EFFECT OF ETHYLENEDIAMINETETRAACETIC ACID PRE-TREATMENT ON BIOFILM DISSOLUTION BY SODIUM HYPOCHLORITE

by

Joshua Ananthan

D.D.S. University of Toronto 2016

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Craniofacial Science)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

August 2018

The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:
Effect of ethylenediaminetetraacetic acid pre-treatment on biofilm dissolution by sodium hypochlorite

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submitted by Joshua Ananthan in partial fulfillment of the requirements for
the degree of Master of Science
in Craniofacial Science

Examining Committee:
Dr. Markus Haapasalo, Department of Oral Biological & Medical Sciences
Supervisor

Dr. Ya Shen, Department of Oral Biological & Medical Sciences
Co-Supervisor

Dr. Adriana Manso, Department of Oral Biological & Medical Sciences
Supervisory Committee Member

Dr. Vincent Lee, Department of Oral Health Sciences
Additional Examiner

Abstract

Introduction: Endodontic treatment is aimed at the management and treatment of pathoses caused by bacterial colonization of the root canal system; this is primarily achieved through mechanical and chemical means. Various irrigants have been introduced in varying degrees, concentrations and combinations to optimize bacterial eradication, however, there is no well established optimal protocol nor a recommended procedure by a regulatory body. The present study aims to evaluate the effect of ethylenediaminetetraacetic acid (EDTA) pre-treatment on sodium hypochlorite's ability to dissolve biofilm.

Materials and Methods: Hydroxyapatite discs were inoculated with subgingival plaque and biofilm was grown in brain-heart infusion broth for three weeks in anaerobic conditions. Samples were exposed to a pre-treatment of either sterile water or 17% EDTA for 30 or 60 seconds, washed with an intermediate sterile water rinse, and subjected to either 2% or 6% sodium hypochlorite. Samples were examined under stereomicroscopy for complete dissolution of the biofilm; time was recorded from initial exposure to sodium hypochlorite to complete dissolution.

Results: Biofilm dissolution time was decreased by increasing the concentration of sodium hypochlorite from 2% to 6% for all matched groups. Pre-treatment with 17% EDTA for 30 seconds decreased the time for biofilm dissolution by sodium hypochlorite while pre-treatment with 17% EDTA for 60 seconds increased the time for biofilm dissolution; this effect was conserved between both 2% and 6% concentrations of sodium hypochlorite.

Discussion: Overall, pre-treatment of biofilms with 17% EDTA will alter sodium hypochlorite's dissolution ability. Shorter exposure times may augment NaOCl biofilm dissolution while long exposure times may be detrimental to sodium hypochlorite's dissolution ability. Regardless, of

length of pre-treatment time, sodium hypochlorite at both 2% and 6% concentrations was eventually able to complete dissolve all biofilm in all samples.

Lay Summary

Endodontic treatment, primarily root canal therapy, is aimed at the treatment and prevention of diseases of the root canal system and its adjacent or contiguous tissues caused by bacterial invasion and colonization within the root canal system. This is primarily achieved via chemical disinfecting solutions and irrigants which act to dissolve, destroy or remove bacteria and their associated community structures. While many different irrigants exist within clinical endodontics, there is no established optimal protocol for the most efficient and predictable cleansing of the root canal system.

The present study was undertaken to evaluate the role of irrigant sequence and exposure time on the ability to dissolve and completely remove bacteria and their associated structures. The use of ethylenediaminetetraacetic acid as a pre-treatment irrigants on biofilms significantly affects the ability of sodium hypochlorite to dissolve biofilm.

Preface

This dissertation, “Effect of ethylenediaminetetraacetic acid pre-treatment on biofilm dissolution by sodium hypochlorite” is an original, independent, and unpublished work by Joshua Ananthan perfomed under the supervision and guidance of Dr. Markus Haapasalo and Dr. Jolanta Aleksejuniene.

All thesis work was completed by Joshua Ananthan including design, experiments, data analysis and writing. Dr. Markus Haapasalo contributed to the study design and thesis editing. Dr. Jolanta Aleksejuniene provided guidance and editing for statistical analysis. The relative contributions to this research project by Joshua Ananthan was 80%.

Approvals for research regarding informed consent and biofilm protocols were granted by the University of British Columbia Clinical Research Ethics Board (H12-02430, entitled “Dynamics of oral biofilms and mechanical characteristics of instruments”).

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List of Abbreviations

ANOVA: Analysis of variance

BHI: Brain heart infusion

EDTA: Ethylenediaminetetraacetic acid

NaOCl: Sodium hypochlorite

RT: Room temperature

Acknowledgements

This thesis is the culmination of a great deal of effort, time and deliberation not only of myself, but those around me and especially the many wonderful people at UBC.

I would like to firstly express many thanks to my supervisor, Dr. Markus Haapasalo, whose tireless assistance and dedication to myself and this project were unquestioningly fundamental in it's inception, progress and completion. I would like to thank Duo Zhang for his assistance in the laboratory and willingness to help through the many months of my experimentation.

Furthermore, I would like to thank Dr. Jolanta Aleksejuniene who graciously and adeptly helped me with the understanding, execution and interpretation of the data and statistics. The three of you surely made this project a reality and greatly facilitated its completion.

I would like to thank the entire graduate endodontics program including the instructors, staff, residents and laboratory personnel who have been helpful, insightful and supportive in my journey and research endeavours at UBC.

Dedication

I dedicate this work to my family and Sabrina; all of you have been patient, supportive and understanding throughout my educational and academic pursuits and without any of you, I would not be where I am today.

I would like to single out my father, whose example as both a person and dentist have provided ideals to which I strive to achieve in life, my education, and in clinical practice; your advice and guidance have always proved instrumental in my success and fortune.

Finally, Sabrina, without your dedication, care and unlimited patience for me over this period of my life, I surely would have failed. You have been the rock to which I gravitate around and my base of stability during this exciting and demanding chapter of my life. My accomplishments and hard work are as much the result of your persistence as they are mine.

Thank you.

Chapter 1: Introduction

1.1 Endodontics and biofilms

Biofilms are organized communities of microorganisms embedded in an extracellular polymeric matrix of secreted macromolecules such as proteins, lipids and polysaccharides (1). Biofilms can develop on any surface after stable attachment of microorganisms and within dentistry have been indicted as the cause for many diseases (1-3). The resident microflora of the oral cavity are prone to and frequently favour growth and development in biofilm masses on the dental hard tissues in the form of dental plaque and can lead to dental disease including caries and periodontal diseases (4). Furthermore, invasion of these bacteria into the pulpal space can lead to endodontic infection and like in the oral cavity, these microbes favour a biofilm organizational model (1).

Treatment of endodontic disease has traditionally aimed at management of these complicated microbial colonies which can not only establish and develop themselves onto the root canal walls, but also within irregularities and dentin tubules and persist leading to sequential inflammation of the pulpal tissue, pulpal necrosis and eventual apical periodontitis (1, 5). Management of these biofilms is typically achieved by orthograde mechanical instrumentation and chemical disinfection of the root canal system (5, 6).

1.2 Endodontic management of biofilms

While mechanical instrumentation of the root canal system presents a significant clinical challenge in endodontic therapy, the ultimate goal of mechanical instrumentation is to facilitate chemical disinfection by allowing sufficient space for irrigant action and penetration as mechanical instrumentation often leaves a significant surface area and volume of the root canal

system untouched (7-9). An ideal irrigating solution provides ample washing action to remove debris, acts as a lubricant to reduce instrument friction during preparation, facilitates dentin removal by the instrument, dissolves the organic matter of pulp tissue, biofilm and dentin, dissolves inorganic tissue in the form of dentin and biofilm, penetrates the anatomic complexities and irregularities of the root canal system, kills bacteria and yeasts in planktonic and biofilm forms, does not irritate or damage vital periapical tissue, has no caustic or cytotoxic effects and does not weaken the tooth structure (5, 10). To date, no single irrigating solution possesses all of these properties, however, the combination of a select few irrigants allows clinicians to achieve their goal of root canal disinfection (10).

Sodium hypochlorite (NaOCl) is one of the most widely used antimicrobial irrigants used in endodontics due to its antimicrobial properties and superior tissue dissolution capability (11, 12). Sodium hypochlorite alone, however, is only able to act on organic tissue matter and cells and is highly caustic when inadvertently extruded into the peri-radicular tissues (11, 12).

Ethylenediamine tetraacetic acid (EDTA) is a chelating agent that is used as an adjunct to sodium hypochlorite for its ability to remove the inorganic components of biofilm and the smear layer (5, 10, 13). Together, the combined usage of these solutions has proven extremely effective both *in vitro* and clinically for chemical disinfection, biofilm dissolution and smear layer removal (14, 15).

While many authors have examined the effects of sodium hypochlorite and EDTA when used alone and in combination on tissue dissolution and microbial killing, none have examined the effects of irrigant sequence on biofilm dissolution (11, 16-18). Clinically, identification of the most effective irrigation sequence is important for clinical success and efficient treatment; both

irrigants are essential for the complete disinfection of the root canal system, however, no established protocol has been identified that optimally addresses the bacterial cause of endodontic disease (5, 10).

Chapter 2: Review of the Literature

2.1 Endodontic pathoses pathophysiology

2.1.1 Microbial etiology of endodontic disease

The etiology and pathogenesis of endodontic pathoses, and specifically the ultimate outcome of apical periodontitis, has been well established and confirmed within the endodontic literature as microbial in origin. While microorganisms were first identified within infected root canals in the late 19th century by Miller (19) and microbes implicated as a pathogenic necessity for endodontic infection, numerous studies since then have demonstrated the role of bacteria and biofilms in the pathogenesis of endodontic infection. Kakehashi et al. (20) first examined the development of apical periodontitis following pulpal exposure in Fischer rats; only rats with a normal oral microflora developed apical periodontitis following pulpal exposure, while germ-free rats exhibited no apical periodontitis and furthermore demonstrated pulpal healing and tertiary dentin formation following pulpal exposure. These results were later demonstrated in monkeys by Moller et al. (21). Thus, insult to the pulp alone is insufficient to stimulate apical periodontitis (22).

