DYNAMIC DISSOLUTION AND INHIBITION OF BIOFILM DEVELOPMENT BY ENDODONTIC DISINFECTING AGENTS

by

Zhejun Wang

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

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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. Hannu Larjava, Department of Oral Biological & Medical Sciences
Supervisory Committee Member

Dr. Babak Chehroudi, Department of Oral Health Sciences
Additional Examiner

Abstract

Many disinfecting solutions have been developed to disrupt the biofilm and to kill biofilm bacteria. However, there is little data so far showing dynamic dissolution on biofilm. The present study aims to establish a standardized model that makes it possible to evaluate the dynamic dissolution of biofilm and inhibition of growth of multispecies biofilm by endodontic irrigation solutions.

Biofilm was grown from plaque bacteria on collagen coated hydroxyapatite (HA) disks in brain-heart infusion broth for 3 days or 3 weeks under anaerobic conditions. Biofilms were stained by the LIVE/DEAD viability stain and subjected to sterile water, 2% sodium hypochlorite (NaOCl), 6% NaOCl, or 2% chlorhexidine (CHX) for 32 minutes. Dynamic change in fluorescence from each biofilm sample after treatment was analyzed using a live-cell imaging confocal laser scanning microscopy (LC-CLSM). Biovolume and proportion of dead bacteria were calculated. The biofilm structures after treatments were visualized by scanning electron microscopy (SEM). The treated biofilms on HA disks were collected and cultured on blood agar plates for the colony forming unit (CFU) test. Another set of sterile HA disks were immersed in 2%, 0.2% or 0.02% CHX solutions for 3 minutes. Plaque biofilm growth on these disks was monitored by LC-CLSM for 12 hours.

For 3-day-old biofilms, 2% and 6% NaOCl reduced the biovolume by 63% and 94% in 32 minutes respectively. For 3-week-old biofilms, 75% and 86% of the biofilm was dissolved by 2% and 6% NaOCl respectively in 32 minutes. Six percent NaOCl was the most effective in dissolving and killing bacteria followed by 2% NaOCl and CHX. CFU results indicated difference in bacterial reduction between biofilm and planktonic culturing after disinfection. SEM showed biofilm bacteria disruption after CHX and NaOCl treatments. The use of 2% CHX and sterile water did

not result in biofilm dissolution. However, prior exposure of HA disks to 2% and 0.2% CHX for 3 minutes prevented biofilm from growing on HA disk surfaces for at least 12 hours.

In conclusion, NaOCl dissolved biofilm effectively, more with higher concentration and longer time of exposure. CHX does not dissolve biofilm but inhibits biofilm growth on HA surface.

Lay Summary

The objective of root canal treatment is to eliminate the microbes living in the root canal system in the tooth. When bacteria invade the root canal system they can grow in a complex form named biofilm. The success of the treatment depends on effective eradication of biofilms. Irrigation is a key part of successful treatment and is also the only way to clean those root canal spaces that can hardly be touched by metal instruments. An optimal irrigation solution is desired to dissolve and kill the biofilm bacteria efficiently. However, there are no established methods so far to accurately measure the dissolution and inhibition of oral biofilm in real-time.

The objective of the present study is to establish a standardized model to evaluate the dynamic biofilm dissolution and inhibition by endodontic disinfecting solutions using real-time imaging function by a confocal laser scanning microscope.

Preface

This dissertation, “Dynamic dissolution and inhibition of biofilm development by endodontic disinfecting agents” is an original, independent and unpublished work by Zhejun Wang. The project was performed under the supervision and guidance of Dr. Markus Haapasalo and Dr. Ya Shen.

All thesis work was completed by Zhejun Wang including design, experiments, data analysis and writing. Dr. Markus Haapasalo and Dr. Ya Shen contributed to the study design and thesis editing. The relative contributions to this research project by Dr. Wang was 85%.

