TIME DEPENDENT EFFECTS OF IODINE POTASSIUM IODIDE (IKI) ON A POLYMICROBIAL BIOFILM

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DEDICATION

To my wife, Beth, for always being there for me and for your never-ending support.

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Introduction

Microorganisms have been demonstrated to be the primary etiology of pulpal and periradicular disease (Kakehashi et al, 1962, Moller et al., 1981, Lin et al, 2006). As such, disinfection of the root canal system is one of the major goals of root canal therapy. Sjogren found that 94% of nonsurgical root canal therapy in which a negative culture was obtained at the time of obturation were successful over a five year period compared to just 68% if a positive culture was obtained (Sjogren et al, 1997). This is indicative that eliminating bacteria from the canal system greatly improves chances of successful endodontic treatment. The primary microbiological goal of the endodontic treatment of teeth is the reduction of the bacterial populations to levels compatible with periradicular tissue healing (Siqueira et al., 2011).

As methods for identifying potential endodontic pathogens advance, the list of species implicated has grown immensely (Siqueira and Rocas, 2005). It has also become apparent that endodontic infections are polymicrobial and even more diverse than originally thought (Chugal et al., 2011; Siquiera et al., 2011).

The microorganisms inhabiting the root canal space can either exist as planktonic, free-floating, cells or attached to each other and the root canal wall in the form of a biofilm (Shen, 2011). With the majority of root canal infections displaying the presence of biofilm, apical periodontitis has been classified as a biofilm induced disease (Ricucci & Siqueira, 2010).

Failure to fully eliminate biofilm is a likely reason for non-healing root canal therapy. Some species of bacteria like *E. faecalis*, are more resistant to standard

treatment techniques, and to be more resistant to calcium hydroxide (Reit and Dahlen, 1988). Hancock showed that *E. faecalis* was the most commonly recovered bacterial species present in cases of failed root canal therapy (Hancock et al, 2001). *E. faecalis* has been isolated in 38 % to 77 % of cases presenting for retreatment (Sudqvist et al., 1998; Siqueira et al, 2004). Resistant species like *E. faecalis* can be even more protected in biofilm form and lead many to attempt to find another protocol to help eliminate these species.

Mechanical preparation of the root canal system fails to completely instrument the entire complex root canal anatomy (Peters, 2003). Therefore, mechanical instrumentation alone is unable to remove all bacteria and their byproducts (Siqueira, 1999), and the clinician must rely on chemical irrigation to debride the remaining portions of the root canal space. The ideal irrigant with broad spectrum antimicrobial activity and high efficacy, the ability to dissolve pulp tissue remnants, inactivate endotoxin, prevent or dissolve smear layer, and be systemically nontoxic and noncaustic to periodontal tissues does not exist (Zehnder, 2006). Sodium hypochlorite has been shown to be one of the most advantageous irrigants available (Spangberg, 1973), but is extremely cytotoxic to apical tissues.

Iodine potassium iodide (IKI) has been demonstrated to be less cytotoxic and irritating than sodium hypochlorite (Spangberg, 1973). It has also been shown to outperform sodium hypochlorite and chlorhexidine at penetrating and disinfecting dentinal tubules (Orstavik, 1990). However, IKI lacks the ability to dissolve tissue so it has been considered inadequate as a primary irrigant (Haapasalo, 2010). IKI has been

used as both an intracanal medicament and as an interapointment irrigant dressing, but the efficacy of IKI on biofilms has not yet been determined.

The purpose of this study is to demonstrate the use of a new polymicrobial biofilm model to test endodontic irrigants, and to measure the susceptibility of a biofilm to different application times of IKI.

Literature Review

Bacteria and Apical Periodontitis

Bacteria as the causative factor in diseases of endodontic origin first emerged as a theory of W.D. Miller in 1894. During his groundbreaking work identifying bacteria in the oral cavity that could produce acid in the presence of sugars, he also noted the presence of bacteria in "great numbers in some pulps, and especially the repeated occurrences spirochaetes" (Miller, 1894). However, the presence of bacteria did not prove pathogenicity. With the use of germ-free, gnotobiotic rats, Kakehashi, Stanley, and Fitzgerald first demonstrated microorganisms to be the cause of pulpal necrosis and apical periodontitis after pulp exposure (Kakehashi et al, 1962). Then, in 1976, Sundqvist also showed bacteria has a fundamental role in the development of apical periodontitis with his bacteriologic studies of necrotic pulps (Sudqvist, 1976). Moller and Lin further confirmed micro-organisms as the primary etiology of apical periodontitis in both monkeys and dogs respectively by aseptically devitalizing teeth and exposing some to the oral environment. (Moller, 1981; Lin, 2006) This showed that sterile devitalized pulp tissue will not cause apical periodontitis until bacteria contaminate the pulp space. With bacteria and other pathogenic microorganisms identified as the ultimate cause of pulpal and apical pathosis, the question then turned to identifying the species responsible and determining how to best eradicate them.

Numbers of species

Early studies relied on culturing and light microscopy to identify the micro-organisms within the root canal system. These studies confirmed that the primary endodontic infection is polymicrobial and that a persistent infection, or infection present after root canal therapy, tended to be less diverse if not a monoinfection. (Sundqvist, 1992; Baumgartner, 2004) These studies showed primary infections present with three to twelve species while persistent infections to present with one to three species. A major drawback to culturing studies is the inability to culture every microorganism. It was not until anaerobic culturing methods came into practice that it was known that approximately 90% of root canal flora were obligate anaerobes (Sundqvist, 1976). The lack of oxygen in the root canal system will select for anaerobic species as the root canal infection progresses (Fabricius, 1982). Many other factors than the presence of oxygen play a role in the ability to culture a microorganism, including specific media and nutritional requirements that are typically unknown and some bacteria will require the presence of other microorganisms (Sundqvist, 2003).

Newer techniques have attempted to circumvent the difficulties found in culturing. These techniques rely on molecular methods including PCR, DNA hybridization, and pyrosequencing (Siqueira, 2002), and give the ability to look at the DNA or RNA present in the root canal system to identify the species. A recent molecular study showed an average of 33 species in a primary infection and 16 species in a persistent infection using PCR techniques (Chugal, 2011). A recent pyrosequencing studying looking at 16S rRNA found 187 species associated with root canal infections

(Siqueira, 2011). While only 23 of those species were found in relative abundance (>1%), it is becoming apparent that endodontic infections are much more diverse than originally thought.

Species Implicated in Endodontic Infections

While there are over 500 species of bacteria present in the oral cavity, only a select group will become involved in an endodontic infection (Sundqvist and Figdor, 2003). The conditions present in the root canal system are drastically different from the bulk of the oral environment and therefore only a subset of that species will be able to inhabit this area. Not surprisingly, the list of species implicated with endodontic infections has followed the same course as number of species. It has been well established that endodontic infections are polymicrobial (Siqueira 2002; Baumgartner 2004; Sundqvist 1992). When research relied on culturing methods, the list of bacteria was much smaller. Classically, the black pigmenting bacteria of the *Bacteroides, Prevotella,* and *Porphorymonas* genera were thought of as the primary pathogens in endodontic infections (Sundqvist 1989, Baumgartner 1999). Enterococcus also became known as the most common pathogen associated with persistent endodontic disease (Sundqvist 1998, Molander 1998). As molecular methods have progressed, other genera have consistently been reported including *Treponema, Fusobacterium, Streptococcus,* and *Peptostreptococcus* in primary infections and *Actinomyces, Streptococcus, Staphylococcus,* and *Propionibacterium* in persistent infections (Siqueira, 2002).

As the lists have continued to grow with advances in bacterial identification, two important ideas become increasingly clear: each endodontic infection is unique and no one species of bacteria is present 100% of the time. The recent study done with pyrosequencing demonstrated the low similarity between each infection combined with the high diversity represented by 84 different genera detected (Siqueira, 2011). It is becoming more and more evident that targeting a specific species or even an entire genera is less important than understanding how these diverse bacterial communities form, grow, and evolve, and how root canal therapy can be adapted to adequately disrupt and kill these communities to create an environment for healing.

Importance of Biofilms

A biofilm is a community of microorganisms embedded in an extracellular polysaccharide matrix and attached to a solid surface (Shen, 2011). This community lifestyle affords a number of advantages over the individual cell in a planktonic or free-floating state including; increased metabolic diversity and efficiency, protection against host defenses, antimicrobial agents, and environmental stressors, and enhanced pathogenicity (Marsh, 2005). It has been estimated that biofilm infections comprise 65-80% of human infections in the developed world (Costerton, 2004). A histopathologic study of apical segments of both root canal treated and untreated teeth with apical periodontitis revealed bacteria in nearly all specimens, but also found that biofilms were present in 77% of the specimens covering the walls of ramifications of isthmuses. Six percent even had biofilm present on the extra-radicular surface of the root. These

findings were consistent with acceptable criteria for the authors to conclude that apical periodontitis is in the set of biofilm induced diseases (Ricucci & Siqueira, 2010). With the majority of bacteria colonizing the root canal space in a biofilm community, the success of endodontic treatment will depend on the proper elimination of these biofilms.

Biofilm Development

Biofilm formation in root canals is likely initiated at some point after the first invasion of the pulp chamber by planktonic oral microorganisms and initial tissue breakdown (Svensater & Bergenholtz, 2004). A conditioning film of various proteins both from host and bacterial sources will form on the dentin surface. Planktonic bacteria will loosely adhere to that surface and will rapidly change their genetic expression to increase adherence and begin formation of an extracellular polysaccharide matrix. This will in turn allow for more colonization by secondary planktonic bacteria and provide protection for replication (Hall-Stoodley, 2004). Cells will then begin to aggregate and form microcolonies, which will continue to grow and mature. The architecture of each biofilm can be complex and can be flat or mushroom shaped depending on the nutrient source (Hall-Stoodley, 2004). High nutritional conditions will favor the filamentous mushroom shaped mass, while low nutritional conditions will favor flat and more primitive biofilms (De Kievit, 2001; Conway, 2002). Biofilms have also been shown to have heterogeneous matrix-enclosed microcolonies interspersed with open water channels (deBeer et al., 1994). These channels can facilitate efficient nutrient uptake by infusing fluid into the biofilm and also allow for waste-product exchange. The final

phase in biofilm is characterized by a return to transient motility where biofilm cells are sloughed or shed either individually or in mass (Hall-Stoodley, 2004), and these cells can then continue the infective process further down the root canal system.