Kakehashi and Moller's work was later expanded by Sundqvist (23) who cultured bacteria in teeth exhibiting pulpal necrosis following a traumatic event. Radiographically evident periapical lesions were only present in teeth where bacteria were culturable; therefore necrotic pulpal tissue alone is unable to simulate apical periodontitis and microbial presence is necessary for its' development (23). Furthermore, the overwhelming majority of cultured bacteria were anaerobic, however, mixed species were generally found in all samples, further reinforcing the complex microbial ecosystem that is established and involved in endodontic disease (23).

2.1.2 Origin and establishment of microbial infection

Microbial invasion to the pulpal space has been a well established pre-requisite for endodontic disease, however, they must travel through tooth structure or artificial restorations and materials to invade the root canal space (24). Initially, the dental hard tissues provide barriers to pulpal invasion, primarily in the form of the outer-most enamel and cementum (25). While dentin may have some barrier ability, its tubule structure significantly limits its ability to resist bacterial penetration (25). Bacterial contamination of the pulp has been demonstrated even with very superficial carious lesions barely interrupting the dentin-enamel junction (25). Intact enamel and cementum may be interrupted with the development of dental disease processes including caries, crack or fracture formation, attrition, abrasion, abfraction or idiopathic damage during dental procedures (24-26). Similarly, this barrier may be compromised at the level of the cemento-enamel junction where a variety of anatomical configurations may exist; specifically in 10% of the population where the enamel and cementum do not meet, leaving an exposed dentin interface susceptible to microbial invasion (27). Furthermore, restorative procedures and materials are subject to leakage at their cavo-surface interfaces allowing bacterial leakage and periodontal procedures, such as scaling and root planing, may damage the cementum layer exposing the underlying cementum (24). All these mechanisms provide potential avenues for bacterial leakage into the pulpal space.

2.1.3 Pulpal defence to microbial invasion

While microbial leakage into the root canal system can occur via many pathways, it does not necessarily indicate the definitive development of endodontic disease or apical periodontitis as the pulp contains many defensive and repair mechanisms to resist bacterial insult (28). Bacteria and their by-products can rapidly diffuse through the dentin tubules into the pulpal space following exposure to dentin either directly from enamel or cementum damage or a carious lesion (29, 30). This process stimulates the pulpal immune response which through a variety of mechanisms can eradicate or manage invading microorganisms or stimulate reparative processes, including the deposition of hard tissue, to resist further bacterial invasion (31). Inherent in the development of the pulpal immune response is pulpal inflammation and if the bacterial insult proves to be too great for the pulp to manage or a compromised pulp from previous insult, damage or trauma cannot overcome a relatively low bacterial insult, an irreversible inflammation will develop resulting the in pulpal necrosis and eventual apical periodontitis (28).

2.1.4 Pathophysiology of established endodontic infection

After establishment of bacteria within the root canal system, a dynamic and constantly changing environment will determine the types of bacteria that dominate and the development of the endodontic disease; this is primarily dictated by the oxygen availability and tension within the pulp which may shift the microbial balance as the infection develops (32). During the early phases of endodontic infection, there is a relatively high level of oxygen, allowing facultative anaerobic bacteria to survive and flourish such as *Streptococci sp., Actinomyces sp. and Lactobacilli sp.*, however, as pulpal tissue dies and blood supply and oxygen tension decreases,

anaerobic bacteria can grow and develop (33). Eventually, as pulpal necrosis becomes widespread throughout the root canal system, anaerobic bacteria such as *Peptostreptococcus sp., Porphyromonas sp., Veillonella sp.* and *Eubacterium sp.* dominate the microflora (34). Furthermore, as the bacterial ecosystem develops and matures, generally it becomes more specific with a lesser variety of bacteria as specific strains are selected by the ecological changes (35). All these species are generally found as native species of the oral microbiome and are routinely cultured in studies of microorganisms of the oral cavity; as such, the origin of bacteria within endodontic infection is generally traceable to leakage or penetration from the oral cavity, through the tooth into the root canal system (36).

Finally, after pulpal necrosis has occurred and the bacterial infection is well established, apical periodontitis may develop (38, 39). This is an immune and inflammatory reaction of the body in an attempt to contain the bacterial infection and limit the spread to within the confines of the tooth structure and root canal system (38). While traditional endodontic literature has contested that bacteria generally remain confined to the root canal space and the peri-radicular tissues remain bacteria-free through the entire pathogenesis, current studies with newer and more accurate culturing and identification methods have identified bacteria within periapical lesions, generally spreading from the portals of exit, including the apical foramen, lateral and accessory canals or root cracks or fractures, into the surrounding lesion (39-41).

Generally, a homeostatic balance is achieved between the bacterial insult within the tooth and the host inflammatory response outside, however, in cases of high infectious load, highly virulent bacteria or compromised host immune response, the formation of an abscess in the peri-radicular tissues may occur (42, 43). This can present in an acute, associated with significant pain,

swelling or pressure developing in a relatively short period of time, or chronic form, clinically evident following the formation of a sinus tract through which the abscess has followed the path of least resistance to drain outside of the bony confines of the maxilla or mandible and generally has little to no pain or discomfort (42, 43). Regardless, both forms originate with the bacterial infection of the root canal system and its' progression to inflammation of the peri-radicular tissues (43).

2.2 Microbial biofilm

2.2.1 Biofilm formation and development

As discussed above, the infected root canal microflora is complex and in a state of dynamic flux during its establishment and development. As many of the bacteria can typically trace their origin to the oral cavity microbiome, they tend to react and behave similarly (36, 44). While many traditional studies have focused on culturing bacteria from within the root canal space for identification purposes, rarely do they exist in isolation or as planktonic, free-floating cells; typically the microorganisms will establish and mature in the form of an organized biofilm, like that formed on teeth as dental plaque (45, 46).

Within the oral cavity, traditional dental plaque biofilm relies on the formation of a pellicle, a thin conditioning film composed of proteins and salivary compounds that adsorbs onto the enamel surface (47). This is an essential process as enamel is a highly inorganic structure with little to no means for attachment of bacteria directly onto its' surface (47). Within the root canal system, however, the innermost dentin is both significantly more organic in composition and typically already coated with organic material including proteins and cellular matter (48).

Furthermore, unlike the relatively harsh oral cavity that is exposed to a variety of environmental changes and delaminating mechanical forces, the root canal system presents a more ideal environment for establishment and growth of a new biofilm. As such invading planktonic pioneer species such as *Streptococcus* and *Actinomyces* can bind and attach this organic matter via adhesins (33, 49). These microbes can subsequently bind to and allow other bacterial species to join the biofilm in a series of progressive layers such as *Fusobacteriun and Eubacterium* species (34). As the biofilm grows and matures, its composition will be dictated by the availability of nutrients and oxygen tension of the surrounding necrotic pulpal tissue, however, once a mature biofilm has developed, it will help actively change the environment to select for the growth of more anaerobic species which begin to dominate the biofilm's composition (2, 45).

2.2.2 Defensive properties of biofilm

As biofilms mature and develop with longer standing and more chronic endodontic infections, they also develop properties and characteristics which make them more resistant (3, 50). This has been demonstrated by various studies including Shen et al. (51) and Du et al. (52). Furthermore, biofilms have been shown to reach their steady state and most resistant form in approximately 3 weeks and from this time point on are significantly more difficult to remove and more resistant to chemical agents (50, 53). Generally, with maturation of the biofilm, the bacteria focus on production of the extracellular polymeric substance and matrix, a complex network consisting of channels and fluid flow pathways, known as an open architecture, allowing intercellular communication and co-ordinated responses of the bacteria (2). This can include synergistic catabolism of macromolecules for aided digestion, nutrient or energy cycling via cross feeding,

co-aggregation and co-adhesion of new bacteria and modulation of the local environment such as lower pH and oxygen tension (33, 50). Not only does this structure allow for more efficient growth and stability of the biofilm community, but it also confers the resistance properties to protect the bacteria from destruction or removal (2).

The extracellular polymeric matrix is produced from the excretion and production of by-products of the bacteria within the biofilm (54). Its primary role is to provide the scaffold upon which the biofilm can adhere, develop and grow, however, by doing so, provides a structure which confers protection to the bacteria to resist removal by mechanical means, exposure to potential hazardous chemical agents or immune cellular access to individual bacteria (55). The open architecture, tubule network, of the extracellular matrix allows for a complex communication pathway allowing fluid flow to not only provide nutrients, but also protect the bacteria from desiccation (56).

The fluid network of the biofilm is essential for a coordinated and organized response of the bacteria, known as quorum sensing (57). Via the secretion of molecules and cell signalling proteins, individual bacteria can act together like a multicellular organism (57). This provides a variety of benefits including a more coordinated and efficient development of the extracellular matrix and biofilm, greater overall resistance to chemicals and antimicrobials as nearby neighbouring bacteria can confer resistance to protect susceptible microbes, cross feeding and enzyme complementation for the use of waste products in other nutrient or metabolic cycles of other bacteria, gene transfer to confer innate resistances to certain antimicrobials or other beneficial traits and enhanced virulence (58, 59).

Finally, the complex biofilm will have an innate spatial and environmental heterogeneity including pH and oxygen gradients which can vary depending on the level and location of the biofilm allowing the addition and co-aggregation of a complex flora of bacteria creating a complex topographical map of microorganisms, each potentially conferring different advantages to the overall biofilm contributing to its survival and resistance from destruction (45, 46).

2.3 Orthograde endodontic management of biofilms

2.3.1 Overall goals of endodontic interventions

The goal of endodontic treatment is to prevent or cure apical periodontitis; as apical periodontitis has been demonstrated to be caused by bacterial infection of the root canal space, endodontic treatment by extension is aimed at the management of the microorganisms invading the root canal system (42). Traditionally this has been achieved through orthograde root canal treatment, involving the mechanical debridement and chemical disinfection of the root canal system with the objective to reduce the bacterial load within the tooth, each of these though, is not wholly effective and cannot completely address the etiology of apical periodontitis (5, 42).

2.3.2 Mechanical instrumentation

Mechanical debridement focuses on the physical removal of infected pulpal tissue, necrotic tissue remnants, bacteria and their by-products (60). Instrumentation alone without the use of irrigation has been shown to increase the amount of debris within the canal (7), however, when a non-disinfecting irrigant is introduced, Bystrom et al. (61) demonstrated a significant reduction in the bacterial load. Many studies have shown, regardless of the type of instrument or how it is

used, that almost one third of the root canal system remains untouched by the instrument (9, 62). This is due to the anatomic complexity of the root canal system including fins, isthmuses and lateral or accessory canals that cannot be physically debrided and therefore may harbour pathogenic bacteria or biofilm (9, 62). Although instrumentation removes debris and tissue, it more importantly enlarges the root canal space to facilitate chemical disinfection with antimicrobial agents (5, 10, 60).