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List of Abbreviations

ANOVA: Analysis of variance

BHI: Brain heart infusion

CFU: Colony forming unit

CHX: Chlorhexidine digluconate

CLSM: Confocal laser scanning microscopy

EDTA: Ethylenediaminetetraacetic acid

EPS: Extracellular Polymeric Substance

DNA: Deoxyribonucleic acid

GW: GentleWave

HA: Hydroxyapatite

HOCl: Hypochlorous acid

LC-CLSM: Live-cell imaging confocal laser scanning microscopy

NaOCl: Sodium hypochlorite

-OCl: Hypochlorite ion

PAD: Photoactivated disinfection

PUI: Passive ultrasonic irrigation

SEM: Scanning electron microscopy

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It has been a long journey for me to arrive at this point in my life. This is not only a destination after three extraordinary years at UBC, but also a new start for my career. So many unforgettable moments are coming to my mind all with different flavors of memories such as excitement, motivation, frustration, pride, and gratitude. The guidance, trust, support and love from people around me are the most valuable gifts I luckily received. These people are the ones who made my journey so unique, meaningful and successful.

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Dedication

I dedicate this work to my beloved parents. Your endless trust and support are the most precious fortune I could have.

I am grateful to my father who always has enlightened my visions throughout my life with unconditional support. I am grateful to my mother who has given me unwavering care and encouragement through my whole education.

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Chapter 1: Introduction

1.1 The role of biofilms in endodontics

A biofilm is a structured sessile community of microorganisms embedded in a self-secreted hydrated matrix of extracellular substances that contains polysaccharides, proteins, extracellular deoxyribonucleic acid (DNA) and lipids (1). Biofilms normally grow in moist environments that originate from the attachment of planktonic bacteria to the substrate surface (2). Biofilms are known to be responsible for many diseases such as cystic fibrosis and chronic sinusitis (2-4). The adherent and aggregated lifestyle of biofilm results in increased adaptive resistance to antimicrobial agents, making biofilm-related infections inherently difficult to treat (5, 6).

This challenge from biofilms exists also in endodontic infections. Bacteria in endodontic infections originate from tooth surface plaque. In the anaerobic microenvironment of necrotic teeth, the microorganisms colonize root canal space, bind to the root canal walls and grow as habitat-adapted multispecies endodontic biofilms (7). The microbial infection in the root canal system can lead to an inflammatory reaction by the periradicular tissues and result in apical periodontitis which can cause pain, swelling, an unhealed sinus tract and persistent lesion (8). Root canal treatment is an effective strategy in endodontics to treat apical periodontitis by mechanical instrumentation as well as chemical disinfection. The goal of the root canal treatment is to effectively eradicate the biofilms in the root canal system (9).

1.2 Evaluation of biofilm eradication by irrigation

Irrigation plays a key role in biofilm eradication in the root canal treatment by facilitating the killing of the microorganisms and dissolving the biofilm. It is a critical part of successful root canal treatment and is also the only way to impact areas of the root canal system that mechanical

instrumentation cannot reach. An optimal irrigation solution would have excellent tissue-dissolving ability and a strong killing effect. Although a number of irrigating solutions with strong antimicrobial efficacy have been used in the endodontic clinic, there are no established methods so far to accurately evaluate and quantitatively measure their ability to dissolve biofilm.

Sodium hypochlorite (NaOCl) and chlorhexidine gluconate (CHX) are two of the most commonly used irrigating solutions in endodontics because of their capability to kill bacteria and/or dissolve organic tissue (10). Hypochlorous acid (HOCl), which forms from NaOCl, penetrates bacterial cell wall and membrane, damages DNA and bacterial proteins and causes bacterial disruption (11, 12). Many previous studies have evaluated the tissue dissolution effect of NaOCl using different substrates including pulp tissue (13-15) and meat (16, 17). Chlorhexidine was reported to have minimal dissolution effect but good killing ability against biofilm bacteria. Previous studies have set up different biofilm models to evaluate the killing effect of NaOCl and CHX (18, 19). However, there is little data so far showing the ability of NaOCl to dissolve biofilm and the reaction dynamics of NaOCl or CHX. One of the major challenges has been to establish an accurate assessment *in vitro* of their effects on biofilms.

