru-Filho M, et al. Antibacterial effectiveness of several irrigating solutions and the Endox Plus system - an ex vivo study. Int Endod J 2012;45(12):1091-1096.

198. Virtej A, MacKenzie CR, Raab WH, Pfeffer K, Barthel CR. Determination of the performance of various root canal disinfection methods after in situ carriage. J Endod 2007;33(8):926-929.

199. Lendini M, Alemanno E, Migliaretti G, Berutti E. The effect of high-frequency electrical pulses on organic tissue in root canals. Int Endod J 2005;38(8):531-538.

200. Maden M, Ertugrul IF, Orhan EO, Erik CE, Yetis CC, Tuncer Y, et al. Enhancing antibacterial effect of sodium hypochlorite by low electric current-assisted sonic agitation. PLoS One 2017;12(8):e0183895.

201. ISEM. EMAT (TM). 2012 2012 [cited 4/6/2019]; Available from: http://emat.jp/english/index.php

202. Cassanelli C, Marchese A, Cagnacci S, Debbia EA. Alteration of membrane permeability of bacteria and yeast by high frequency alternating current (HFAC). Open Microbiol J 2008;2:32-37.

203. Del Pozo JL, Rouse MS, Patel R. Bioelectric effect and bacterial biofilms: A systematic review. Int J Artif Organs 2008;31(9):786-795.

204. Costerton JW, Ellis B, Lam K, Johnson F, Khoury AE. Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. Antimicrob Agents Chemother 1994;38(12):2803-2809.

205. Jass J, Lappin-Scott HM. The efficacy of antibiotics enhanced by electrical currents against Pseudomonas aeruginosa biofilms. J Antimicrob Chemother 1996;38(6):987-1000.

206. Khoury AE, Lam K, Ellis B, Costerton JW. Prevention and control of bacterial infections associated with medical devices. Asaio j 1992;38(3):M174-178.

207. Stewart PS, Wattanakaroon W, Goodrum L, Fortun SM, McLeod BR. Electrolytic generation of oxygen partially explains electrical enhancement of tobramycin efficacy against Pseudomonas aeruginosa biofilm. Antimicrobial Agent Chemotherapy 1999;43(2):292-296.

208. McLeod BR, Fortun S, Costerton JW, Stewart PS. Enhanced bacterial biofilm control using electromagnetic fields in combination with antibiotics. Methods Enzymol 1999;310:656-670.

209. Forough Reyhani M, Rezagholizadeh Y, Narimani MR, Rezagholizadeh L, Mazani M, Barhaghi MHS, et al. Antibacterial effect of different concentrations of sodium hypochlorite on Enterococcus faecalis biofilms in root canals. J Dent Res Dent Clin Dent Prospects 2017;11(4):215-221.

210. Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, et al. Inhibitory effects of cranberry polyphenols on formation and acidogenicity of Streptococcus mutans biofilms. FEMS Microbiology Letters 2006;257(1):50-56.

211. Guerreiro-Tanomaru JM, de Faria-Junior NB, Duarte MA, Ordinola-Zapata R, Graeff MS, Tanomaru-Filho M. Comparative analysis of Enterococcus faecalis biofilm formation on different substrates. J Endod 2013;39(3):346-350.

212. Kishen A, Shrestha A, Del Carpio-Perochena A. Validation of biofilm assays to assess antibiofilm efficacy in instrumented root canals after syringe irrigation and sonic agitation. J Endod 2018;44(2):292-298.

213. Stocks SM. Mechanism and use of the commercially available viability stain, BacLight. Cytometry Part A 2004;61A(2):189-195.

214. Berney M, Hammes F, Bosshard F, Weilenmann H-U, Egli T. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. Applied and environmental microbiology 2007;73(10):3283-3290.

215. Arias-Moliz MT, Ferrer-Luque CM, Espigares-García M, Baca P. Enterococcus faecalis biofilms eradication by root canal irrigants. J Endod 2009;35(5):711-714.