2.3.3 Sodium hypochlorite

Traditionally, sodium hypochlorite has been the irrigant of choice during endodontic treatment due to its excellent antimicrobial effect and ability to dissolve tissue and organic matter (63). The mechanism of action of sodium hypochlorite is multi-fold depending on the tissue or chemical it comes in contact with (64). There are three primary reactions that occur upon contact with organic tissue including saponification, amino acid neutralization and chloramination (64). Saponification occurs if sodium hypochlorite reacts with a fatty acid which will yield a soap and glycerol. If it interacts with an amino acid, it will form a salt and water (64). Finally chloramination results when hypochlorous acid within the sodium hypochlorite solution interacts with an amino acid, releasing chloramine which prevents cellular metabolism, is a strong oxidizer and inhibits bacterial enzymes (64). These reactions together act to damage microbial membranes, inhibition cellular replications and destroy macromolecules such as DNA and proteins (65). Furthermore, and to a lesser extent, sodium hypochlorite yields the weak acid hypochlorous acid which can cause hydrolysis of amino acids or direct acid attack and sodium hypochlorite itself is a strong base, with a pH greater than 11, which can change the ecological

conditions around the biofilm potentially affecting some bacteria (64). Clinically, sodium hypochlorite has been used at varying concentrations, typically between 0.5% and 6%, with conflicting evidence with respect to concentration and efficacy (66). Bystrom et al. (67) and Siqueira (68) have showed no difference between 0.5% and 5% solutions and 1% and 5% solutions respectively while Haapasalo et al. (69) and Du et al. (52) have showed higher concentrations more completely and more quickly dissolve biofilms; however it should be noted many of these studies vary greatly in terms of methodology and measured outcome.

2.3.4 EDTA

While sodium hypochlorite has many of the properties of an ideal irrigant including broad and effective antimicrobial activity and tissue dissolution ability, it does not possess the ability to remove the inorganic component of biofilm and the smear layer generated on canal walls following mechanical instrumentation (10). Due to this limitation, chelating solutions are often required should complete biofilm and smear removal be an objective in root canal treatment (70). Ethylenediaminetetraacetic acid (EDTA) is a chelator that has free electron pairs to bind inactive metallic ions to form a stable complex (71). As many solids, including dentin and biofilm, will exist in a state of equilibrium between deposition and release of ions, within a solution of EDTA, the calcium and other metal ions will be bound by the chelator and removed from the solution (70). Furthermore, the use of EDTA may break up inorganic bonds within the biofilm, potentially detaching fragments or increasing the surface area for exposure to other antimicrobial agents. The combined actions of sodium hypochlorite and EDTA allow for complete dissolution of

smear and all its components when sufficient concentrations are used for an adequate amount of time (72, 73).

While the combination of these irrigants is often advocated within the literature, irrigation of the root canal system still presents many challenges limiting our efficacy and ability to completely disinfect the root canal system including anatomic complexities, dentin erosion, complete biofilm removal and microbial resistance, the smear layer, and efficacy versus safety issues (5, 10).

2.3.5 Limitations of endodontic treatment

The complexity of root canal systems has been highly analyzed with historic methods including dye penetration and sectioning and recently with micro computed tomography (micro-CT) and electron microscopy (74-78). The clinical treatment of root canal spaces is often limited to the accessible canal, while in actuality the root canal system is comprised of a series of complicated branches, connections, irregularities and accessory canals; each of these provides a potential haven for bacteria and biofilm to develop without being subjected to chemomechanical cleansing of root canal treatment (62). Furthermore, dentin tubules may harbour bacteria away from the root canal proper and if the smear layer is not removed, allow continued survival of these bacteria following endodontic treatment (79, 80). These areas do not allow effective penetration of antimicrobial solutions and even those that may seep into these spaces may be of insufficient volume to effectively destroy the biofilm (5). Finally, mechanical instrumentation may pack debris and necrotic pulpal tissue into these anatomic complexities, further protecting the bacteria and biofilm from effective removal (7).

2.4 Irrigation in endodontics

2.4.1 Limitations of current irrigants

While a combination of irrigants has been shown most effective for removal of the most amount of biofilm, a prolonged use or repeated switching between sodium hypochlorite and EDTA has been shown to contribute to dentin microerosion (81, 82). The removal of organic and inorganic tissue by these irrigants is not only limited to the bacterial infection, but also acts on the dentin walls of the root canal (81). Repeated exposure to sodium hypochlorite and EDTA has been shown to cause topographic changes in the dentin including significant decreases in calcium and phosphorus ions and long term exposure to high concentrations of sodium hypochlorite has been associated with a significant reduction in the flexural strength of dentin (81). These effects are most pronounced when sodium hypochlorite irrigation is used following the action of a chelator or demineralizing agent as the chelator has exposed the collagenous matrix of the dentin which is liable to sodium hypochlorite dissolution if it is re-introduced into the canal (82). While the long term consequences of microerosion have yet to be demonstrated or understood, a tenet of endodontic treatment is to retain as much tooth structure and residual strength as possible (42). While many *in vitro* studies have demonstrated the efficacy of sodium hypochlorite and EDTA in their ability to dissolve biofilm, the clinical reality of endodontic treatment acknowledges the inability to render the root canal completely void of bacteria (83, 84). The biofilm will inherently provide some resistance to dissolution if inadequate concentration or time of irrigant exposure occurs as the multi-layer structure will provide protection to the deepest layers attached to the dentin wall (45). Furthermore, depending on the antimicrobial used, such as tetracycline-containing irrigants, certain bacteria may have innate or acquired defence mechanisms conferring

resistance to the irrigant by that bacteria and the associated biofilm, preventing the irrigant's action (50, 85).

2.4.2 The smear layer as a barrier to disinfection

Similar to the anatomic complexities of the root canal system, the smear layer acts as a potential protective harbour for bacteria. A smear layer is an amorphous, irregular and granular layer, approximately 1-5 microns in thickness, of dentin debris, odontoblasts, pulpal remnants and bacteria that is formed during mechanical instrumentation by the translocating and burnishing action of the instrument against the canal wall (72). This complex structure provides several challenges as it acts as a physical barrier to irrigants preventing penetration into the dentin tubules or uninstrumented anatomy that may harbour bacteria or act as an avenue for micro-leakage and post-endodontic treatment disease (1). This is due to the extensive reticular network of microchannels that provide an avenue for microbial leakage or due to the organic component of smear which is liable to proteolytic degradation by bacteria such as *A. viscosus* and *S. sanguis* which can deteriorate the root canal filling to tooth interface (73). Due to these potential negative implications of leaving the smear layer behind, many authors have advocated the removal of the smear layer by use of sodium hypochlorite followed by EDTA for complete dissolution of the organic and inorganic constituents, including Bystrom et al. who demonstrated its clinical implications and success (14).

2.4.3 Efficacy versus safety

Finally, clinical efficacy of the irrigant must be balanced with patient safety (5, 10). While EDTA is relatively harmless when extruded into the peri-radicular tissues during treatment, sodium hypochlorite can cause significant pain, swelling and bruising if introduced beyond the apical foramen (5, 12). Copious and frequent exchange of solution of adequate antimicrobial concentration must be accomplished during endodontic treatment to ensure optimal microbial eradication, however, a thorough understanding of the fluid dynamics during irrigation and method of irrigation is critical for safe and effective treatment outcomes (5). Not only must the safety of each irrigant used in isolation be considered, but irrigants used in combination must be evaluated for any potential synergistic or caustic interactions. The combined use of sodium hypochlorite and chlorhexidine without an intermittent saline rinse will yield a reaction forming an insoluble precipitate parachloroaniline (86) which not only presents as an issue of a potential solid obstructing the root canal space, but has been shown by Chhabra et al. (87) to be cytotoxic and carcinogenic in rats and mice. Several authors have reported the effects of adding chelating agents, such as EDTA to sodium hypochlorite, and of primary concern as noted by Zehnder et al. (18) is the reduction in sodium hypochlorite pH and reduced availability of hypochlorite ion for interaction with organic tissue (17). Furthermore, Irala et al. (88) demonstrated a significantly reduced ability of NaOCl to dissolve tissue by sodium hypochlorite when mixed with EDTA and Grawehr et al. (89) showed a significantly reduced antimicrobial efficacy against *E. faecalis* and *C. albicans* when the irrigants were combined. The direct mixture of sodium hypochlorite and EDTA releases chlorine gas which is potentially hazardous to humans (90). However, it should be noted that while the chelating and inorganic dissolution ability of EDTA is not compromised

when used in combination with sodium hypochlorite, there is a possible concentration dependant effect on the interaction and all experiments have focused on the effects of mixed solutions as opposed to sequential usage mimicking the clinical reality of endodontic therapy (17, 18, 91).

2.4.4 Needle irrigation

Traditionally, clinical endodontics has relied on the action of irrigants and needle delivery to both introduce the irrigant into the root canal system and provide a turbulent flushing action to liberate debris and biofilm (5). Such delivery, however, is limited by the depth of penetration of the needle into the canal and the fluid dynamics within the root canal space (5, 92). Chow et al. (92) has demonstrated the limited ability of fluid to flow beyond the tip of the needle and furthermore, Tay et al. (93) showed irrigation is often limited by the formation of air bubbles in the region apical to the needle preventing any fluid from penetrating the deepest areas of the root canal space. Several advances and devices have been introduced to facilitate irrigant delivery and penetration.

2.4.5 Energy

The utilization of energy to assist canal disinfection was first introduced by Martin (94) as a means to augment our available protocols. Ultrasonic energy moving freely within the canal space allow acoustic streaming and cavitation processes which has been shown to significantly improve debris removal from canal irregularities and improve irrigant penetration (95, 96). Acoustic streaming is the movement of the fluid due to the oscillatory movement of the ultrasonic instrument which helps generate a small disturbing force to the biofilm and replenish

the irrigant in different regions of the root canal space (97-99). Cavitation is the process by which microscopic air and vacuum bubbles formed within the base liquid collapse and explode releasing a localized area of head, energy and pressure which forms shear forces to assist in removal of the biofilm (100). Similarly, sonic energy has been utilized as the lower frequency and oscillation amplitude is possibly safer with lesser potential for irrigant extrusion, however, primarily acts by acoustic streaming with no cavitation and mainly assists debris removal, irrigant movement and renewal of irrigant to improve irrigation efficacy (101-103).