1.3 A new model for dynamic biofilm dissolution

As it is difficult to weigh biofilms without causing structural damages and to obtain accurate measurements since they are too light to be measured on a balance, the application of a novel protocol that allows real-time analysis of biofilm volume variation with fluorescent staining would greatly assist the evaluation of the biofilm dissolution and inhibition. The live cell imaging technique has been extensively applied in mammalian cell studies to obtain a better understanding of biological function through cellular dynamics (20, 21). A confocal laser scanning microscopy (CLSM) with live-cell imaging function maintains the physiological body temperature for bacterial

growth and enables continuous monitoring of the biofilm treated by the disinfecting solutions. This new platform may have the potential to help the profession to better understand antibiofilm properties of existing and upcoming, new irrigation solutions and eventually lead to clinical application on patients.

The present study aims to establish a standardized biofilm dissolution model to non-invasively evaluate the effect of different endodontic irrigation solutions on oral multispecies biofilm.

Chapter 2: Review of Literature

2.1 Etiology of endodontic disease

2.1.1 Microorganisms in endodontic infection

The infectious etiology of apical periodontitis has been well established over the last century and a number of studies have contributed to associate microorganisms with the endodontic disease. Since Miller first observed microorganisms in infected root canal in 1894 (22), microorganisms have been implicated in infections of endodontic origin. In 1965, Kakehashi et al. (23) demonstrated that apical pathology only developed after pulp exposure to the oral cavity in conventional rats with normal oral microorganisms, while no apical periodontitis developed following pulpal exposures in germ-free rats. Moller et al. (24) noted similar results using monkey models.

The important role of bacteria in the etiology of apical periodontitis was further confirmed by Sundqvist's study in 1976 (25). This study used anaerobic culturing techniques to evaluate the bacteria existence in trauma-induced necrotic pulp. Bacteria were found only in the root canals of teeth with radiographic periapical lesion. Ninety percent of the isolates were anaerobic bacteria. In the absence of infection, the necrotic pulp tissue itself in the root canal cannot induce apical periodontitis.

The root canal system is a well-sealed space protected by overlying enamel or cementum. To be able to cause pulpal inflammation and infection, microbes need to overcome a series of barriers. Supra- or subgingival plaque bacteria can invade dentinal tubules if dentin is exposed to the oral cavity. The interruption of the protective barrier can be caused by dental caries, restorative or periodontal procedures, enamel or dentin cracks, tooth abrasion/attrition or traumatic injuries (26-

28). When the pathogenic microbes invade the normally sterile tissue, the bacteria and their byproducts can cause excessive and deleterious pulp immune responses, tissue necrosis and microbe dissemination in the root canal (29).

Oral microbial communities are some of the most complex in the human body. Over 500 species of bacteria have been cultured from the oral cavity (30). More than 700 species have been identified at the DNA level using molecular techniques (31), and many of the microbes are difficult to culture on routinely used media. These include species such as *Dialister pneumosintes*, *Treponema maltophilum*, *T. amylovorum*, *T. medium* and *T. lecithinolyticum* (32-34). *Streptococcus* and *Lactobacillus* species have been implicated as chief pathogens in the progression of caries together with *Actinomyces*, *Bifidobacterium* and *Eubacterium* (35, 36).

Bacterial invasion of the dentinal tubules under decay can cause the initiation of innate immune responses to the bacterial antigens, irreversible acute inflammation, and eventually resulting in liquefactive necrosis of the pulp tissue (31). The major ecological factors that determine the composition of the root canal microbiota include oxygen tension, nutrients amount and types, and bacterial interactions (37). The initial phases of the pulpal infection process are dominated by facultative bacteria. After a few days or weeks, oxygen is depleted within the root canal as a result of pulp consumption. Oxygen supply is further interrupted with loss of blood circulation and growth of obligate anaerobic bacteria is initiated. With the passage of time, the pulp canal space becomes a reservoir with low oxygen levels for the rapid growth of anaerobic bacteria and the release of their byproducts. Anaerobic conditions become even more pronounced; as a result, anaerobes dominate the microbiota instead of facultative bacteria (38). Previous studies showed that in addition to *Streptococci*, *Actinomyces*, and *Lactobacilli*, obligate anaerobic species of *Fusobacterium, Propionibacterium, Peptostreptococcus, Porphyromonas, Veillonella, Prevotella*,

and *Eubacterium* dominate the microflora in heavily infected pulp (24, 39, 40). Other microorganisms such as yeasts and *Spirochetes* also have been recovered from necrotic pulp (41, 42).