216. Frough-Reyhani M, Ghasemi N, Soroush-Barhaghi M, Amini M, Gholizadeh Y. Antimicrobial efficacy of different concentrations of sodium hypochlorite on the biofilm of Enterococcus faecalis at different stages of development. J Clin Exp Dent 2016;8(5):e480-e484.

217. Gabrilska RA, Rumbaugh KP. Biofilm models of polymicrobial infection. Future Microbiol 2015;10(12):1997-2015.

218. Bradley CR, Fraise AP. Heat and chemical resistance of enterococci. J Hosp Inf 1996;34(3):191-196.

219. Montero-Miralles P, Martín-González J, Alonso-Ezpeleta O, Jiménez-Sánchez MC, Velasco-Ortega E, Segura-Egea JJ. Effectiveness and clinical implications of the use of topical antibiotics in regenerative endodontic procedures: a review. Int Endod J 2018;51(9):981-988.

ABSTRACT

USE OF ELECTROMAGNETIC STIMULATION ON AN *ENTEROCOCCUS FAECALIS* BIOFILM IN ROOT CANAL TREATED TEETH *IN VITRO*

by

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Introduction: Nonsurgical root canal therapy procedures aim to reduce the total microbial load within an infected root canal system through chemomechanical debridement of the root canal system via instrumentation in conjunction with an antibacterial irrigating solution. The most commonly used irrigant is sodium hypochlorite, often at concentrations toxic to human cells. Electromagnetic wave irradiation is a novel method of disinfection that has been shown to be bactericidal against planktonic microorganisms in solution, but its efficacy against an established

biofilm is unknown. Pilot studies have demonstrated a synergistic killing effect with sodium hypochlorite through a process termed electromagnetic stimulation (EMS). If confirmed, lower concentrations of the current gold standard of 6.0-percent sodium hypochlorite could be used to irrigate infected root canals during endodontic treatment, resulting in less toxicity to human cells. There are also regenerative implications as EMS could be used to disinfect the root canals of immature teeth using 1.5-percent sodium hypochlorite, as recommended by the American Association of Endodontists.

Objectives: The purpose of this *in-vitro* study was to evaluate the anti-biofilm effect of EMS against an established biofilm of *Enterococcus faecalis.*

Materials and Methods: Single rooted teeth were cut to a standardized length (12 mm) and instrumented with a 45.05 Wave One Gold reciprocating file. Specimens were sterilized and inoculated with *E. faecalis*, which grew for two weeks to form an established biofilm. There were five treatment groups: 1) 6.0-percent sodium hypochlorite; 2) 1.5-percent sodium hypochlorite; 3) 1.5-percent sodium hypochlorite with EMS; 4) 0.9-percent saline with EMS and 5) 0.9-percent saline. Samples were collected, plated, and incubated for two days. The number of CFUs/mL was determined and converted to log_{10}. The effect of treatment group on bacterial counts was made using Wilcoxon Rank Sums Test. One sample per group was scored and split for confocal imaging.

Null Hypothesis: Teeth treated with EMS in combination with 1.5-percent sodium hypochlorite or 0.9-percent saline will not demonstrate a significant anti-biofilm effect in comparison to those treated with 6.0-percent sodium hypochlorite alone.

Results: 0.9-percent saline and 0.9-percent saline with EMS were significantly

higher than 6.0-percent NaOCl, 1.5-percent NaOCl, and 1.5-percent NaOCl with EMS. 0.9-percent saline was significantly higher than 0.9-percent saline with EMS. The three groups that included treatment with NaOCl were not significantly different from each other. Confocal imaging confirmed the CFU findings.

Conclusion: Because there was no growth in any of the NaOCl groups, the null hypothesis cannot be rejected. However, there was an antibiofilm effect when comparing the two saline groups, demonstrating that EMS has an antibiofilm effect. Future studies should focus on determining what concentration of NaOCl is most effective in combination with EMS.

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