2.4.6 GentleWave

GentleWave (Sonendo Inc, Laguna Hills, CA) is a novel cleaning system that uses a wide spectrum of sound waves during irrigant delivery to clean with minimal instrumentation of the root canal system (69). Unlike energy augmented irrigation, the GentleWave system simultaneously delivers irrigant, uses multi-sonic sound waves and removes used irrigant for replenishment (69). A handpiece is placed over the access cavity into the chamber and delivers fluid into the system (104). This results in the benefits of fluid dynamics and shear forces during fluid movement as well as cavitation and energy release from sound wave energy and has been shown to be significantly more effective in rate of tissue dissolution as well as clinically effective (69, 104, 105). Furthermore, Haapasalo et al. (106) demonstrated that GentleWave creates negative pressure in the apical region of the root canal system, decreasing the risk of irrigant extrusion. Sigurdsson et al. (108) demonstrated a 97.7% success rate in patients with periapical lesions 1 year following treatment and Sigurdsson et al. (107) showed a 92.9% success rate in patients with pulpal necrosis and 98.4% success in patients with irreversible pulpitis.

GentleWave is a promising system with potential for effective and safe irrigant delivery with minimal instrumentation to the root canal system without compromising chemical disinfection.

2.5 Biofilm models for investigation of irrigants

2.5.1 Open culture model

An open culture biofilm model uses a base, such as dentin or hydroxyapatite, to grow a culture of bacteria into an established biofilm (109). The *in vitro* model can be regulated with respect to type of culture, from a single species or mixed, and environmental conditions to regulate the type of biofilm under investigation; this technique allows for the development of a consistently standardized biofilm with rigid control of the environmental conditions (109). This model has been proven by Wang et al. (110) and Zhang et al. (111) as an effective means to evaluate the response and efficacy of irrigant exposure to biofilm and allows for analysis of the biofilm by fluorescence microscopy, scanning electron microscopy, stereomicroscopy or confocal laser scanning microscopy.

2.5.2 Dentin canal model

A dentin canal biofilm model aims to more accurately reflect the clinical reality of endodontic treatment by more closely simulating the root canal space following mechanical instrumentation (112). Unlike an open culture model, anatomic complexities including canal irregularities and dentin tubules can also be studied; briefly, the canals are instrumented and cleaned to open the dentin tubules and bacteria are centrifuged into the canal for subsequent treatment or experimentation (112). Several studies have used this model to examine bacterial penetration, the

role of the smear layer, irrigant penetration and medication placement or removal (112-114). While this model more closely mimics an *in vivo* model, end point analysis and measured outcomes are often more difficult and technically demanding as aside from fluorescence microscopy, biofilm measurement by stereomicroscopy or scanning electron microscopy (SEM) require invasive sectioning of the model (115).

2.5.3 Microscopy

Fluorescence microscopy has been demonstrated as an effective means for analysis of bacteria on an open culture biofilm or dentin tube model including presence or live-dead staining, however, is potentially problematic depending on the study design and outcome measure (111, 116). Berney et al. (117) demonstrated EDTA in particular can create porosities in bacterial cell membranes, allowing 'dead stain' penetration into the cell and an artificially increased dead cell count. It is therefore likely more prudent, when studying the effects of EDTA, to examine biofilm dissolution or bacterial clearance for a more accurate reflection of the irrigant's effects.

Chapter 3: Aim and Hypotheses

3.1 Aim

The primary goal of endodontic therapy is the prevention or treatment of apical periodontitis; as apical periodontitis is caused by bacterial infection of the pulpal tissue, the goal of endodontic therapy by extension is the disinfection of the root canal space of bacteria (42). A variety of protocols and techniques exist to facilitate cleaning of the root canal system, however, each possesses its own inherent limitations and setbacks and none can completely render the root canal bacteria free. Due to the highly adaptive and resistant nature of bacteria, especially when organized into a biofilm as in endodontic infection (2, 50), treatment should be aimed at the most effective and complete biofilm destruction. Furthermore, there is no general consensus or guideline established for a protocol as to the best practice and most effective antimicrobial action in terms of irrigants used, sequence of irrigants or techniques to enhance chemo-mechanical disinfection. As chemical disinfection is the primary means of bacterial eradication, with mechanical instrumentation providing a means to more effective irrigation, an understanding of the most effective combination and sequence of irrigants is essential for clinical success (5, 10, 60). While many studies have examined the effects of mixed irrigant solutions, none have looked at the outcomes of irrigant sequence on antimicrobial efficacy and biofilm dissolution, specifically with respect to sodium hypochlorite and EDTA.

The present study aims to study the effect of EDTA pre-treatment on sodium hypochlorite's biofilm dissolution ability in an open culture model. The study will evaluate whether the reported inhibitory effect of EDTA on sodium hypochlorite's tissue dissolution efficacy remains when the solutions are used sequentially as opposed to mixed, if there is a concentration dependent

relationship on the inhibitory effect of EDTA on sodium hypochlorite and if there is a time dependent relationship on the inhibitory effect of EDTA on sodium hypochlorite.

3.2 Hypotheses

The null hypotheses of this study are that 1) pre-treatment of open culture biofilm with EDTA will not alter the dissolution time of the biofilm by sodium hypochlorite; 2) varying the time of pre-treatment of open culture biofilm with EDTA will not alter the dissolution time of the biofilm by sodium hypochlorite and; 3) varying the concentration of sodium hypochlorite will not alter the dissolution time of open culture biofilm pre-treated with EDTA.

Chapter 4: Materials and Methods

4.1 Biofilm culturing

The open culture biofilm models were grown on hydroxyapatite discs using a previously established model. Hydroxyapatite discs (9.65mm diameter x 1.52mm thickness; Clarkson Chromatography Products, Williamsport, PA) were inspected for any irregularities or deformations and subsequently cleaned in an ultrasonic bath and sterilized in an autoclave. Any discs with significant scratches, dents or deformations were discarded. The discs were allowed to cool to RT and were incubated in 24 well culture plates (Corning Inc., NY) with a 1mL solution of 3mg/mL bovine dermal type I collagen (Cohesion, Palo Alto, CA), 0.012N HCl and distilled water for a period of 24 hours at 4°C.

Subgingival plaque was collected form an adult volunteer (University of British Columbia Clinical Research Ethics Board H12-02430) and suspended in brain-heart infusion broth (BHI; Becton Dickinson, Sparks, MD) and vortex mixed for 30 seconds; the suspension was adjusted to optical density (OD_{405}) 0.10 above the control using 150µL measured in a 96 well microtiter plate in a microplate reader (Model 3350; Bio-Rad Laboratories, Richmond, Ca). The plaque suspension was diluted by a factor of 10 times in BHI. The collagen solution was pipetted from the 24 well culture plates containing the coated hydroxyapatite discs and 2mL of the BHI-plaque suspension mixture was pipetted into each well. The discs were incubated in an anaerobic bag under anaerobic conditions (AnaeroGen COMPACT, Oxoid Ltd., Hampshire, UK) at 37°C for 3 weeks with fresh medium changed only on day 14.

4.2 Biofilm dissolution

After three weeks of anaerobic incubation a total of 96 samples were randomly divided into eight groups for experimental analysis. The culture medium suspension was pipetted from the wells and the discs were transferred to a new 24 well culture plate. Each disc was subjected to a pre-treatment of 1mL of 17% EDTA (Sigma-Aldrich, St. Louis, MO) or 1mL of sterile water (H_2O), distilled water which had been autoclaved and allowed to cool to RT and was introduced by micropipette. After 30 seconds or 1 minute of EDTA pre-treatment, the irrigant was pipetted out and the disc was transferred to a new 24 well culture plate. 1mL of sterile water was then pipetted into the well for 1 minute for all samples and was subsequently pipetted out and the disc transferred to a new 24 well culture plate. Finally, 1mL of either 2% or 6% sodium hypochlorite (EMD Chemical Inc, Darmstadt, Germany) was pipetted into the well. Samples were analyzed under a stereomicroscope (Zeiss, Oberkochen, Germany) and timed to the nearest whole second for time from complete submersion in sodium hypochlorite to complete biofilm dissolution. Samples were divided into one of the following eight groups: 1) 1 minute sterile H_2O - 1 minute sterile H_2O - 2% NaOCl; 2) 1 minute 17% EDTA- 1 minute sterile H_2O - 2% NaOCl; 3) 30 seconds sterile H_2O - 1 minute sterile H_2O - 2% NaOCl; 4) 30 seconds 17% EDTA- 1 minute sterile H_2O - 2% NaOCl; 5) 1 minute sterile H_2O - 1 minute sterile H_2O - 6% NaOCl; 6) 1 minute 17% EDTA - 1 minute sterile H_2O - 6% NaOCl; 7) 30 seconds sterile H_2O - 1 minute sterile H_2O - 6% NaOCl and; 8) 30 seconds 17% EDTA - 1 minute sterile H_2O - 6% NaOCl.

Table 1. Pre-treatment Conditions and Strength of Sodium Hypochlorite that the Biofilms Were Exposed To.

Group	PreTreatment Irrigant	Pretreatment Time	NaOCl Strength
1	Sterile water	30s	2%
2	17% EDTA		
3	Sterile water	60s	
4	17% EDTA		
5	Sterile water	30s	6%
6	17% EDTA		
7	Sterile water	60s	
8	17% EDTA		

Ten hydroxyapatite discs were randomly selected following complete biofilm dissolution, as determined under the stereomicroscope, and subjected to SEM analysis to confirm complete biofilm dissolution. Samples were fixed with 2.5% glutaraldehyde for 10 minutes, followed by 1% osmium tetroxide (pH=6.8) for 1 hour and serial dehydration with increasing concentrations of ethanol (70%, 80%, 90%, 95%, 100%) for 15 minutes each. Samples were dried using a critical point dryer (Samdri-795; Tousimis Research Corporation, Rockville, MD), mounted on stubs with silver (Pelco Conductive Silver Paint; Ted Pella, Redding, CA), sputter coated with a 10nm layer of iridium (Leica EM MED020 Coating System, Tokyo, Japan) and examined by SEM (Helios Nanolab 650, FEI, Eindhoven, the Netherlands) across the entire disc surface to ensure no residual biofilm using 35,000X magnification at 2.0kV (Figure 4).