The bacterial species specificity may apply to different types of infections and symptoms of pain. The primary infections, those that occur in untreated necrotic teeth, involve a larger number of species than secondary infections from previously treated teeth. Figdor and Sundqvist (43) reported that primary infections consisted of an equal mix of gram-positive and gram-negative bacteria and contained mostly obligate anaerobes, while secondary infections contained mostly gram-positive bacteria with a more equal distribution of facultative and obligate anaerobes. *Enterococcus faecalis* and *Streptococcus epidermidis* were found to be the most prevalent species in secondary infections (44). Siqueira et al. (45) reported increased prevalence of *Fusobacterium* in symptomatic infections. Gomes et al. (46) showed an increased prevalence of *Peptostreptococci* and *Prevotella melaninogenica* in the presence of pain. Sabeti et al. (47) found that cytomegalovirus and Epstein-Barr virus were also associated with painful endodontic infections.

As a sequel to microbial infection of the root canal space, apical periodontitis can be developed as an inflammatory process at the apex of the root. Traditional studies in 1970s supported the opinion that the microorganisms only harbor within the root canal system, whereas the periapical lesion is bacteria free (48-50). However, more recent studies have demonstrated the presence of microorganisms in the periapical tissues (51, 52). The microorganisms in the periradicular tissues are established either by adherence to the external root surface (extraradicular biofilm) or by formation of actinomycotic colonies within the body of the periapical lesion (53). In the former situation, some bacterial species that overcome host defenses accumulated near or beyond the

apical foramen as an extension of the intraradicular infection through apical foramen or apical ramifications (54).

Extraradicular infections could also be independent from intraradicular infection and can persist after non-surgical root canal treatment (55). One possibility for the periapical biofilm formation may be that bacteria in acute apical abscesses persist in the periradicular tissues following resolution of the acute response, sometimes resulting in a draining sinus tract (44). Another possibility for the pathogenesis of periapical biofilms is that the bacteria reach the periapical tissue during over-instrumentation. Bacteria embedded in dentinal chips persist in the periradicular tissues and sustain periradicular inflammation (56).

It has been suggested that the main bacterial species implicated in independent extra-radicular infections are *Actinomyces* species and *Propionibacterium propionicum* (52). These bacteria can form cohesive colonies that are resistant to phagocytosis. Anaerobic cultivation also demonstrated the survival of anaerobic and facultative anaerobic bacteria such as *Prevotella* and *Porphyromonas* in periapical inflammatory lesions (51).

2.1.2 Plaque biofilm formation

A large variety of endodontic microorganisms can be isolated from endodontic infections, however, most of them do not exist independently as monoinfections nor as freely floating single cells, but rather grow in spatially organized, coordinated and metabolically integrated multispecies biofilm communities (57). Bacteria in endodontic infections originate from dental plaque consisting mainly of gram-positive and gram-negative facultative and anaerobic bacteria (58).

The early stage of plaque biofilm formation involves the adsorption of macromolecules in the planktonic phase by the dentin surface, forming a conditioning film, pellicle, typically composed

of organic molecules (59). Numerous microorganisms in the planktonic phase then move close to the surface and bind to the acquired pellicle receptors *via* adhesins (60). Certain bacteria such as *Streptococcuci*, *Actinomyces*, *Capnocytophaga*, and *Veillonella parvula* are the primary colonizers. The micro-colonies from primary colonizers progressively enlarge and coalesce to form the first layer of biofilm cells covering the hydroxyapatite surface of dentin (61). Following this, multiple layers of bacterial cells pile up on the primary colonizers, resulting eventually in a structurally organized mixed microbial community. The late colonizers in the dental plaque biofilm include *Fusobacterium nucleatum*, *Prevotella intermedia*, *Eubacterium nodatum*, and *Treponema denticola*, and numerous other species (62). At this maturation stage the biofilm is characterized by a primitive circulatory system balancing the multiplying and detaching bacteria. The nature of the microenvironment influence growth and succession of microorganisms in biofilm.