Endodontic Biofilm Models

Testing the effectiveness of preparation techniques or irrigation protocols in a controllable environment is important for the development of new protocols and verifying the effectiveness of existing ones. In an effort to mimic conditions within a root canal during infection, many *in vitro* or *ex vivo* biofilm models have been attempted. Some of the more basic models rely on a single species of bacteria like *E. faecalis* that is known to have the ability to form a single species biofilm. These have been grown and tested on a variety of surfaces including glass plates (Elliot, 2005), plastic pegs (Ceri, 1999), or dentin (George, 2005). However, since most endodontic infections are polymicrobial, other researchers have placed importance on developing a polymicrobial biofilm model. Shen, Stojicic, and Haapasalo (2010) used a subgingival plaque sample and grew the biofilms on collagen coated hydroxyapatite discs for 3-12 weeks under anaerobic conditions. This method allowed for biofilm thicknesses of 57μm at 2 days to 201μm at 12 weeks (Shen, 2011). Clegg & Vertucci (2006) employed a similar method with bacterial samples harvested from endodontically involved teeth. They then grew the biofilm anaerobically on saliva coated root dentin over seven days. Neither of these methods employed any shear forces in the production of the biofilm and had variable biofilm thickness depending on length of incubation.

Recently, the University of Minnesota has employed the use of a bioreactor with shear forces to grow thick (300-500μm), polymicrobial biofilms over 48-72 hours (Chen et al., 2012). This system was intended to test how bacterial biofilms grow on dental composites and how they affect the interface between tooth and composite (Lenton et al., 2012). Bacterial samples were taken from the interface of dental composites on pediatric dental patients and were grown on saliva coated hydroxyapatite or composite discs. The biofilms were grown under predominantly aerobic conditions but have been shown to retain a number of anaerobes (Rudney et al.). They have been shown to be very complex, and depending on conditions, they can be expected to have an average of 25 to 39 species with some supporting more than 60 species (Rudney et al.). While it has not been used to test endodontic applications, it does have potential as it is a suitable method to reproducibly grow complex biofilms in a short time course.

Endodontic Irrigants

Siqueira (1999) has shown that mechanical instrumentation of the root canal space alone can eliminate over 94% of bacterial contamination. However, thousands of bacteria can remain in the canal system that may continue to replicate and continue to cause infection and apical periodontitis. Peters (2003) has also shown using micro CT during root canal instrumentation that nearly 40% of the walls of the root canal system are untouched by mechanical preparation, and these areas are all potential harborers of biofilm and bacterial byproducts. Practitioners must rely solely on the endodontic

irrigant for disinfection of those areas, which makes antimicrobial endodontic irrigants essential for a successful outcome.

In a review of endodontic irrigants, Zehnder (2006) discusses the ideal properties of an endodontic irrigant. The ideal irrigant should have a broad antimicrobial spectrum and high efficacy against anaerobic and facultative microorganisms organized in biofilms; should dissolve necrotic pulp tissue remnants; should inactivate endotoxin; should also prevent the formation of a smear layer during instrumentation or dissolve the latter once it has formed; should be systemically nontoxic, noncaustic to periodontal tissues, and have little potential to cause anaphylactic reaction. Currently, however, there is not an irrigant that can fulfill all of the ideal requirements. As such, many clinicians have employed the use of multiple irrigants during the root canal procedure. Sodium hypochlorite is the most commonly used endodontic irrigant and fulfills the bulk of the properties of an ideal irrigant (Zehnder, 2006). Chlorhexidine and Iodine Potassium Iodide (IKI) are also commonly used for their antimicrobial attributes.

Sodium Hypochlorite

Sodium hypochlorite (NaOCl) is the active ingredient in household bleach. It was first used in medical applications in Dakin's solution (0.5% NaOCl) as an antimicrobial disinfectant of wounds during World War I (Dakin, 1915). A. Walker was the first to employ it was an endodontic irrigant in 1936 (Walker, 1936). The antimicrobial action of sodium hypochlorite stems from the formation of hypochlorous acid (HOCl) in the presence of water (Estrela, 2002), and this is a powerful oxidant that will react with

amino and sulphydryl groups of bacterial enzymes. It will also create a saponification reaction with fatty acids and lipids causing dissolution of organic tissue (Estrela, 2002).

Sodium hypochlorite is a broad spectrum antimicrobial due to its mechanism of action, and the same mechanism is responsible for its ability to inactivate endotoxin. It has been proven to be excellent at dissolution of necrotic tissue (Hand et al., 1978) and can also dissolve vital tissue (Rosenfeld et al., 1978). It has been shown to be effective at disinfection and removal of biofilms (Clegg et al., 2006). Six percent sodium hypochlorite was the only concentration that was shown to completely remove the biofilm. However, different concentrations of sodium hypochlorite have been advocated due to its major drawback, cytotoxicity. Its non-selective qualities make it potentially dangerous if expressed beyond the apex of the tooth into the periodontal ligament or surrounding alveolar bone. An early study recommended a 0.5% concentration after studying toxic effect to tissues (Spangberg et al., 1973). A review of complications during irrigation outlines the risks of and extreme reactions to sodium hypochlorite (Hulsmann et al., 2007). Case reports have also demonstrated a risk of paresthesia resulting from extrusion of sodium hypochlorite (Reeh & Messer, 1989). The other potential drawback to use of sodium hypochlorite is allergic reaction, and case reports demonstrate the importance of ruling out sodium hypochlorite allergy before clinical use (Kaufman et al., 1988). The incidence of sodium hypochlorite accidents is very low (Hulsmann et al., 2007), which is why it is still widely used. However, the severity of the potential reactions has fueled the search for alternatives like chlorhexidine or IKI.

Chlorhexidine

Chlorhexidine has gained popularity as an endodontic irrigant as it is generally less caustic than sodium hypochlorite (Spangberg et al., 1973), and it was first developed in the 1940s in an attempt to create an anti-viral substance. However, it was found to be much better as an antibacterial agent (Zhender, 2006). It is sold in the form chlorhexidine digluconate as either a 0.21% or 2.0% solution, and the more dilute concentration found its way into dentistry as an oral antimicrobial rinse. Chlorhexidine is a potent bisguanide. It is believed to adsorb onto negatively charged surfaces like bacterial cell walls and strongly adsorb to phosphate containing compounds. It will bind to the phospholipids of the inner membrane leading to increased permeability and leakage of low-molecular weight components like potassium ions (Jones, 2000). This mechanism may explain why chlorhexidine is less effective on Gram-negative than on Gram-positive bacteria (Emilson, 1977). Chlorhexidine also lacks the ability to dissolve necrotic tissue remnants (Naenni et al., 2004) and may be more suited as a final irrigant than the main irrigant.

Iodine Potassium Iodide History

Iodine Potassium Iodide (IKI) first came into use in 1829 when the French physician J.G.A. Lugol attempted it use to treat scrofula, a tuberculosis infection of the cervical lymph nodes, and gout (Neuzil,2002). This solution of 5% iodine, 10% potassium iodide, and 85% distilled water became known as Lugol's solution and is still available today. Different concentrations have been available with the iodine ranging from 1-5% and the potassium iodide ranging from 2-10%, but the ratio of iodine to

potassium iodide generally is about 1:2. IKI has been used in multiple applications as an antiseptic and disinfectant, for treatment of thyroid disorders or iodine deficiencies, for emergency disinfection of drinking water, as a stain, and as a reagent for starch detection in laboratory and medical tests (Gottardi, 1991). In dentistry it has been used as an endodontic irrigant and can be used to stain for dysplastic cells in oral cancer screening or margin detection (Haapasalo et al., 2010; Petruzzi 2010). Iodine products were first used as an irrigant in endodontics in 1927.

Iodine Potassium Iodide Mechanism of Action

Iodine in solution can generally be described with the following equations:

(1) $I_2 + H_2O \leftrightarrow H_2OI^+ + I^-$

(2) $H_2OI^+ + HOI \leftrightarrow HOI + H^+$

(3) $HOI \leftrightarrow OI^- + H^+$

(4) $3\ HOI \leftrightarrow IO_3^- + 2\ I^-$

(5) $I_2 + I^- \leftrightarrow I_3^-$

In pure iodine solutions at least seven different iodine containing ions or molecules are present (Gottardi, 1991). Only three of these actually have strong germicidal properties (Black 1968; Gottardi, 1991); the molecular iodine (I_2), the hypoiodic acid (HOI), and the iodine cation (H_2OI^+). The iodine cation however, is more than four powers below the concentration of the HOI and it plays virtually no role in disinfection (Gottardi, 1978).

IKI has potassium iodide added as a stabilizing agent to prevent formation of iodate which has no antimicrobial properties. The potassium iodide causes a shift in the

equilibrium of equation 5 above to create the tri-iodide ion (I_3^-) as a major constituent. The tri-iodide ion has an inferior antibacterial activity because it is a weak oxidant (Kramer, 1952). Due to the shift in equilibrium, IKI has an effective concentration of 0.34% iodine, which still has considerable antimicrobial properties (Gottardi, 1991). The presence of organic materials like dissolved proteins will result in some decrease in active iodine concentration as it reacts with the protein. However, IKI has a comparatively low reactivity with proteins compared to its ability to kill living microorganisms, and combined with near independence of disinfecting ability with pH gives iodine excellent degerming properties (Gottardi, 1991).