4.3 Statistical analysis

Sample size was determined based on the results of a pilot study using G-Power 3.1 software (University of Düsseldorf, Germany; http://www.gpower.hhu.de/en.html) and a priori power analysis with the test family of F test (Analysis of variance, ANOVA) was applied resulting in the power of 0.90 and a required minimum sample size of 12 for each group. Statistical analysis was performed with SPSS 24.0 (SPSS Inc., Chicago, IL) for Mac. The mean and standard deviation time of biofilm dissolution was calculated for each group, normality of the data distribution was confirmed and multiple linear regression for significance of independent variables.

Chapter 5: Results

5.1 Biofilm dissolution

5.1.1 Multiple linear regression

Statistical analysis revealed that all three independent variables, pre-treatment exposure time, strength of sodium hypochlorite and type of pre-treatment irrigant, significantly affected the biofilm dissolution time ($p<0.001$) and therefore the null hypothesis is rejected. Multiple linear regression was run to account for the impact of each variable on the dissolution time and normality and no multi-collinearity criteria were satisfied validating the model (Table 3); the adjusted R squared value of 0.807 indicated 80.7% of variance in the biofilm dissolution time was accountable to the three independent variables.

5.1.2 Sodium hypochlorite

Increasing the concentration of sodium hypochlorite significantly decreased the time for biofilm dissolution ($p<0.001$) for all matched pre-treatment conditions. The use of 6% sodium hypochlorite almost halved the dissolution time when pre-treatment conditions remained the same as shown in Table 2.

5.1.3 Time

Pre-treatment of biofilms with 17%EDTA for longer periods of time prior to dissolution with sodium hypochlorite significantly increased the time of dissolution ($p<0.001$). Doubling the time of EDTA pre-treatment more than doubled the time of dissolution for 6% sodium hypochlorite samples versus 2% sodium hypochlorite samples (Table 2, Fig. 1, Fig. 2).

5.1.4 EDTA

The effect of EDTA as a pre-treatment irrigant on sodium hypochlorite's dissolution ability remained the same regardless of concentration of sodium hypochlorite used. With all variables controlled for and looking at EDTA pre-treatment as an independent factor, the use of EDTA for 1 minute prior to sodium hypochlorite dissolution significantly increases the dissolution time of biofilm over the control sterile water ($p<0.05$). A pre-treatment regimen of EDTA for 30 seconds significantly decreased the time for sodium hypochlorite dissolution ($p<0.001$), however, pretreatment with EDTA for 60 seconds significantly increased the time for sodium hypochlorite dissolution ($p<0.001$). Regardless of pre-treatment irrigant or time of exposure, all samples were completely dissolved when sufficient time was allowed.

Table 2. Biofilm Dissolution Time with Sodium Hypochlorite Following EDTA Pre-Treatment.

Group (Pre-treatment/Sodium Hypochlorite Strength)	Dissolution Time (sec) [Mean, (SD)]
30 sec Sterile Water/2% NaOCl	585.17 (78.52)[a]
30 sec 17%EDTA/2% NaOCl	578.17 (72.67)[b]
60 sec Sterile Water/2% NaOCl	547.17 (53.43)[a]
60 sec 17% EDTA/2% NaOCl	744.58 (69.66)[c]
30 sec Sterile Water/6% NaOCl	250.00 (17.41)[d]
30 sec 17%EDTA/6% NaOCl	188.83 (21.85)[e]
60 sec Sterile Water/6% NaOCl	267.17 (21.54)[d]
60 sec 17% EDTA/6% NaOCl	475.83 (63.04)[f]

Different superscript letters indicate statistically significant differences between groups ($p<0.05$). Repeated measures ANOVA was applied for statistical analysis.

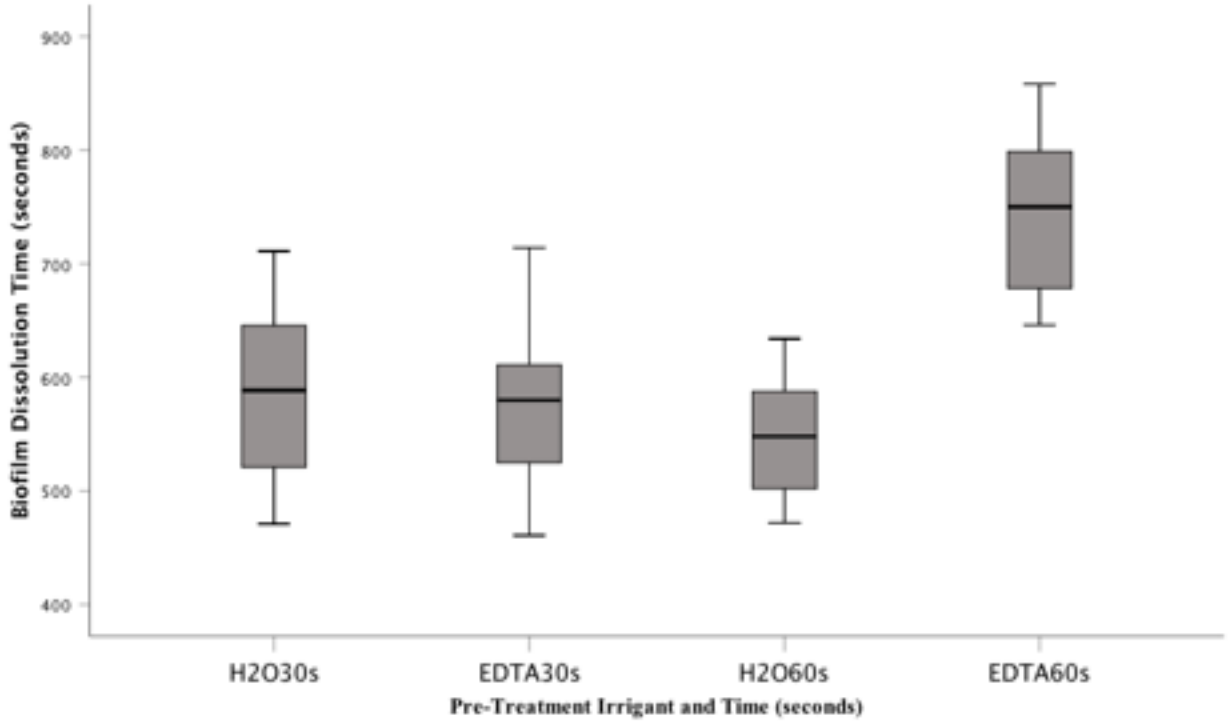

Figure 1. Distribution of times for complete biofilm dissolution with 2% NaOCl following pre-treatment with sterile water or 17% EDTA. H2O= Sterile water. EDTA= 17% EDTA.

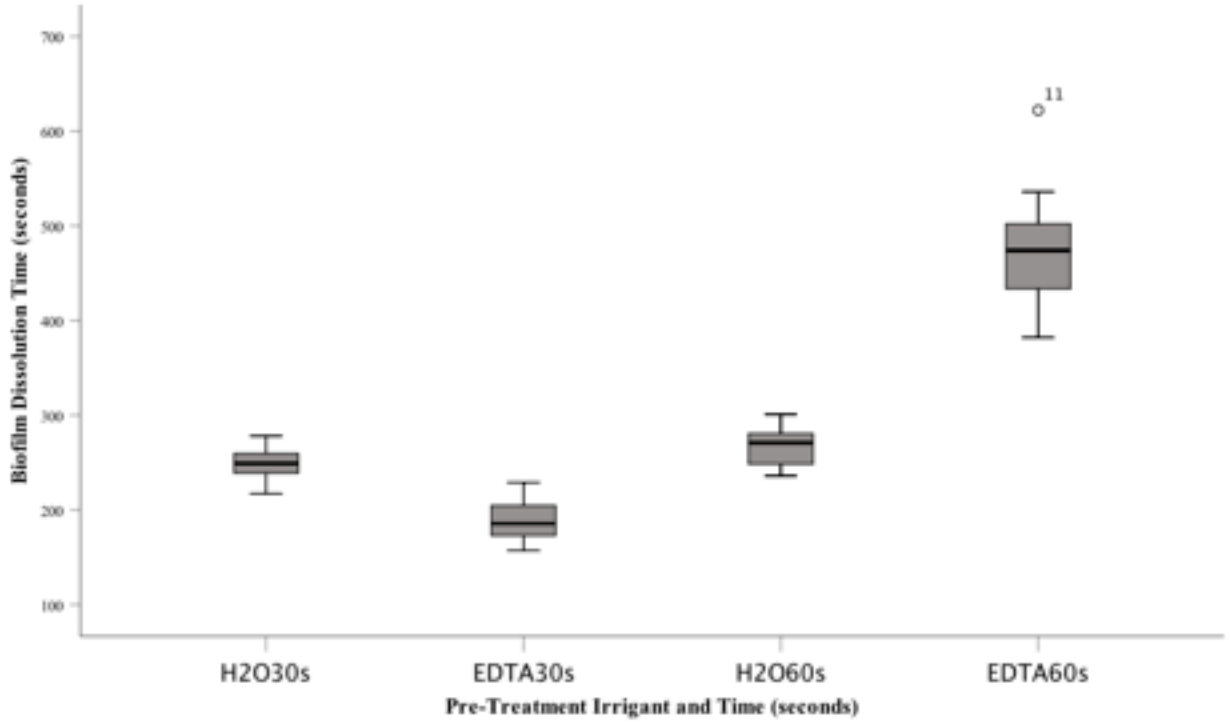

Figure 2. Distribution of times for complete biofilm dissolution with 6% NaOCl following pre-treatment with sterile water or 17% EDTA. H2O= Sterile water. EDTA= 17% EDTA.