The supra-gingival plaque is dominated by gram-positive bacteria while the subgingival plaque is primarily composed of gram-negative anaerobic bacteria (57). The dental plaque remains relatively stable under normal circumstances. However, when the bacteria follow certain pathways to invade a sterile pulp canal space and change the local ecological environment (lowering the pH and redox, consuming nutrients), the new environment may selectively favor the growth of anaerobic proteolytic bacteria, which outcompete other species in the micro-community of the plaque to become pathogenic by virtue of a numerical dominance (63).

2.1.3 Young and old biofilms

Bacterial infections may exist in root canal lasting from months to years. Multiple previous studies have shown that bacteria in young biofilms were more susceptible to disinfectants than cells in old biofilms (64-66). Anwar et al. (67) reported substantial difference between 2-day and 7-day *P.*

aeruginosa biofilms in biofilm accumulation. The 7-day-old biofilms were significantly more resistant to antimicrobial agents (68). Dental plaque biofilms were considered to be mature from 3 weeks onward with regard to their structural development (66). Stojicic et al. (18) reported that mature plaque biofilms (3-week-old) from 6 different donors were substantially more resistant to three different disinfecting agents than younger biofilms. Shen et al. (66) showed that bacteria in mature plaque biofilms were more resistant to chlorhexidine digluconate (CHX) killing than in young plaque. Du et al. (65) showed that significantly more *E. faecalis* cells were killed in young biofilms than in old biofilms.

Biofilm age and bacterial cell density are usually strongly correlated (64). In young biofilms, more bacteria are in the active and exponential growth phase with extracellular polymeric substance (EPS) formation. The later step of biofilm maturation controls the thickness and architecture of the biofilms, helping biofilms reach a steady state of accumulation. This maturation process is regulated by cell-to-cell communication signals, and at the same time, reflects the physiology of the bacterial cells (69). Mature biofilms might develop their own localized environments that dictate the metabolic activities of the cells and protect them to some extent against changes in the environment (66). The nutrients can produce changes within the environment of mature biofilms as well, so that the ability to survive or adapt to nutritional, pH and other changes within mature biofilms remains an important aspect of the ecology of biofilm.

2.1.4 Mechanisms of biofilm resistance

Dental plaque, which is comprised of diverse bacteria in the biofilm state, displays increased resistance to antimicrobial agents in comparison to planktonic bacteria (70, 71). The protective mechanisms of biofilm resistance are not fully understood. However, several mechanisms have been proposed including physical or chemical diffusion barriers (EPS formation), cell-to-cell

communication (quorum sensing), presence of persister cells and physiological heterogeneity within the biofilm population (61).

Extracellular polymeric substance

Extracellular polymeric substance is a self-produced extracellular matrix holding biofilm cells together. EPS plays a major role in maintaining the integrity of the biofilm and promoting bacterial adherence to the tooth surface. It not only prevents desiccation in biofilm, but also acts as a diffusion barrier contributing to the biofilm resistance to disinfectants (72). Such protection can be due to physical hindrances in antimicrobial diffusion or direct binding of the antimicrobial agents to bacterial cells (61). Bacterial cells at the surface of the biofilm may be easier to kill because of proximity to exposure of antimicrobial agents, whereas bacteria embedded deep in the EPS are more prone to survive. A previous study reported that EPS is responsible for the protection and virulence of *Streptococcus mutans* biofilm on pellicle coated hydroxyapatite disks (73). A more recent investigation used a mathematic model to demonstrate the resistance of EPS produced by plaque biofilm against CHX (2). The EPS matrix can also be considered a chemically active barrier. It has been reported that anionic EPS can bind and sequester toxic heavy metals, cationic peptides and positively charged antibiotics (74).

Cell-to-cell communication

Cell-to-cell communication within biofilms also plays an important role in biofilm resistance. Quorum sensing is a regulatory mechanism involving cell-to-cell communication mediated by signal molecules. This concept was first introduced by Davies et al. (75) in 1998 showing the role of quorum sensing system in *Pseudomonas aeruginosa* biofilm formation. Later research has also shown quorum sensing in dental plaque biofilm (76). In the process of quorum sensing, bacteria communicate with each other by using autoinducers to regulate gene expression in response to

fluctuations in cell density (77). Two types of quorum sensing systems have been recognized in biofilms including intra-species communication and inter-species communication (78). In intra-species communication, gram-positive bacteria utilize the products of oligopeptides, while gram-negative bacteria usually use acyl homoserine lactone as the signal molecule. In inter-species communication, bacteria use autoinducer-2 as signal molecules. Besides biofilm resistance, quorum sensing also has been reported to be responsible for bacterial attachment, maturation of biofilm and dispersion of cells (61).