The iodine component of IKI seems to be the predominant source of antimicrobial activity. It is able to rapidly penetrate the cell wall of microorganisms (Chang, 1971). Once inside the microorganism, there are four ways that the iodine reacts, although the exact mechanism of killing is not known (Gottardi, 1991). The most important mechanism is likely by oxidizing the S-H group of cysteine which will result in the inability to form disulfide bridges and prevent correct microbial protein synthesis (Kruse, 1970). Another potential mechanism is that iodine will react with basic N-H functions in amino acids and form N-iododerivatives, which will block important positions for hydrogen bonding and again result in lethal disorder of protein structure (Gottardi, 1991). Iodine can also react with the phenolic group of tyrosine and cause steric hindrance in hydrogen bonding. The final theorized interaction is iodine reacting with the carbon-carbon double bond of unsaturated fatty acids leading to a change in the physical properties of microbial lipids and result in membrane immobilization (Apostolov, 1980).

In general, IKI's antimicrobial activity results from the iodine component acting to disrupt protein synthesis and structure and to change physical properties of lipids causing membrane immobilization.

Range of Action

Iodine and iodine containing preparations like IKI are broad spectrum antimicrobials that have been shown to be effective on a large variety of microorganisms (Gottardi, 1991). Time of application and concentration does and will affect the spectrum of action, and iodine has been shown to be effective against health-related microorganisms like enteric bacteria, enteric virus, bacterial viruses, and protozoan cysts (Hoehn, 1976). Mycobacteria and the spores of bacilli and clostridia can also be killed by iodine (Wallhausser, 1978). Iodine also exhibits fungicidal and trichomonacidal activity (Knolle, 1975).

Iodine Toxicity

IKI relies on elemental iodine for its antimicrobial activity, however, toxicity of the free iodine for living tissue must be considered. Application of IKI to healthy skin causes little to no irritation but on a burn, will be irritating as more iodine will be absorbed by the body. A study on treating burns with iodine containing compounds showed a sharp increase in plasma iodine but claimed that thyroid function was not altered (Kuhn, 1987). High doses of free iodine will be highly toxic and cause swelling and bleeding of mucous membranes if brought into body cavities, and consuming 30g of iodine tincture (similar in iodine concentration with IKI) can be fatal (Wirth, 1967). In

an endodontic application, little uptake of iodine can be expected if contained within the root canal system. A study using povidone-iodide preparations as mouth antiseptics, vaginal gels, and liquid soaps, showed an increase in iodine supply (Gloebel, 1984). The authors measured thyroid hormones and thyroid stimulating hormone and did not note any developments of hyperthyroidism or hypothyroidism. Also, they found that the bulk of the iodine was converted to iodide and organic bound iodine that left the body by urinary excretion with a biologic half-life of approximately 2 days (Gloebel, 1984).

Iodine Potassium Iodide in Endodontics

Iodine disinfectants have been used in endodontics since 1927 (Johnston, 1927) when Johnston used an iodine tincture. IKI became more prominent in endodontics in the 1970's after a series of papers by Spangberg researching the various antimicrobial effects and toxicities of potential irrigants and medicaments (Spangberg et al., 1973). He approved of IKI as an adequate irrigant due to its relative low toxicity compared to high antimicrobial abilities when compared to other irrigants, but felt that it could be diluted up to ten times to lower the potential toxicity while maintaining adequate antimicrobial activity. He reported that the staining potential of IKI is low after washout and that its effect is of short duration, so it will not interfere with taking cultures (Spangberg et al., 1973). Another potential strength of IKI is the potential for substantivity as it has been shown to have residual antimicrobial activity for 24 hours after a one minute application to skin (Gottardi, 1989). However, it has not been proven in a tooth model, and it has been shown to be less cytotoxic and irritating than both sodium hypochlorite and

chlorhexidine (Spangberg et al., 1979). However, IKI has a higher allergy potential than sodium hypochlorite and chlorhexidine (Popescu et al., 1984).

IKI has the potential to be utilized in two ways in endodontics; one is as an intracanal medicament (ICM) potentially with calcium hydroxide, and the other is as an irrigating solution. Maddox first showed IKI to have no significant difference in post-op pain when compared to other ICMs in 1977 (Maddox, 1977). Fuss showed that adding IKI to calcium hydroxide improved disinfection of *E. faecalis* in dentin tubules at increasing depths from 200 to 500µm (Fuss, 2002). Haenni demonstrated that the addition of IKI did not affect the pH increase across dentin from calcium hydroxide but noted no additive antimicrobial effect on *E. faecalis* and *C. albicans* using an agar diffusion test (Haenni et al., 2003). Siren showed that IKI alone was the best ICM when antimicrobial ability on *E. faecalis* was measured at 24 hours, effectively killing all bacteria to the 700µm depth in dentin tubules (Siren et al., 2004). However, there was no difference between IKI alone and with calcium hydroxide when measured at seven days, disinfecting to the full 950µm depth (Siren et al., 2004). Interestingly, both IKI and IKI with calcium hydroxide were more effective than calcium hydroxide alone and with 0.5% chlorhexidine (Siren et al., 2004). A recent study confirmed this when they showed the IKI and calcium hydroxide combination to be better than the calcium hydroxide alone or with 0.5% chlorhexidine on dentin blocks infected with *E. faecalis* at both the 24 hour and 7 day time points (Prabhakar et al., 2012). The same study also showed no effect on the pH or dentin fracture resistance with the addition of IKI (Prabhakar et al., 2012). The addition of IKI to the calcium hydroxide as an ICM shows promise as it appears to

increase the antimicrobial effects over calcium hydroxide alone while also maintaining the benefits of a high pH that many clinicians expect from their ICM.

The other major use of IKI is as an irrigant. Spangberg first advocated its use but due to its lack of tissue dissolving capabilities, he recommended it if a positive culture was obtained after cleaning and shaping with sodium hypochlorite (Spangberg et al., 1973). Other reviews have expressed interest in IKI where they advocate its use as a possible irrigant treatment after instrumentation with sodium hypochlorite (Haapasalo, 2010; Siqueira, 2011; Zehnder 2006). Orstavik found that 5 minutes of IKI was able to outperform both 5.25% sodium hypochlorite and 0.21% chlorhexidine in disinfection depth of *S. sanguis* infected dentinal tubules (Orstavik et al., 1990). IKI was found to penetrate more that 1000µm compared to sodium hypochlorite at 200-300µm and chlorhexidine at 100-300µm (Orstavik et al., 1990). Safavi incubated dentin with *S. faecium* for either 27 days (infected) or 10 minutes (contaminated) and tested IKI at different time points for disinfection. After culturing, no growth occurred after only a one minute treatment of IKI on contaminated dentin, however, 10 minutes was required to obtain no further growth on the infected dentin (Safavi et al., 1990). Peciuliene incorporated IKI as a final rinse when comparing a one visit endodontic retreatment protocol to a two-visit protocol with calcium hydroxide (Peciuliene et al., 2001). The single visit protocol resulted in 19 of the 20 samples having a negative culture compared to 15 of the 20 two visit protocol samples. The IKI was only required as a final rinse in 5 of the single visit protocol groups that had a positive culture after initial cleaning and shaping. Therefore, the IKI was effective in disinfecting 4 of the 5 samples with its 5

minute application (Peciuliene et al., 2001). A similar study was done that compared a single visit root canal treatment with a 10 minute intra-appointment dressing of IKI to a two visit treatment with calcium hydroxide ICM in a randomized control trial model (Kvist et al., 2004). They found no significant difference in percentages of samples with recovered microorganisms between the two groups, with 29% of the IKI treated samples and 36% of the two-visit calcium hydroxide group with recovered microorganisms (Kvist et al., 2004). A recent study evaluated the effect of adding a detergent to IKI and found an increased percentage of killed *E. faecalis* in dentin tubules (Wang et al., 2012). Additionally, 6% sodium hypochlorite was found to be superior to both IKI and IKI with 0.1% cetrimide at 1 minute and 3 minute application times (Wang et al., 2012). In general, IKI as an irrigant has shown some promising results, however, it has yet to be tested on a polymicrobial biofilm model.

Methods of Measuring Effectiveness

There have been many methods for measuring the effectiveness of antimicrobial agents in endodontics. Typically a sample is infected, and the antimicrobial agent is applied. Then a method for measuring the viability of remaining cells needs to be employed. The most common method tends to be culturing. Culturing can be done either by looking at turbidity of a broth or media as an all or none component (Orstavik et al., 1990; Safavi et al., 1990); otherwise an attempt to quantify it can be made by plating the sample and looking at colony forming units (Coldero et al., 2002). This method can work rather well in the case of an *in vitro* single species model where proper growth conditions are known. It can potentially be a problem in *in vivo* or *ex vivo* situations

where viable bacteria may be present but will not grow in the allotted nutrient or atmospheric conditions.

Staining and immunofluorescence are a potential way to circumvent this issue. A common method is a LIVE/DEAD stain that will fluoresce at different wavelengths depending on the viability of the cell (Shen et al., 2011). Generaly, the live stain is a nucleic acid stain that will be taken up by live cells and when excited at 485 nm will fluoresce in the green spectrum at 530 nm. The dead stain is typically propidium iodide which cannot permeate an intact membrane and will only then be taken up by cells presumed dead due to membrane permeability. It will emit light in the 630 nm or red spectrum when excited at 485 nm. These stains can be modified slightly depending on the type of cell, and assays exist specifically for bacterial viability testing. The fluorescence can be quantified and a measurement can be made as a percentage of dead or living cells remaining (Shen et al., 2011). A confocal laser scanning microscope is typically required for imaging of these stains (Wang et al., 2012). These methods can be rather time consuming to obtain enough planes of images across the sample to properly assess the entire population. Another potential problem is the in potential issue of stain penetration to deeper layers in thicker specimens, especially a thick biofilm model.

The MTT assay is very common in cell culture for quantifying cell viability. MTT stands for the yellow tetrazolium salt 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The assay relies on reductase enzymes to convert the MTT salt into formazan, a purple crystal that is generally insoluble in water (Zhang et al., 2002). These enzymes are only active in live cells and therefore the amount of formazan

created will correspond to the number of living cells present. In order to quantify the amount of formazan produced, it must be dissolved into solution and optical density or absorbance can be measured with a spectrophotometer. Different solutions can be used to dissolve the foramazan including dimethyl sulphoxide or sodium dodecyl sulfate, and the solution used will adjust the best wavelength for spectrophotometry (Zhang et al., 2002). This method can become rather efficient as the optical densities can be recorded with the use of a microplate reader. The MTT assay can provide a consistent measure of cell viability in bacterial cells that is quantifiable and can be measured in a timely fashion.