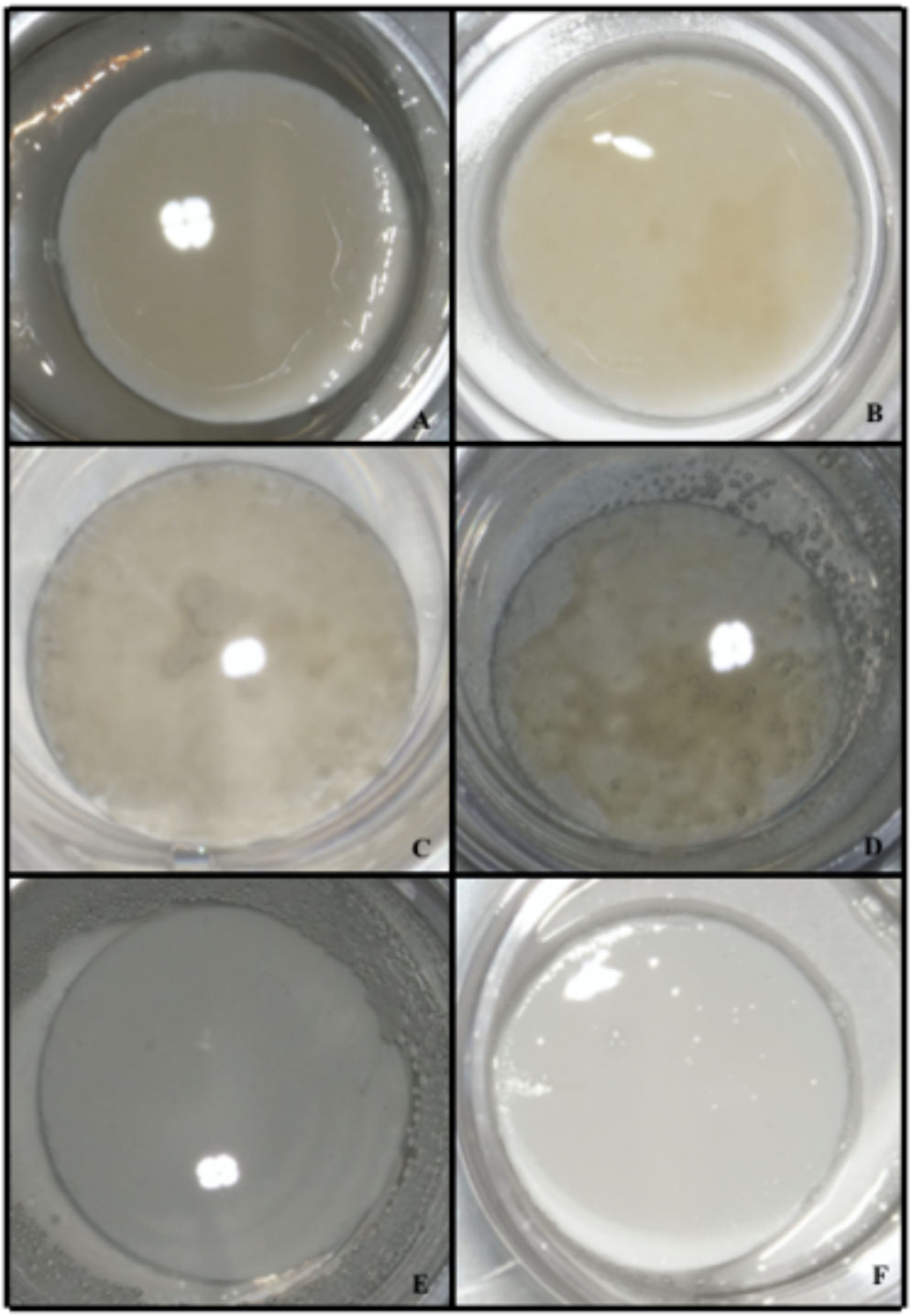

Figure 3. Biofilm dissolution progression. A-Mature 3 week old biofilm on a hydroxyapatite disc. B-Biofilm following exposure to 17% EDTA for 30 seconds. C-Disc immediately after starting 6% NaOCl exposure. D- Disc 90 seconds following 6% NaOCl exposure. E-Complete biofilm dissolution on hydroxyapatite disc. F-Confirmed complete biofilm dissolution following removal of irrigant.

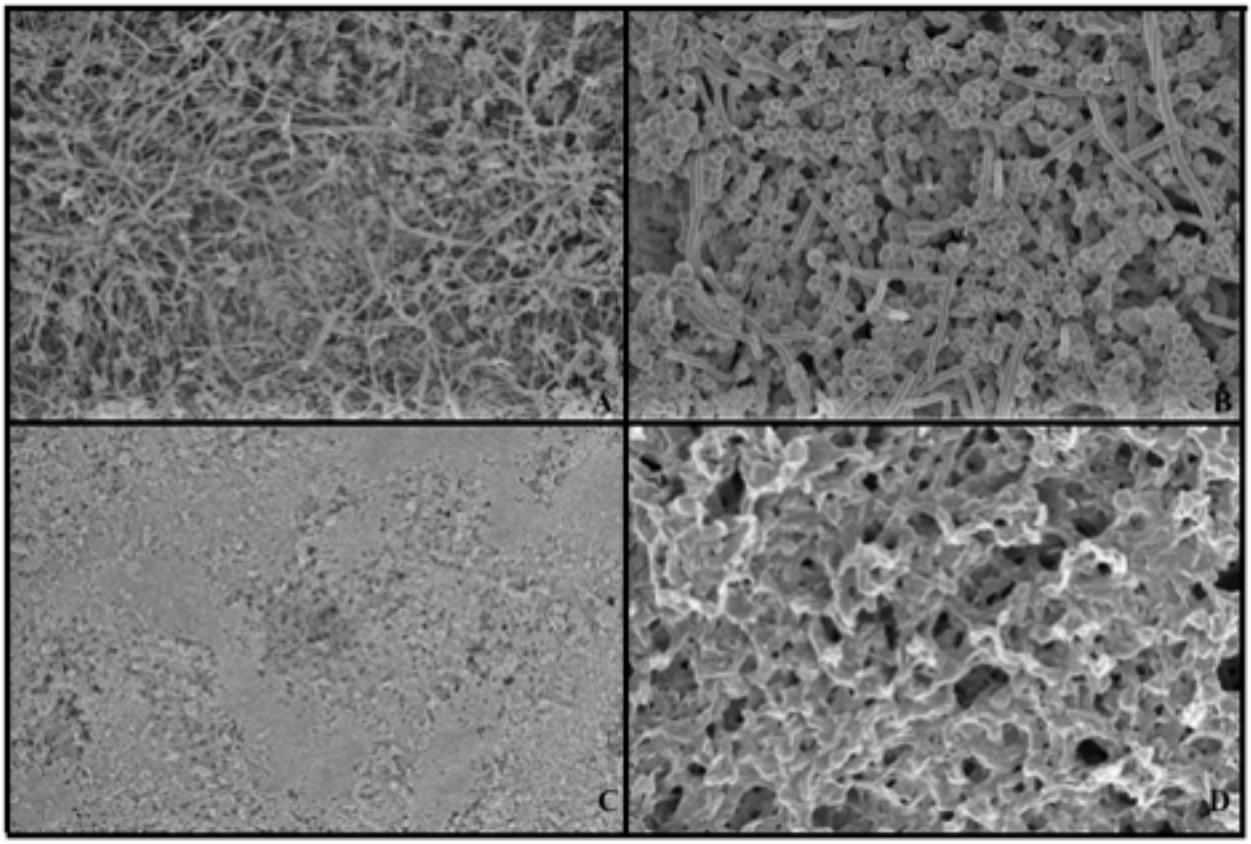

Figure 4. SEM images of hydroxyapatite discs. A-Mature biofilm on hydroxyapatite disc at low magnification (X2000). B- Mature biofilm on hydroxyapatite disc at high magnification (X6500). C-Hydroxyapatite disc free of biofilm and bacteria at medium magnification (X5000). D-Hydroxyapatite disc free of biofilm and bacteria at high magnification (X35000).

Table 3. Effect of EDTA Pre-Treatment Time, Sodium Hypochlorite Strength and Pre-Treatment Irrigant on Biofilm Dissolution.

Predictors	Outcome: Biofilm Dissolution		
	Standardized Regression Coefficient β (95% CI)	Tolerance	Significance
Pre-Treatment Time	0.281 (73.78, 142.51)	1.000	p<0.001
Sodium Hypochlorite %	-0.828 (-352.68, -283.95)	1.000	p<0.001
Pre-Treatment Irrigant	0.220 (50.12, 118.84)	1.000	p<0.001
			Adjusted R^2=0.807

Multiple linear regression was applied for statistical analysis.

Table 4. Effect of EDTA Pre-Treatment Time, Sodium Hypochlorite Strength and Pre-Treatment Irrigant on Sodium Hypochlorite's Time to Dissolve Biofilm.

Predictors		Dissolution Time (sec) [Mean, (SD)]	Significance
Pre-Treatment Time	30 seconds	400.54 (191.96)	$p<0.01$
	60 seconds	508.69 (180.55)	
Sodium Hypochlorite %	2%	613.77 (102.55)	$p<0.001$
	6%	295.46 (114.73)	
Pre-Treatment Irrigant	Sterile Water	412.38 (163.30)	$p<0.05$
	17% EDTA	496.85 (212.39)	

Independent samples T test was applied for statistical analysis.

5.2 Intraexaminer reliability

Intraexaminer reliability was calculated for 10 randomly selected samples by measuring two separate end point times for each sample and demonstrated excellent correlation (Pearson=1.000, $p<0.001$) as shown in Figure 5. Furthermore, tested samples showed no significant difference between means of calculated dissolution times ($p<0.05$) as shown in Table 5.