Persister cells

The formation of persister cells is another mechanism of biofilm resistance. The presence of persisters was firstly noted by Bigger (79) in 1944. A small group of live bacteria in the biofilm community known as non-growing persisters exists. This type of bacteria can enter a state of deep dormancy. The mechanism may be based on a deliberate expression of toxic proteins in a small fraction of cells to maintain them in a dormant state, as a result of stochastic expression of a broad variety of genes (80). Biofilm bacteria produce persister cells that neither grow nor die in the presence of microbicidal antibiotics and other antimicrobial substances. The killing rate for persisters is usually very low. Persisters can resuscitate from their dormant state when antimicrobial substances are removed and fresh nutrients become available (81). A recent study used a mathematic model to analyze the recovery process of plaque biofilm after CHX treatment (2). It was found that when the efficacy of CHX dropped below a threshold that was no longer fatal to the bacteria, the persisters became metabolically active and began to convert back into susceptible cells and to resume a variety of active cell functions including cell division.

2.2 Root canal disinfection

Endodontic infections originating from the dental pulp can spread into the alveolar bone. Infections may eventually spread also through fascial spaces and the underlying tissues and organs to potentially dangerous locations and cause a life-threatening condition (82). Debridement of the root canal by instrumentation and irrigation is considered the most important part in the prevention and treatment of endodontic diseases (9). The goal of instrumentation and irrigation is to remove and kill all microorganisms in the root canal system.

2.2.1 Strategies on root canal disinfection

Mechanical instrumentation

Mechanical instrumentation is the core method for bacterial reduction in infected root canals. Mechanical instrumentation alone without irrigation has been shown to result in 70% more debris in the canal than when irrigation is used (83). Bystrom and Sundqvist (84) showed that instrumentation and irrigation with physiologic saline substantially reduced the bacterial load. However, mechanical preparation with instruments and irrigation with saline cannot predictably eliminate the bacteria from the infected root canals and the antimicrobial efficacy of instrumentation with saline was still considered poor (85). When an antimicrobial irrigation solution was used instead of saline, the antibacterial effect was much more effective (86). Moreover, it has been demonstrated that endodontic instrumentation leaves as much as 35% of the canal walls untouched (87). Therefore, using irrigating solutions with strong antibacterial activity has been regarded as the necessary supplement to mechanical preparation. It is also important to be able to consistently deliver irrigants to the full length of the root canal in order to achieve biofilm and debris removal.

Sodium hypochlorite

Sodium hypochlorite is the most commonly used irrigating solution in endodontic treatment by the majority of endodontists because of its antimicrobial effect, tissue dissolution capacity, and biocompatibility when confined to the canal (88). Since biofilm, pulpal remnants, and predentin are mainly organic matter, NaOCl is the key irrigant in the cleaning of the untouched parts of the root canal. When NaOCl is in contact with organic tissue, NaOCl reacts with fatty acids creating soap and glycerol known as saponification reaction. It also reacts with amino acids creating salt, chloramine and water, leading to liquefaction of the organic tissue. Hypochlorous acid (HOCl) uses bacterial porins as channels to evade the bacterial defenses and cause more damage to internal structures (89). It also causes damage to cell membranes and bacterial division. Likewise, it interacts with DNA and bacterial proteins disrupting their proper operation (90). Both HOCl and the hypochlorite ion (-OCl) are strong oxidizing agents. HOCl and -OCl have been reported to react with a wide variety of biological molecules such as proteins, amino acids, peptides, lipids, and DNA (91). Therefore, the main molecular mechanisms of action of the NaOCl on bacterial structures include: a) penetration of bacterial porins, b) bacterial DNA damage and c) oxidation of sulfhydryl groups, which are in the structure of proteins and enzymes essential for the growth and functions of bacteria (92).