Specific Aims

1. To create a model system for testing microbial efficacy of root canal irrigants against a complex biofilm on hydroxyapatite discs.
2. To evaluate the effectiveness of IKI on biofilm disinfection.
3. To determine what the minimum time point is for proper disinfection of the model system with IKI.
4. To provide the clinician a time point that they may use for their protocol in the use of IKI during disinfection of the root canal system.

Hypothesis

As the exposure time of IKI decreases, the antimicrobial activity will also decrease as measured by MTT absorbance.

Null Hypothesis

There will be no difference between the antimicrobial activity of IKI at different time points as measured by absorbance of MTT.

Materials and Methods

Bacterial Sample

Saliva and plaque samples were collected from pediatric dentistry patients that presented with a history of early childhood caries during an ongoing project approved by the University of Minnesota's Institutional Review Board. Five milliliters of saliva was collected and stored on ice. Then a plaque sample was collected from the interface around tooth structure and existing composite restorations and placed in 1.0 mL of liquid dental transport medium (AS-916 Anaerobe systems) and stored on ice.

Biofilm Reactor

The biofilm reactor is the same as described in Chen et al, 2012, and it is an *ex vivo* model designed to assess the proliferation of oral biofilms on dental materials. The preparation of the experiment is carried out in a AC600LFUV HEPA clean workstation in a fume hood. The whole biofilm reactor is a closed system and all vent tubes are capped with a 0.2μm AERVENT cartridge filter and vented inside the fume hood as shown in Figure 1 below. This reactor design allows growth media to be flowed through a glass vessel which is stirred to produce shear forces.

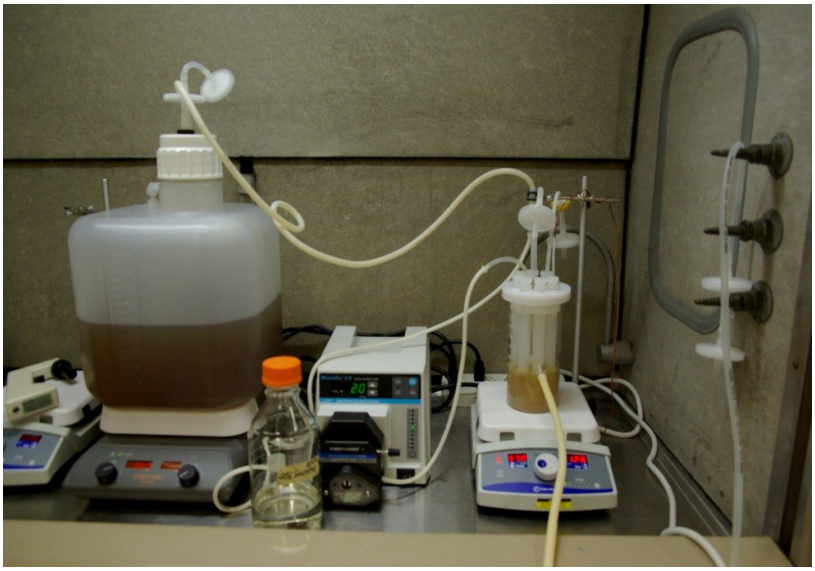

Figure 1: Bioreactor courtesy Dr. Joel Rudney, University of Minnesota

The biofilm is grown on removable hydroxyapatite discs (HA, Clarkson Chromatography, Williamsport, PA) measuring 12 mm in diameter and 3 mm thick. Three discs are mounted into special rods that allow the discs to be suspended in the reactor vessel (Figure 2).

Figure 2: Rods to suspend discs in the bioreactor. The lowest of the rods contains the hydroxyapatite discs used in this experiment. Courtesy Dr. Joel Rudney, University of Minnesota

Sample Preparation

The saliva sample is centrifuged at 10,000 rpm for 10 minutes at 4º C twice. The supernatant is then diluted with Gibbon's Buffer (Appendix 1), a buffer simulating the ionic composition of saliva, at a 1:1 ratio and filtered sterile with a 0.2 µm Sterile Millex Filter Unit (Millipore, Bellerica, MA). The plaque sample is then vortexed for 20 seconds. One sample was obtained from a frozen stock prepared from a previous biofilm grown on up to 12 hydroxyapatite discs in the same reactor from a single subject.

Biofilm Formation

The HA discs are sterilized by autoclave and then coated with 30 µL of the saliva solution to simulate a salivary pellicle to facilitate colonization and then coated with 30 µL of the plaque sample solution. 350 mL of basal mucin medium (BMM) (Appendix 1) is placed in the glass biofilm reactor vessel. The rods holding the HA discs are suspended in the medium. Samples are then incubated at 37º C overnight with a stirbar set to stir at 125 rpm. This allowed the biofilms to establish themselves on the surface of the disc under shear conditions.

The next day the biofilm has fresh medium flowed into the vessel at a set rate of 17 mL/minute to allow for removal of planktonic bacteria. Temperature is maintained at 37º C and stirred at 125 rpm. The reactor was pulsed with 20 % sucrose five times to simulate fermentable carbohydrate consumption. At approximately 48 total hours of biofilm growth, the discs are removed from the reactor. Each disc was placed in its own 50 mL centrifuge tube for treatment with each irrigant protocol.

Irrigant Protocols

Three irrigants were used for testing on the biofilms. Phosphate Buffered Saline (PBS) was used as a negative control group. Original Clorox (The Clorox Company, Oakland, CA) at full strength was used for 6.0 % sodium hypochlorite (NaOCl) as the positive control group. The final irrigant used was 2 % IKI (iodine 2 %; potassium-iodide 4 %; and distilled water 94 %) (Ricca Chemical Company, Arlington, TX). The six total treatment groups are as follows:

1X PBS for 10 minutes
6.0 % NaOCl 10 minutes
2 % IKI for 1 minute
2 % IKI for 5 minutes
2 % IKI for 10 minutes
2 % IKI for 20 minutes

Treatment times were established during multiple pilot studies.

Antibacterial Testing

One biofilm coated disc was subjected to a treatment group. Two milliliters of each irrigant was added to the 50 mL centrifuge tube containing the disc. The 2 mL was enough to completely submerge the disc. After the allotted time depending on the treatment group, the irrigant was aspirated and each disc was washed three times with 1X PBS to completely remove any residual irrigant.

Biofilm Assessment

The treated biofilm was mechanically removed from each disc and resuspended in 1.5 mL of spun down media (SDM). SDM was created by centrifuging BMM at 10,000 rpm for 5 minutes to remove the mucin component. SDM was used based on pilot studies that showed increased viability of remaining bacteria without adverse optical density issues. This cell suspension was centrifuged at 2,600 rpm for 2 minutes and resuspended in 1.0 mL of SDM to create a stock solution.

Viability Testing

Viability testing was done with the Vybrant MTT Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA). A 12 mM stock MTT solution was made according to manufacturer's directions with sterile PBS. A 1:10 dilution of the biofilm cell suspensions was created by diluting 40 µL into 360 µL of SDM. This dilution was necessary based on previous pilot studies. 40 µL of stock MTT was added to each cell suspension per manufacturer's instructions. A negative control was also created by adding 40 µL of MTT to 400 µL of SDM. Cells and a corresponding blank with no MTT were incubated at 37ºC for 2.5 hours. After the incubation cells were pelleted by centrifuging at 2,600 rpm for 2 minutes. 300 µL of the supernatant was removed and 200 µL of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) was added and triturated. (Zhang and Liu, 2002) This was incubated at 37º C for 10 minutes and then 100 µL was placed in a clear 96-well plate in triplicate. Optical density was read by a

Synergy HT microplate reader (Biotek Instruments Inc., Winooski, VT) at 530 nm. (Zhang and Liu, 2002)

Crystal Violet

Crystal Violet was used to indicate the amount of remaining bioburden present after treatment by the irrigants. The remaining biofilm stock solution (0.920 mL) was centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed, and 0.5 mL of 0.1 % crystal violet solution was added to each tube and incubated at room temperature with shaking at 125 revolutions per minute. Samples were again centrifuged at 10,000 rpm for 5 minutes and rinsed twice with 1X PBS. 1.0 mL of 30 % acetic acid was then added in each tube and incubated for 15 minutes at room temperature to allow the dye to solubilize. A 1:10 dilution was made and 300 μL was added to a well in a clear 96-well plate in triplicate. Optical density was read by a Synergy HT microplate reader (Biotek Instruments Inc., Winooski, VT) at 600 nm.

Scanning Electron Microscopy

One experiment was devoted to imaging with scanning electron microscopy (SEM). Biofilms were grown on discs and treated by each group exactly as above. After treatment by each irrigant protocol and 3 washes with 1X PBS, the discs were fixed with primary fixative (Appendix 1) at room temperature for 60 minutes. Discs were washed with 0.1M sodium cacodylate buffer for 5 minutes. A secondary fixation was done with 1 % OsO_4 in 0.1M sodium cacodylate buffer for 60 minutes. Discs were washed with 0.1M sodium cacodylate buffer for 5 minutes. The discs were then dehydrated by placing

in increasing concentrations of ethanol for 5 minutes at each concentration. The concentrations of ethanol were 50 %, 70 %, 80 %, 95 % and 100 %. Critical point drying with carbon dioxide was then done with the Samdri-780 Critical Point Dryer (Tousimis, Rockville, MD). Samples were then coated with platinum for 30 seconds using a DV502A Au/Pd sputter coating unit (Denton Vacuum, LLC, Moorestown, NJ). Imaging was then done with a S-4700 SEM unit (Hitachi High Technologies America, Inc., Irving, TX).