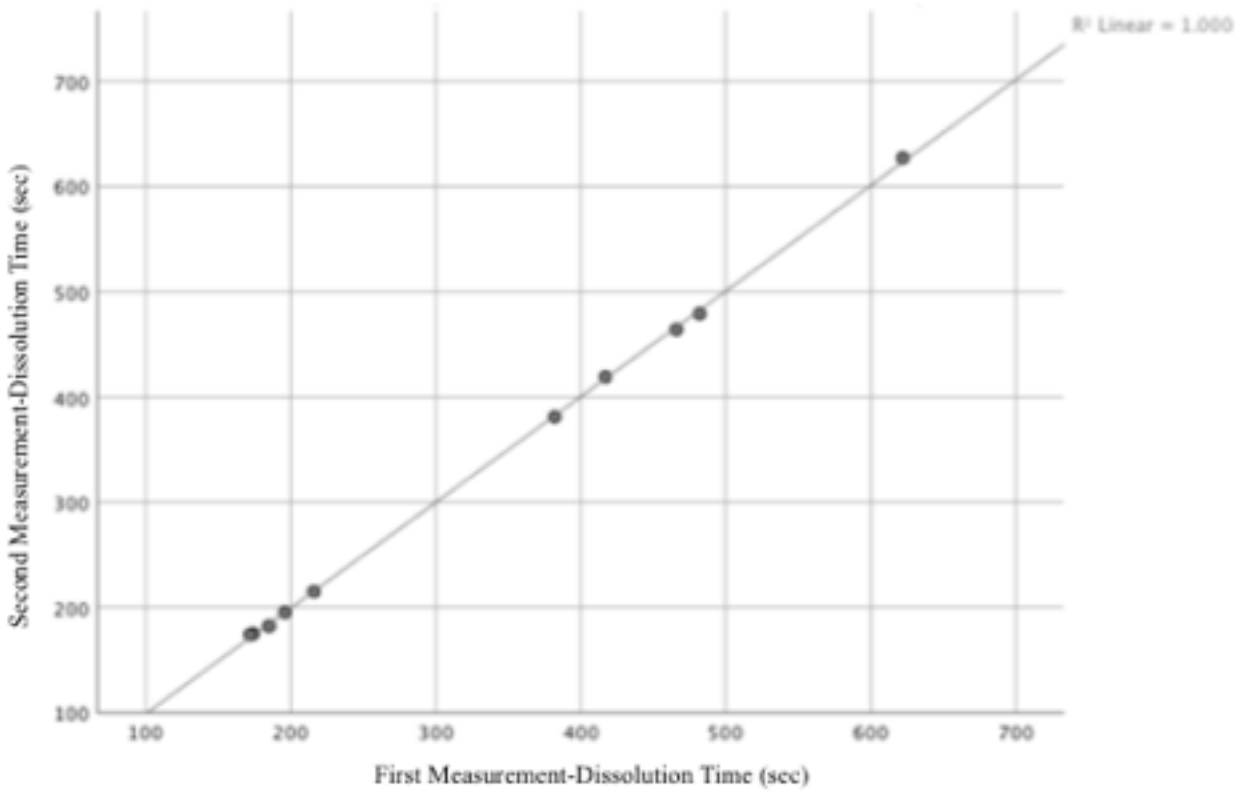

Figure 5. Correlation between matched samples of independently recorded biofilm dissolution samples. Pearson score (1.00) demonstrated excellent intraexaminer correlation. Linear and Pearson correlation testing was applied for statistical analysis.

Table 5. Comparison Between Means of First and Second Measurement of Randomly Selected Biofilm Dissolution Samples.

Group	Outcome: Biofilm Dissolution (sec)	
	Mean (SD) (sec)	Significance
First Measurement	331.20 (162.75)	p>0.05
Second Measurement	331.10 (163.50)	

Paired samples T test was applied for statistical analysis.

Chapter 6: Discussion

Chemical disinfection of root canal systems with endodontic infections has been identified as one of the most significant prognostic factors for endodontic treatment, as such identification of the most potent and clinically effective irrigants has become a major point of investigation (118). While a variety of models have been investigated for biofilm dissolution, including dentin canal, open culture models provide a more accessible and consistently standardized model to examine biofilm growth and development, irrigant disinfection efficacy and biofilm dissolution (109). Furthermore, unlike soft tissue dissolution models, the open culture biofilm model presents the ability to more closely mimic the clinical matter the irrigant will be used on; multispecies subgingival plaque biofilm models have been used in many studies to test irrigant efficacy and changes or killing of biofilms and biovolumes as endodontic biofilms originate from the oral microflora (109). It should be noted that the open culture model does present its own limitations. Of primary importance is that the model less closely approximates the *in vivo* clinical situation than a dentin canal model, however, as discussed in section 2.5.2, this model would require destructive methods both before and after experimentation to confirm biofilm development, is more difficult to standardize between samples and if destructive or SEM imaging was not utilized, fluorescence staining would likely be required which when examining the effects of EDTA, especially when live-dead staining is used, can be misleading (109). Furthermore, the model is relatively static and does not account for fluid dynamics or irrigant accessibility, two issues better simulated in the dentin canal model. Considering the aims and measured outcomes of the experiment, however, these limitations do not undermine nor compromise the experimental model and investigated questions (109).

Stereomicroscopy allows for high magnification and accurate visualization of biofilm, especially in an open culture model and has been demonstrated as an effective means for biofilm investigation and identification (119). As discussed in section 2.5.3 live-dead fluorescence staining has inherent limitations when EDTA is used as an irrigant. Furthermore, the desired measured outcome of this study was complete biofilm dissolution, rendering this method of observation unnecessarily demanding. SEM characterization of biofilm has been well documented (120-122), however, would have several drawbacks when studying irrigation phenomenon. First and foremost is the necessity for biofilm fixation and coating for pre-experimental characterization, a process that would completely inhibit or else compromise and alter the irrigant action as well as desiccate and possibly alter the biofilm. Furthermore, SEM analysis would not allow real-time examination of irrigant dissolution, the samples would have to be subjected to an irrigant for a pre-determined amount of time and subsequently verified with SEM, undermining the accuracy of the dissolution times. Finally, during the study, samples were randomly selected when complete biofilm dissolution was determined on the stereomicroscope for SEM evaluation to confirm the validity of the technique and complete biofilm dissolution. Sodium hypochlorite's biofilm dissolution ability is dependent on the concentration of the solution and time of exposure; both of which positively correlate with dissolution (123). While some studies have reported the potential negative consequence of mixing sodium hypochlorite with a chelator, no studies have examined biofilm pre-treatment with EDTA before sodium hypochlorite exposure (71, 88-90). As discussed in section 2.3 many authors recommend the sequential usage of sodium hypochlorite and EDTA during orthograde endodontic treatment, and while sodium hypochlorite is an excellent antimicrobial, it possesses its own risks during usage if

extruded into the peri-radicular tissues (124). Furthermore, as endodontic treatment relies primarily on chemical disinfection of anatomically inaccessible areas and dentin tubules, the most potent and efficient combination and sequence would be ideal for maximal biofilm eradication as well as clinical efficiency (5, 10). It has been well documented that smear layer generated from mechanical instrumentation may harbour bacteria both directly within the smear (125) and indirectly by making areas, such as tubules and complicated anatomy, inaccessible for irrigant (7), it is therefore is prudent for smear layer removal prior to use of the final antimicrobial irrigant, however, frequent altering between sodium hypochlorite and a chelator, such as EDTA, has been shown by Qian et al. (81) to cause dentin microerosion due to the systematic and repeated removal of organic and inorganic matter sequentially. Therefore, the use of EDTA as a final irrigating agent, as often suggested, leaves the potential for residual bacteria and biofilm to remain within the root canal system. The use of EDTA during instrumentation would provide many of the irrigant advantages as sodium hypochlorite including flushing action, lubricant ability for mechanical instruments and facilitates dentin removal as well as removing the inorganic matter of the pulp and biofilm, removal of the smear layer, and possible detachment of biofilm form the dentin walls or disruption of inorganic bonds within the biofilm extracellular polymeric matrix. This would be followed by a final irrigation of sodium hypochlorite which would have better penetrating ability into anatomic irregularities and dentin tubules previously blocked by the smear layer and avoids the repeated switching between sodium hypochlorite and chelator that is known to lead to dentin microerosion.

Within the limitations of this study, several important outcomes were observed with respect to the interaction between sodium hypochlorite and EDTA and the ability of sodium hypochlorite to dissolve biofilm. As expected, regardless of the pre-treatment conditions of the biofilm, when matched pairs are compared against each other, meaning the same pre-treatment irrigant and time of exposure, increasing only the concentration of the sodium hypochlorite significantly decreased the biofilm dissolution time. Improved tissue dissolution by increasing the concentration of sodium hypochlorite is a well documented phenomenon resulting from the increased availability of hypochlorite allowing for greater reaction with the organic tissue (126); similarly, increasing the time of exposure (123), energy activation (127) or increasing the temperature (128) of the sodium hypochlorite have been researched strategies to effectively increase the organic dissolution and anti-bacterial properties of sodium hypochlorite. Thus, the utilization of high concentrations of sodium hypochlorite, regardless of the conditions the dentin has been exposed to beforehand, will definitely improve organic material dissolution. The benefits of increased sodium hypochlorite concentrations, when used clinically, should always be weighed with the risks for each practitioner and in each clinical scenario as the risks of potential for sodium hypochlorite accident and dentin micro-erosion may also increase in likelihood with greater sodium hypochlorite concentrations depending on the location, conditions and mechanism of irrigant utilization.

The effect of EDTA and time of pre-treatment must be examined concomitantly as the effect of each variable may play a role on the other. Within the groups exposed to 30 seconds of 17% EDTA pre-treatment, there was a reduction in the time for complete biofilm dissolution with

sodium hypochlorite as compared to the groups exposed to 30 seconds of sterile water. This effect was independent of the concentration of sodium hypochlorite used. The potentiating effect of 17% EDTA on sodium hypochlorite's dissolving ability may be explained by several factors. Firstly, and likely most significantly, is the impact EDTA will have on the biofilm attachment to the hydroxyapatite disc surface. As in Figure 6, many of the samples exhibited slight detachment of biofilm mass around the edges of the hydroxyapatite disc. The effect of this is potentially two-fold; detachment allows exposure of a greater surface area to the sodium hypochlorite allowing more rapid dissolution and it exposes the deepest layer of the biofilm which is potentially more susceptible to the actions of sodium hypochlorite. Sodium hypochlorite can have a variable effect on biofilm depending on the composition and maturity; time in particular has a significant impact on dissolution ability as immature 1 week-old biofilms have been shown to have greater susceptibility and more microbial killing by sodium hypochlorite versus established 3 week -old biofilms (52). Similarly, biofilm composition is stratified and subject to change during the maturation and development process that occurs after establishment; superficial layers may be more resilient to external destructive forces and environmental changes while deeper pioneer species are not as well protected (2). The exposure of the bottom, possibly less well protected, surface of the biofilm may allow sodium hypochlorite to dissolve and penetrate the biofilm more readily, hastening dissolution. This effect became more pronounced with more exposure time to sodium hypochlorite as greater sections of the biofilm delaminated from the hydroxyapatite disc.