In endodontic clinically related research, NaOCl presents high antimicrobial activity and dissolves biofilm, pulpal tissue and organic components of dentin when used in concentrations between 0.5% and 6% (93). Bystrom and Sundqvist (86) did not show any significant difference in antibacterial efficiency between 0.5% and 5% NaOCl solutions *in vivo*. Siqueira (94) showed no difference among 1%, 2.5%, and 5% NaOCl solutions *in vitro*. Other studies showed that higher concentrations of NaOCl have superior antimicrobial and tissue dissolution activity than lower

concentration (17, 65, 95, 96). The difference in results reported by different studies may be due to different biofilm growing conditions, bacterial species/strains used, and detection methods. Sodium hypochlorite is also effective in killing yeast. Waltimo et al. (97) showed that 5% and 0.5% NaOCl eliminated *Candida albicans* in 30 seconds *in vitro*. Despite the strong antimicrobial ability of NaOCl, it is still difficult to eliminate all bacteria in the root canal system. Du et al. (65) used 6% NaOCl to attack *E. faecalis* biofilm in dentinal tubules for 30 minutes, 12% and 22% bacteria were still alive in 1-day and 3-week-old biofilm respectively after treatment.

Sodium hypochlorite only removes organic tissue, therefore the smear layer cannot be completely removed using NaOCl alone. The smear layer should be removed as it may contain microorganisms and weaken the effects of disinfecting agents in dentin; and the quality of the root filling and bonding to the canal wall may be compromised (98). Ethylenediaminetetraacetic acid (EDTA) and different combination products with EDTA such as QMiX (Dentsply Tulsa Dental, Tulsa, OK) and SmearClear (Vista Dental, Racine, WI) have no tissue-dissolving effect on organic matter, but they can be used after NaOCl to remove smear layer and facilitate root canal disinfection during the final rinse (99).

Chlorhexidine digluconate

Chlorhexidine digluconate is a cationic bisbiguanide with broad antibacterial activity and strong affinity for binding to skin and mucous membranes. The CHX molecule reacts with negatively charged groups on the cell surface, causing an irreversible loss of cytoplasmic constituents, membrane damage, and enzyme inhibition (66). High concentrations of CHX can cause coagulation of intracellular constituents. A 2% CHX solution is widely used as an endodontic irrigating solution in the final rinse of root canal irrigation. A series of previous studies have evaluated the antimicrobial efficacy of CHX and compared to NaOCl (100). Killing of microbes

by CHX was time-dependent, and CHX was unable to kill all microbes in the young and old biofilms (66). Chlorhexidine digluconate killed planktonic *E. faecalis* cells in 30 seconds in concentrations of 0.2–2% (101). It required a much longer time to kill *E. faecalis* than the corresponding concentration when CHX was used in a gel form (101). Compared with NaOCl, CHX was reported to be superior to NaOCl in killing of *E. faecalis* in planktonic culture in one study (102). Jeansonne and White (103) found CHX was as effective as 5.25% NaOCl in terms of antibacterial activity. In other studies using the dentin infection model, 2% CHX showed a killing effect similar to that of 2% NaOCl but was less effective than 6% NaOCl against *E. faecalis* and plaque biofilm (19, 104).

Chlorhexidine digluconate also possesses the property of substantivity (105). CHX binds to dentin allowing its antimicrobial activity to persist for long time (106). Shen et al. (107) applied a mathematic model to demonstrate the inhibitive effects of CHX on plaque formation. Chlorhexidine digluconate exerted the substantivity effect by an immediate bactericidal action followed by prolonged bacteriostatic effect of the CHX which bonded onto the hydroxyapatite surface. Another study evaluated the substantivity of 2% CHX within the root canal system of bovine, reporting that CHX retained antimicrobial activity against *E. faecalis* as long as 12 weeks after a 10-minute treatment (108). However, despite the excellent antimicrobial activity and substantivity of CHX, CHX lacks the ability to dissolve organic and inorganic matter (9).