Statistical Analysis

Means and standard deviations were calculated for the outcomes. Analysis of variance (ANOVA) was used to compare the group means for each measure type. If the overall ANOVA was significant ($p < 0.05$), pairwise comparisons were made using a Tukey-Kramer adjustment. P-values less than 0.05 were considered statistically significant. SAS V9.1.3 (SAS Institute Inc, Cary, NC) was used for the analysis.

Results

MTT

Average optical density values for viability shown in Table 1 were analyzed in two ways. The first method was to subtract the overall blank, MTT in SDM, from each optical density value, and the second way was to add an extra measure of control so that each group had its own blank of the cell suspension in SDM but no MTT. The n for the 20 minute IKI group was only 4 as one run of the experiment did not include this treatment group. After ANOVA and pairwise comparisons, both showed a significant difference between the PBS treated control group and all other groups ($p < 0.0001$). No significant differences were found between any of the other groups with p-values ranging from 0.0947 to 0.99 (Appendix 2). As measured by MTT under the conditions of this experiment, there was no significant difference in viability of the biofilm after treatment with 1 to 20 minutes of 2 % IKI and 10 minutes of 6.0 % sodium hypochlorite. However, while not statistically significant, a trend can be observed in Figures 3 and 4. The shorter the IKI treatment time, the larger the optical density tended to be.

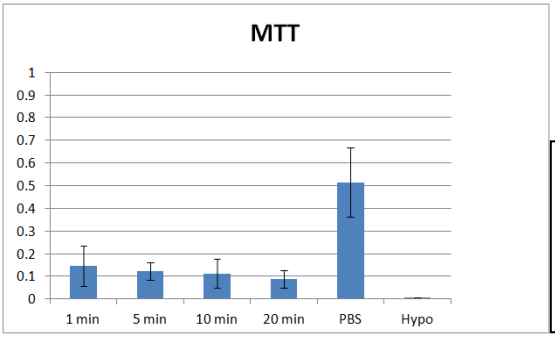

Figure 3: MTT Optical density at 530nm of IKI treated biofilm with PBS and Hypochlorite controls.

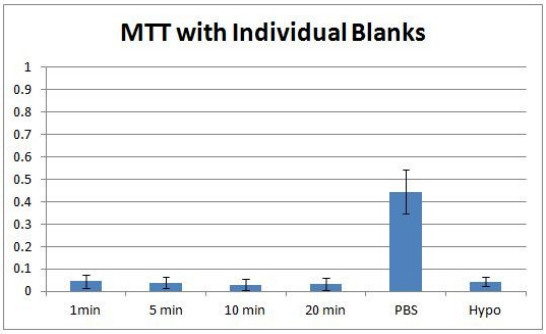

Figure 4: MTT Optical density at 530nm of IKI treated biofilm with PBS and Hypochlorite controls with individual blanks per treatment group.

Table 1. Mean (SD) by Type and Group

Type	Group	N	Mean	SD
MTT	1min	5	**0.146**	0.088
	5 min	5	**0.122**	0.038
	10 min	5	**0.113**	0.064
	20 min	4	**0.088**	0.039
	PBS	5	**0.514**	0.154
	Hypo	5	**0.003**	0.003
MTT individual blank	1min	5	**0.044**	0.030
	5 min	5	**0.039**	0.025
	10 min	5	**0.029**	0.024
	20 min	4	**0.031**	0.028
	PBS	5	**0.445**	0.099
	Hypo	5	**0.044**	0.019
Crystal Violet	1min	5	**0.466**	0.185
	5 min	5	**0.450**	0.189
	10 min	5	**0.424**	0.151
	20 min	4	**0.403**	0.107
	PBS	5	**0.402**	0.156
	Hypo	5	**0.080**	0.056

Crystal Violet

Average values for crystal violet optical densities measured at 600 nm are shown in Table 1. The crystal violet number will correspond to the remaining bioburden remaining after each treatment. After ANOVA and pairwise comparisons, the sodium

hypochlorite group was significantly lower than all other treatment groups ($p < 0.04$ for each comparison). No significant differences were found between any of the other groups with p-values greater than 0.98 (Appendix 2). Again while not statistically significant, there was a slight decrease in mean optical density of crystal violet as the application time of 2 % IKI increased (Figure 5). 2 % IKI, regardless of time of application, had little effect on removal of bioburden, while 10 minutes of sodium hypochlorite was significantly successful.

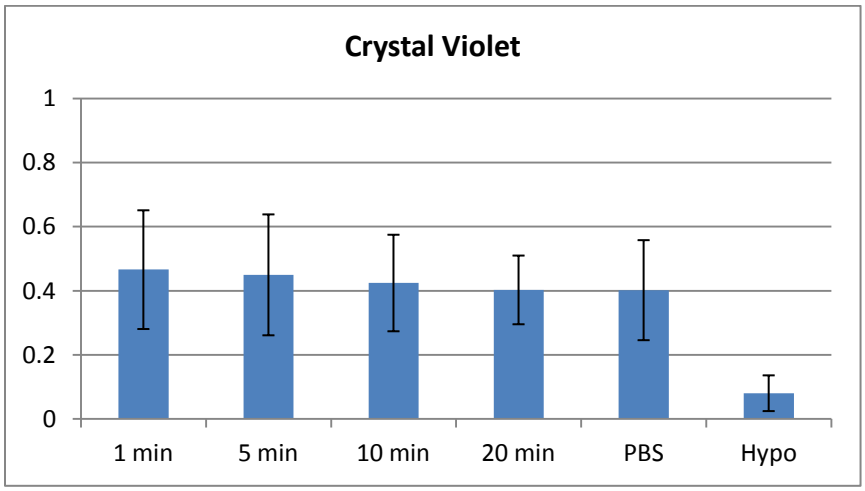

Figure 5: Crystal Violet Optical density at 600nm of IKI treated biofilm with PBS and Hypochlorite.

SEM

Figures 6 through 11 are SEM images at different magnifications for each treatment group. Figure 6 is the PBS treated control that demonstrates the extensive and thick nature of the biofilms. Cracking between large segments of biofilm is artifact from the dehydration process. Chains of cocci are readily apparent in a thick network of other

biotypes and extracellular matrix. Due to the large volume of biofilm present, increased magnification past 1,000X was difficult due to surface charging. A striking difference is noted in Figure 7 with the sodium hypochlorite treated discs. Very little remnants of the original biofilm remain. Increased magnification shows a small amount of debris of the extracellular matrix and with no intact bacteria present. Figure 8 are images from the disc treated for 1 minute with IKI. The 30X image is representative of the side, however, the remaining images which show nice cocci and diplococci are not representative of the entire disc and were taken from a portion of the side where less surface charging was present. Figures 9 and 10 are of the 5 and 10 minute IKI treatments and do not show many differences from the PBS control group. Figure 11, representing the 20 minute IKI group, showed an interesting phenomenon. At the gross 30X view, the biofilm looks similar to control, but when magnification was increased, the surface layer showed areas of lighter shaded debris that may be from some breakdown of the outer layer of the biofilm from the extended exposure to IKI. SEM analysis like the crystal violet data revealed the inability of IKI to remove biofilm regardless of treatment time. However, 10 minutes of 6 % sodium hypochlorite was highly effective at biofilm removal.

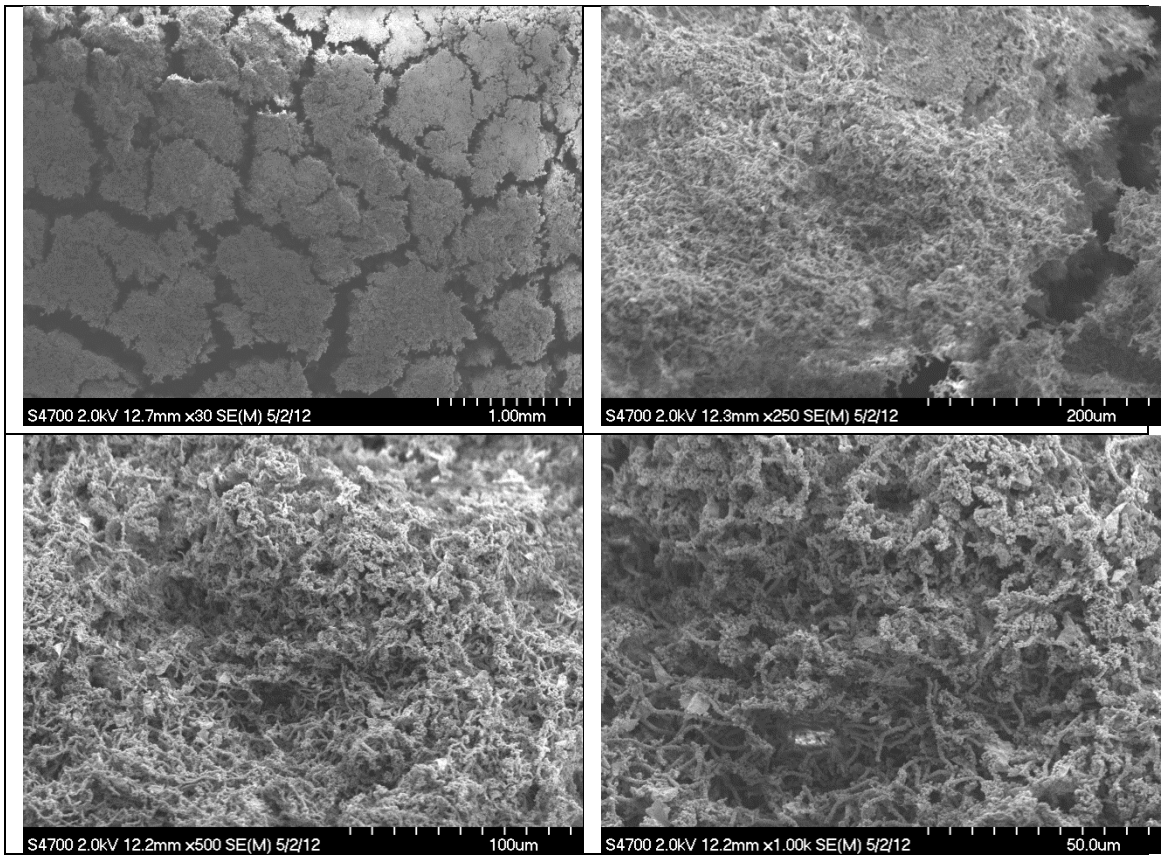

Figure 6: SEM images of biofilm PBS control showing extensive biofilm formation. Top Left: 30X. Top Right: 250X. Lower Left: 500X. Lower Right 1,000X.