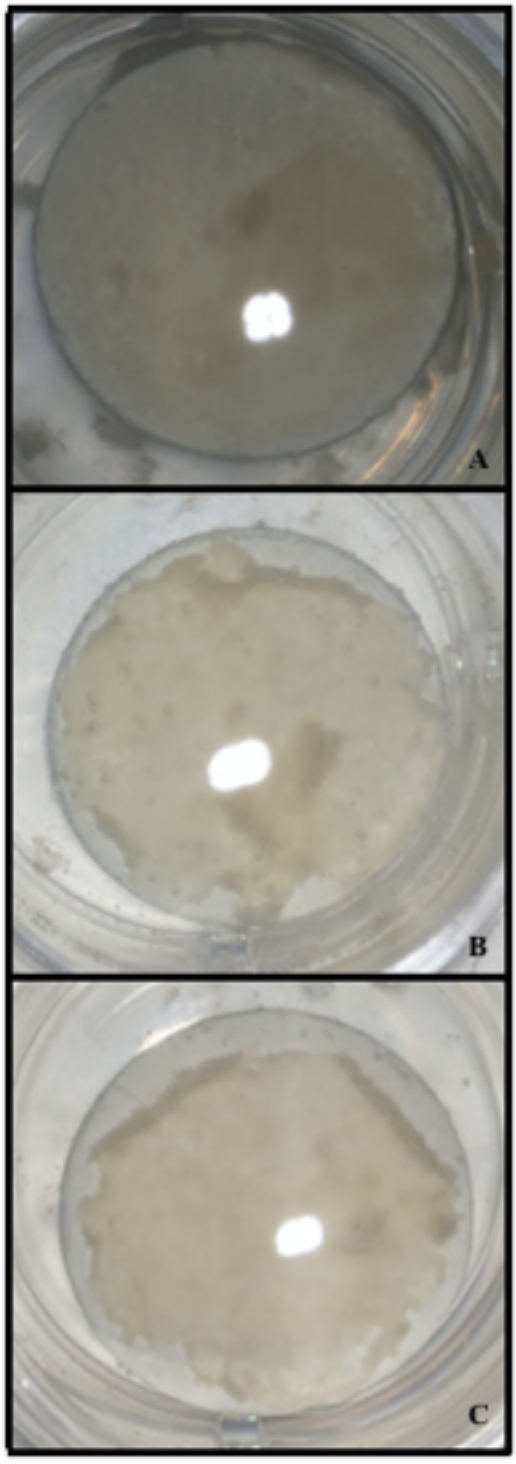

Figure 6. Detachment of the biofilm mass from the hydroxyapatite disc following 17% EDTA treatment. A-30 seconds sterile water pre-treatment. B-30 seconds 17% EDTA pre-treatment. C-1 minute 17%EDTA pretreatment.

Secondly, the use of EDTA may have played an important role in the destruction of vital inorganic bonds of the extracellular matrix, facilitating biofilm break up, sodium hypochlorite penetration into the biofilm and ultimately, dissolution. Biofilms are the result of complex interactions between bacteria and the extracellular matrix including organic components and inorganic nutrients derived from nitrates and phosphates (55); the removal of the inorganic components first may ease the process by which sodium hypochlorite can act on the remaining organic biofilm. Overall, the sequential use of EDTA for 30 seconds followed by sodium hypochlorite had a significant effect on the biofilm dissolution time, decreasing the time for complete dissolution.

When the pre-treatment time was increased from 30 seconds to 60 seconds, the biofilm dissolution time significantly increased relative to the sterile water control groups. Unlike the 30 seconds pre-treatment group, the use of 17% EDTA prior to sodium hypochlorite had a negative effect on dissolution time; like the 30 second groups, however, the effect was independent of the sodium hypochlorite concentration used. The effect of combining sodium hypochlorite and EDTA simultaneously has been demonstrated to decrease the pH of sodium hypochlorite, compromising its' high pH mechanism of action, and reduce the availability of hypochlorite ions in the solution for interaction with organic tissue, the primary form of sodium hypochlorite's chemical reactivity (88). Similar to the 30 second groups, a comparable amount of biofilm detatchment was noted around the edges of the hydroxyapatite disc following exposure to 17% EDTA for 60 seconds; however, this was a qualitative observation and not a measured outcome of the experiment. During the experiment, an intermediate sterile water rinse was utilized for all

groups to separate the pre-treatment irrigant and sodium hypochlorite and discs were moved to new, dry, clean wells prior to exposure to a new irrigant. These two intermediary steps were taken to reduce the likelihood of direct irrigant interaction as there is a known deleterious effect when EDTA, regardless of the composition or formulation, is mixed with sodium hypochlorite (89). A sterile water rinse to the biofilm, prior to sodium hypochlorite exposure was aimed at removing excess EDTA from the biofilm and hydroxyapatite disc surfaces. Similarly, placing the discs in new wells ensured there was no residual irrigant that would likely be unable to be completely removed if simply removed by micropipette. Therefore, the impact of prolonged biofilm exposure to 17% EDTA on sodium hypochlorite's biofilm dissolution ability may be due to a small scale chemical interaction within the biofilm as opposed to macro-effects such as biofilm detachment or irrigant mixing. As EDTA is allowed to a greater time of exposure in these samples, there could be greater sorption of the EDTA and incorporation into the fluid capillary network of the biofilm proper. When the sodium hypochlorite was introduced, its penetration, and therefore progression and dissolution of the biofilm, may be hindered as breakdown of superficial layers of biofilm expose EDTA from the fluid channels, inhibiting the directly exposed solution and requiring replenishment from the body of the irrigant before further dissolution progress could be made. As there was no energy activation or agitation of the samples or solutions, this process would be relatively slow in a stagnant solution, however, further manipulation or agitation of the solution may overcome this factor. It should be noted that while this study did not look specifically at the anti-bacterial properties of sodium hypochlorite following EDTA pre-treatment, a mixture of these solutions has been shown to compromise

sodium hypochlorite's antimicrobial activity (88-90), but the effects when used in pre-treatment specifically have not been examined before now.

Even though the effects of EDTA exposure to the biofilm were conserved between different concentrations of sodium hypochlorite, the effects were more pronounced in the 6% sodium hypochlorite group. It should be noted that complete biofilm dissolution was noted in every sample when the reaction was allowed to continue. Due to the lesser availability of chlorine ions, it may be expected that lower concentrations of sodium hypochlorite would be more sensitive to the negative effects of prolonged EDTA exposure to the biofilm, however, the 6% solutions demonstrated a greater variation from the control in both absolute time and as a proportion of killing time. While there have been limited studies looking at mixtures of EDTA with varying concentrations of sodium hypochlorite (88), there was no reported data with respect to relative outcome between they varying solutions and a further investigation, possibly using a range of sodium hypochlorite concentrations, would be required to determine the exact nature of the relationship.

Although sodium hypochlorite has traditionally been used as the irrigant of choice for orthograde endodontic treatment, it has inherent limitations including inability to dissolve inorganic tissue and poor peri-radicular biocompatibility (5). The use of EDTA as the irrigant during mechanical instrumentation may provide some advantages over sodium hypochlorite due to its superior biocompatibility and safety (129), inorganic tissue dissolution (81) and removal of the inorganic component of the smear layer (130). Furthermore, EDTA may allow complete detachment of or

disruption of biofilm bonding facilitating its' removal from the canal, increasing the surface area upon which sodium hypochlorite can act or improving sodium hypochlorite's penetration into the deeper layers of the organic biofilm structure responsible for endodontic pathoses.

Although EDTA pre-treatment of biofilm decreased the time for dissolution by sodium hypochlorite, this relationship seems dependent on the time of EDTA pre-treatment and the concentration of sodium hypochlorite used. Furthermore, pre-treatment for up to 1 minute increased the time for complete biofilm dissolution by sodium hypochlorite regardless of the concentration used. This study is limited in its clinical applicability due to the nature of the experimental model; future avenues of research examining the effects of EDTA pre-treatment on biofilm should be aimed at a closer *in vivo* approximation. Physical replication of the root canal system may be rectified by a dentin canal biofilm model, however, as discussed, this poses issues with respect to standardization of pre-experimental biofilms and measurement of experimental outcomes. The second aspect is with respect to irrigant volume and delivery. The volume of 1mL used in each sample was chosen for standardization purposes and to ensure there was an adequate hypochlorite ion amount at lower concentrations to guarantee complete biofilm dissolution. Clinical endodontics typically involves the utilization of larger volumes of irrigant over the course of mechanical instrumentation as well as continuous flushing and replenishing of the irrigant with possible augmentation in the forms of heat, agitation or sonic and ultrasonic activation (5). As this study was primarily focused on investigating the effects of EDTA pre-treatment on the ability of sodium hypochlorite to dissolve biofilm, the introduction of continuous movement or replenishment of the irrigant or the addition of augmented irrigation

techniques, such as agitation ultrasonic activation, would have been difficult to standardize between samples and added a confounding variable to the measured outcome.

Furthermore, the mechanism of interaction between EDTA and biofilms should be investigated as to whether there is a breakdown of inorganic bonds between bacteria and the extracellular polymeric matrix or if the EDTA allows only for gross detachment of the biofilm from the dentin surface increasing the surface area of contact and penetration of sodium hypochlorite into the biofilm's architecture. Several studies have investigated bacterial penetration into simulated canals and anatomic irregularities (112); should EDTA pre-treatment facilitate early smear layer removal within an instrumented root canal system, sodium hypochlorite's ability to penetrate root canal irregularities following EDTA pre-treatment should be investigated including whether or not it is better able to disinfect dentinal tubules harbouring bacteria or if an increased volume of biofilm is removed from such complexities. Finally, studies on dentin micro-erosion have focused on alternating solutions from sodium hypochlorite to EDTA and back to sodium hypochlorite; a characterization of a dentin surface irrigated with sodium hypochlorite as a final irrigant following pre-treatment with EDTA should be undertaken to determine if the deleterious effects are due to the scripted sequence of irrigants or simply the result of alternating back and forth resulting in repeated organic-inorganic tissue removal cycles.

Chapter 7: Conclusions

The use of 17% EDTA irrigant as a pre-treatment of biofilms on hydroxyapatite discs significantly affects sodium hypochlorite's biofilm dissolution ability. The null hypotheses are all rejected. Shorter pre-treatment times, of 30 seconds, decreased the time to complete dissolution whereas longer pre-treatment times, of 60 seconds, increased the time to complete dissolution by sodium hypochlorite; this effect was conserved between varying concentrations of sodium hypochlorite, but increasing the concentration of sodium hypochlorite significantly decreased the dissolution time.

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