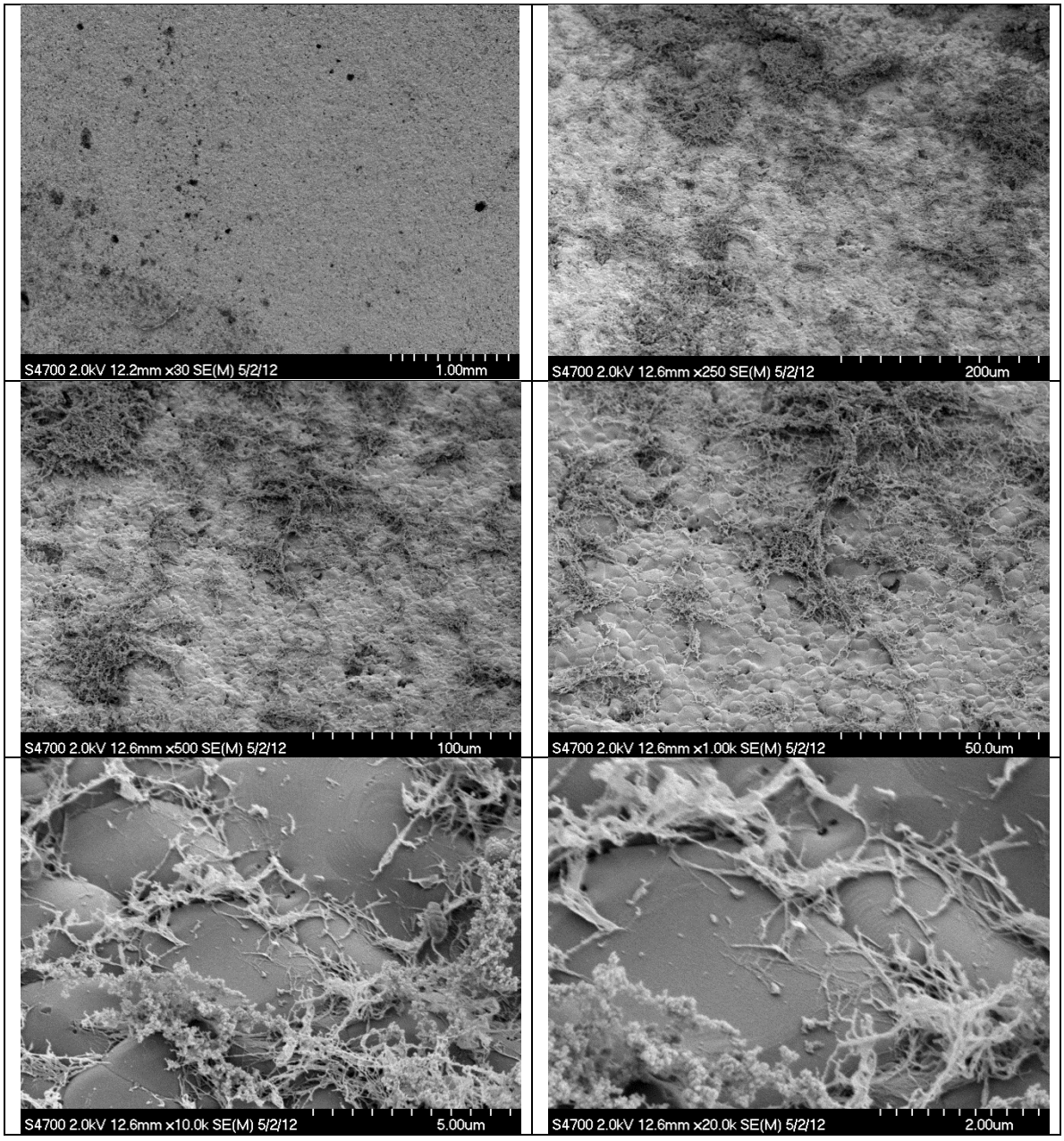

Figure 7: SEM images of biofilm treated with 6% NaOCl demonstrating excellent ability to remove biofilm with some residual debris present. Top Left: 30X. Top Right: 250X. Middle Left: 500X. Middle Right 1,000X. Lower Left: 10,000X. Lower Right: 20,000X.

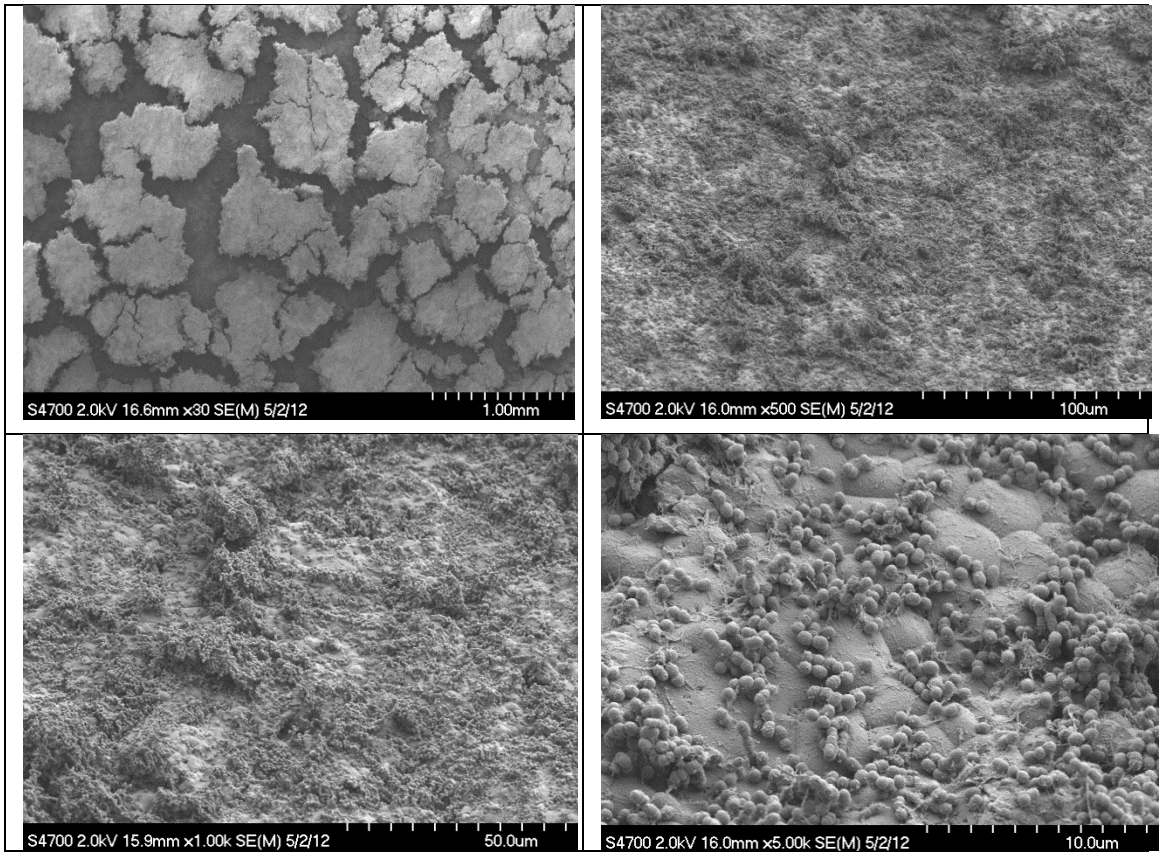

Figure 8: SEM images of biofilm treated with 2% IKI for 1 minute showing extensive biofilm remaining with magnified images of cocci and diplococci from a non-representative portion of the hydroxyapatite disc. . Top Left: 30X. Top Right: 500X. Lower Left: 1,000X. Lower Right: 5,000X.

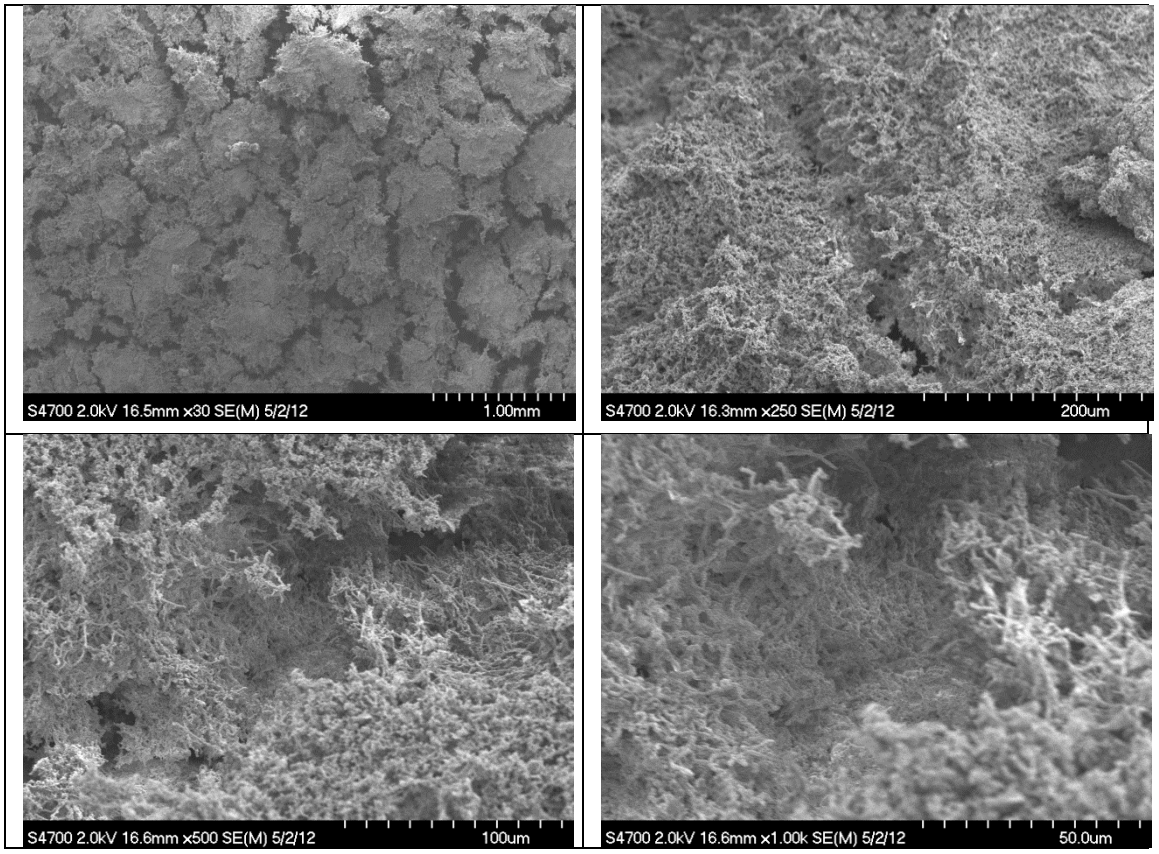

Figure 9: SEM images of biofilm treated with 2% IKI for 5 minutes showing extensive biofilm remaining. Top Left: 30X. Top Right: 250X. Lower Left: 500X. Lower Right: 1,000X.

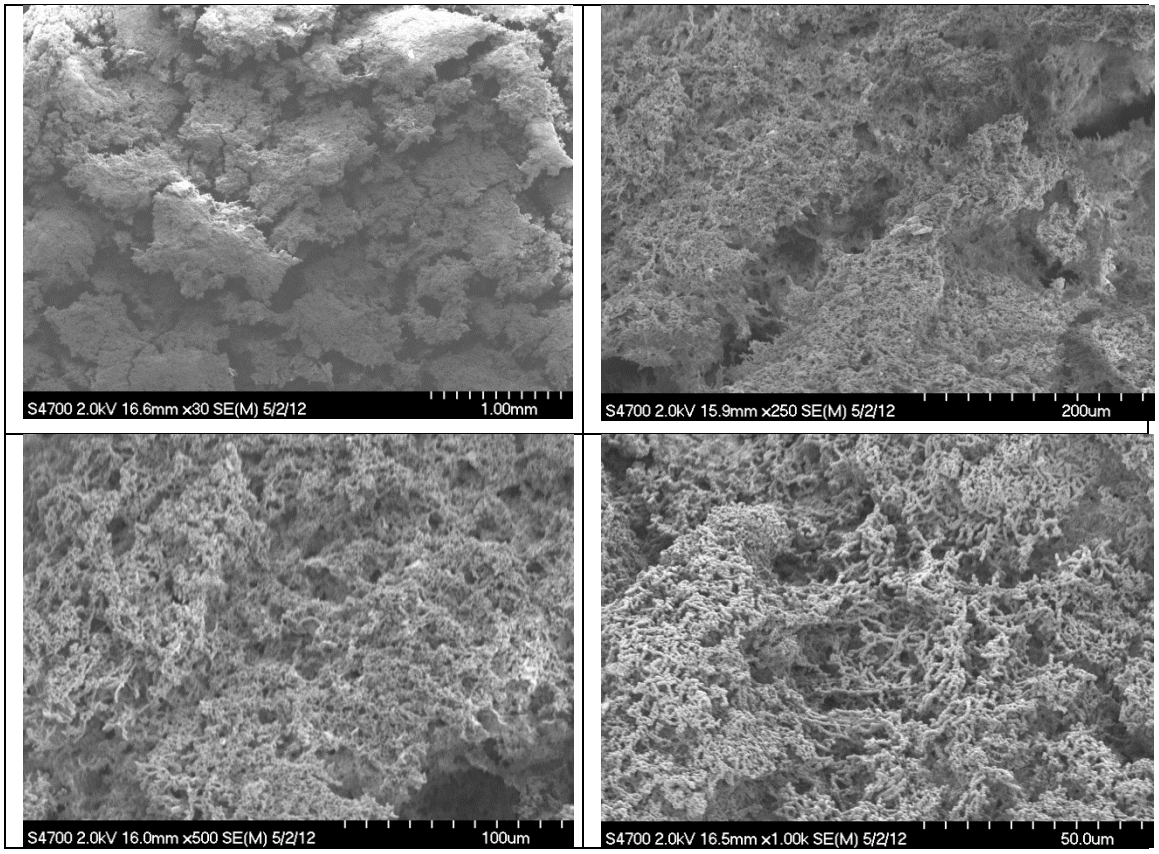

Figure 10: SEM images of biofilm treated with 2% IKI for 10 minutes showing extensive biofilm remaining. Top Left: 30X. Top Right: 250X. Lower Left: 500X. Lower Right: 1,000X.

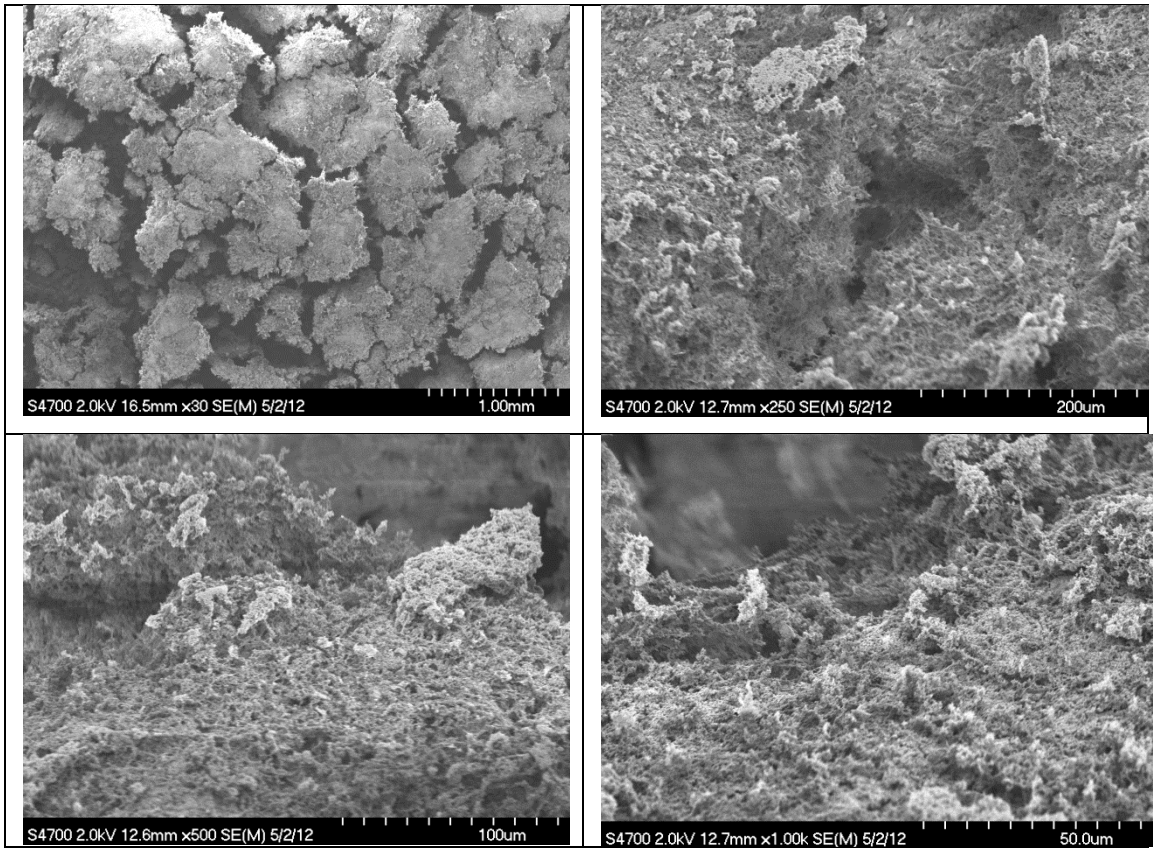

Figure 11: SEM images of biofilm treated with 2% IKI for 20 minutes showing extensive biofilm remaining. Magnified images reveal surface debris that may have resulted from breakdown of the outer biofilm layer with the extended exposure time. Top Left: 30X. Top Right: 250X. Lower Left: 500X. Lower Right: 1,000X.

Discussion

The goal of this study was to demonstrate the ability of the bioreactor at the University of Minnesota to serve as a model to test endodontic irrigants. Within the scope of the study the model did prove successful. It is able to provide a complex and diverse biofilm in a short amount of time (48 hours) that irrigants can be easily applied without dilution for analysis of viability. The biofilms used in this study are reported to have an average of 25 species and grow to a thickness of 300-500 μm as measured with cross-polarization optical coherence tomography (Rudney et al.; Chen et al, 2012). The species represented in our biofilm, however, may not reflect a true endodontic biofilm. Clegg grew their biofilms from paperpoint samples of necrotic teeth with lesions (Clegg et al, 2006) which is more likely to be representative. Shen sampled from subgingival plaque which also may not be completely representative of an endodontic biofilm, but will likely contain a higher population of anaerobic species (Shen et al, 2010). Our biofilm was sampled from supragingival plaque on the margins of composite restorations in pediatric patients to take advantage of an ongoing project. As such, our biofilm make-up may less resemble an endodontic biofilm. Another possible disadvantage of this biofilm system is that it is not grown under anaerobic conditions. Despite the aerobic conditions, the biofilms produced do support growth of anaerobic species (Rudney et al.). This is likely due to initial protection by facultative partners followed by anaerobic growth as the thickness of the biofilm increased and oxygen is consumed by the facultative species (Rudney et al.). The addition of sucrose pulses during the growth of this biofilm may have not been ideal for representing an endodontic biofilm. While some

species increased in relative abundance, some species were lost from the biofilm. The average number of species present drops from 39 to 25 with the sucrose pulsing (Rudney et al.). In addition, potentially important species like *Actinomyces* and *Prevotella* are lost during pulsing (Rudney et al, in publication). Future studies for endodontic irrigants or medicaments should likely omit the sucrose pulsing aspect. This biofilm does share some common species with endodontic infections. *Prevotella, Actinomyces, Streptococcus, Fusobacterium, Tannerella,* and *Veillonella* were all prevalent in the majority of microcosms grown with this reactor system (Rudney et al.). Even though this biofilm may not fully represent an endodontic biofilm, it likely provides much more insight into the efficacy of a medicament or irrigant than studies that rely on a single species or a limited number of species.

Thickness and maturity of a biofilm may also be important factors in testing irrigants on biofilm. In biofilms created with no shear forces it took 3 weeks to grow biofilm to 155 μm thick and 12 weeks to reach 201 μm (Shen et al, 2011). This biofilm reactor will grow biofilms in excess of 300μm in 2 days. The thickness of the biofilm and the extracellular polysaccharide matrix will definitely play a role in the time required for an endodontic irrigant to diffuse through and disinfect or dissolve that mass. It is unknown how thick the typical endodontic biofilm is, and there is likely a wide range depending on the tooth and dominant species involved. Maturity of the biofilm may also be a question at only 48 hours of growth. Shen found that 3 weeks was required for a mature biofilm in their stagnant biofilm model (Shen et al., 2011). This can be a potential problem because mature biofilms are more resistant to antimicrobial action than

immature (Shen et al., 2011). The thickness of this biofilm however, perhaps compensates for that issue.

These biofilms were grown with fresh media and a surplus of nutrients to encourage growth. Nutrient starved biofilms have been shown to be more resistant (Shen et al., 2010). Nutrient deprivation may be more representative of an endodontic biofilm due to the nature of the root canal environment. The longer the tooth has been necrotic, the more likely nutrient deprivation is a factor. It may be prudent in future studies to limit the nutrients for a period of time before testing.

IKI had not been previously tested on a biofilm model. This study confirmed that its broad spectrum and powerful antimicrobial actions demonstrated in previous studies (Spangberg et al., 1973; Orastavik et al., 1990; Safavi et al., 1990) are effective at killing bacteria in a thick biofilm. There was no significant difference between the viability of biofilms treated with IKI for any time point and 6 % sodium hypochlorite for 10 minutes as measured with MTT.

While no statistical significance was found under the conditions of this study, there was a noticeable trend in the data as shown in Figure 3. The lower the time of application of IKI, the higher the viability appeared. The sodium hypochlorite had the lowest of the MTT optical density values. In an effort to compensate for a potential increase in background of the IKI treated samples due to the presence of more bioburden and the possibility that the IKI increased background by staining the cells, each sample also had a blank with no MTT applied. Figure 4 represents the data with that extra

control factor. This brought the optical density values of the IKI treated samples into the same range as the sodium hypochlorite control. That however did not remove the trend across the 1 minute, 5 minute, and 10 minute application times. This trend is concerning because it may reflect a small number of viable bacteria present in the lower treatment time groups that the MTT assay was not sensitive enough to detect.

If a small amount of viable bacteria do remain, it is likely due to the time required for the IKI to diffuse through the thick biofilm. In many endodontic situations the biofilm will likely not be as thick as in this study. The most common applications of IKI are as an intracanal medicament mixed with calcium hydroxide where exposure time is more than adequate for diffusion through a biofilm, or as an intra-appointment dressing applied after initial cleaning and shaping with use of sodium hypochlorite and EDTA where a significant portion of the biofilm has already been removed or disrupted.

MTT was chosen for this study due to its ability to quantify the amount of viable cells remaining after treatment. Colony forming unit counts are less reliable due to the unknown growth conditions of many of the bacteria present. Viability fluorescence staining with the use of a confocal laser scanning microscope has been shown to be more reflective of true viability since stresses on a bacterial cell that may still be viable can prevent it from replicating (Shen et al., 2010). Viability staining was not a practical option for this study due to difficulties encountered with the stain penetrating to the deepest layers of the biofilm. Therefore, MTT was an acceptable alternative that allows for more efficient data collection and because it is a common and proven assay for determining viability of bacteria (Zhang and Liu, 2002).

It is possible that the MTT testing conditions of this study were not sensitive enough to distinguish between the small numbers of bacteria left across the treatment groups. The conditions were originally chosen to accurately reflect the positive and negative control differential. Future MTT testing conditions in this biofilm model would need to be altered either by increasing the cell concentration treated with MTT or perhaps by increasing the incubation time with the MTT to allow for more formazan crystal formation.

While IKI successfully killed the biofilm, it failed to remove it as evidenced by the crystal violet measurements of remaining bioburden. There was no significant difference between any of the IKI treatment groups and the PBS treated control group (Figure 5). 6.0 % sodium hypochlorite performed well as an effective irrigant for dissolution of the biofilm as demonstrated in previous studies (Clegg et al., 2006). This was confirmed with SEM that showed a large biofilm mass present on the PBS control and all of the IKI treatment groups (Figures 6-11). The sodium hypochlorite treated disc showed no intact biofilm remaining (Figure 7). It had large areas with bare hydroxyapatite present with some small areas of debris. No intact bacterial cells were noted. The debris was likely remnants of bacterial cells and the polysaccharide extracellular matrix. Clegg showed that 15 minutes of 6 % sodium hypochlorite completely removed their biofilm from dentin disks (Clegg et al., 2006). It is likely that a slightly longer application time of the sodium hypochlorite would have dissolved the last remaining remnants of the biofilm in this study.

While not statistically significant, a trend was noted across the IKI treatment groups with the crystal violet results that was not expected (Figure 5). All of the IKI treated samples had an average crystal violet optical density higher than the PBS control. This may have resulted from extra background absorbance from IKI stained cells. As the time of IKI application increased, the average crystal violet optical densities decreased. This was not expected because IKI is not thought to have tissue dissolution abilities (Spangberg et al., 1979; Haapasalo et al, 2010). It is possible that the longer IKI treatments made the outer layers of the biofilm easier to detach and were lost during the PBS washes and fixation process. The SEM results do not demonstrate dissolution of the biofilm except for possibly in the 20 minute IKI disc (Figure 11) which shows brighter appearing areas of debris on the surface of the biofilm. These areas may be remnants of bacterial cells and the extracellular polysaccharide matrix that clumped on the surface. If IKI does have any tissue dissolution properties it appears to be minimal especially when compared to the sodium hypochlorite results.

This study was able to demonstrate the superior antimicrobial ability of IKI on biofilms and confirmed its poor ability to dissolve tissue and remove biofilm. Due to its inability to remove biofilm or tissue remnants, IKI is not advised for use as a primary irrigant. Its powerful antimicrobial activity combined with lower comparative toxicity makes it a suitable choice for further disinfection protocols after cleaning and shaping with an irrigant like sodium hypochlorite. We sought to define a time point for application of IKI in this fashion and found no statistically significant difference between application times ranging from 1 to 20 minutes. However, due to the trend of increasing

viability with decreasing application time, it would be advisable to use IKI for as long as is clinically prudent.

Conclusions

1. The bioreactor at the University of Minnesota can be utilized for growth of thick and complex polymicrobial biofilms suitable for testing the efficacy of antimicrobial and biofilm removing endodontic irrigants.
2. The antimicrobial efficacy of 1, 5, 10 and 20 minute applications of 2 % IKI was statistically not significantly different from 10 minutes of 6 % sodium hypochlorite.
3. 6 % sodium hypochlorite for 10 minutes was superior to all time points of 2 % IKI in removal and dissolution of biofilm.
4. Due to the inability of 2 % IKI to remove biofilm it is not advised for use as the primary endodontic irrigant but may be a successful as an adjunctive irrigant to disinfect any residual biofilm that remains after cleaning and shaping.
5. IKI may show potential as an intracanal medicament for species like *E. faecalis* that may be resistant to calcium hydroxide due to its powerful antimicrobial activity through biofilms.

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Appendix 1 – Media preparations

Courtesy Ruoqiong Chen

1x Gibbon's Buffer:

KCl	50mM
$CaCl_2$	1.0mM
$MgCl_2$	0.1mM
K_2HPO_4	1.0mM

BMM medium:

Chemicals:		
Partially purified pig gastric mucin	2.5 g/l	50.0 g/20L
Triplicate peptone	5.0 g/l	100.0 g/20L
Protease peptone	10.0 g/l	200.0 g/20L
Yeast extract	5.0 g/l	100.0 g/20L
KCl	33.5 mmol	49.95 g/20L
Hemin	2.5 mg/l	0.05 g/20L
autoclave		
Menadione	5.8 mmol	19.98 mg/20L
urea	1.0 mmol	1.20 g/20L
arginine	1.0 mmol	3.48 g/20L

Primary Fixative

2% glutaraldehyde

0.1M Sodium cacodylate buffer

0.15% alcian blue

Appendix 2: Statistical Analysis

MTT Blanks: ANOVA p-value <0.0001. In the pairwise comparisons, the mean for the PBS group was statistically significantly different from all other group means ($p < 0.0001$ for each comparison). P-values for all other comparisons were greater than 0.99.

MTT No Blanks: ANOVA p-value <0.0001. In the pairwise comparisons, the mean for the PBS group was statistically significantly different from all other group means ($p < 0.0001$ for each comparison).

1 min vs. Hypo: p=0.0947

5 min vs. Hypo: p=0.2240

10 min vs. Hypo: p=0.3022

20 min vs. Hypo: p=0.6260

P-values for all other comparisons were greater than 0.89.

CV: ANOVA p-value =0.0044. In the pairwise comparisons, the mean for the Hypo group was statistically significantly different from all other group means ($p < 0.05$ for each comparison).

1 min vs. Hypo: p=0.0055

5 min vs. Hypo: p=0.0082

10 min vs. Hypo: p=0.0153

20 min vs. Hypo: p=0.0395

PBS vs. Hypo: p=0.0262

P-values for all other comparisons were greater than 0.98.