2.2.2 Other strategies to facilitate effective irrigation

Ultrasonics and EndoVac

Various methods have been developed through years to facilitate irrigation and disinfection in the root canal, particularly in difficult-to-reach areas. Passive ultrasonic irrigation (PUI) was developed to improve the cleanliness of both the main canal and isthmuses areas by creating

acoustic streaming (109, 110). Malki et al. (111) reported that PUI is more effective than needle irrigation in removing debris from depressions in the canal space. PUI can also help NaOCl penetrate into the lateral canals better than regular positive pressure irrigation (112). Beus et al. (113) showed that PUI was as effective as a final rinse with CHX in eliminating bacteria.

EndoVac (Discus Dental, Culver City, CA) system uses negative pressure to achieve safe irrigation of the apical canal, and without fear of irrigant extrusion to the periapical area. By using EndoVac system, the irrigant placed in the pulp chamber is sucked down the root canal and back up again through a thin needle. Negative pressure method offers significantly better cleaning than the conventional positive pressure syringe-needle irrigation at 1 mm from the working length (114).

Photodynamic therapy

Photoactivated disinfection (PAD) has been recommended as an adjunctive procedure to kill residual bacteria in the root canal system after standard endodontic debridement (115). It involves the use of a photosensitizer (e.g. methylene blue dye) that is activated by light in the presence of oxygen. Photosensitizer binds to bacterial membrane surface and enters into cytoplasm. When exposed to the light of a specific wavelength, a photosensitizer can react with either oxygen or other biomolecules to create free radicals, resulting in cell death (116). PAD has been claimed to target microorganisms with no collateral damage to human cells and tissues (117). Promising results using conventional PAD against *E. faecalis* biofilms was demonstrated in a previous study (117). George and Kishen (118) demonstrated that bacterial killing can be improved by modifying photosensitization and irradiation medium and by introducing the dual stage approach. Stojicic et al. (119) modified PAD by adding low concentrations of EDTA, hydrogen peroxide or CHX in various combinations to the methylene blue solution to facilitate bacterial killing. The results

showed that modified PAD was superior to conventional PAD against both *E. faecalis* and plaque biofilm bacteria.

GentleWave system

A multisonic ultracleaning system, the GentleWave (GW) system (Sonendo Inc, Laguna Hills, CA), has recently been developed for cleaning of the root canal system (17). The GW system was designed to deliver a broad spectrum of sound waves within the irrigant to effectively clean the root canal system with minimal or no instrumentation. It has been reported that the GW system can thoroughly clean the root canal system even in the apical third (120). The mechanism of the cleaning by GW is attributed to the interplay between propagating multisonic energy and fluid dynamics (121). The system disperses fluids from the tip of the handpiece into the pulp chamber, causing cavitation effect (122). The cleaning of the root canal walls is a result of locally released energy, shear forces and hydrodynamic cavitation in the fluid (123). A recent study on the GW system showed it had eight times faster tissue dissolution rate compared to other irrigation systems including ultrasonic irrigation, EndoVac and needle irrigation (17). It has also been shown to create negative pressure at the apical foramen (124) with no extrusion of the irrigating solutions (123). In a multicenter clinical study, the 1-year success rates of necrotic teeth and irreversible pulpitis were 92.9% and 98.4%, respectively, by using GW system (125). In a recent case series study, the GW procedure resulted in a success rate of 97.7% at 1-year recall for the patients with periapical lesions (126). Above all, the GW system seems to be a promising novel cleaning system to be widely used in the future endodontics.

Antibiofilm peptides

Antibiofilm peptides have recently received great attention for possible use in a series of therapeutic applications against oral biofilms. Antibiofilm peptides exert activity against a broad

spectrum of microorganisms including gram-negative, gram-positive bacteria, drug-resistant strains, and fungi (127). The general mechanism for antibiofilm peptides is that the peptides can permeabilize the membrane of the bacterial cells, resulting in either large-scale damage or small defects that dissipate the transmembrane potential leading to cell death (128).

Many studies have identified antibiofilm peptides as the potential next-generation alternative to traditional antimicrobial therapy in the oral cavity. Root canal disinfection has been recognized as a potential area for antibiofilm peptide application (6). Several previous studies have used antibiofilm peptides to evaluate their efficacy against microbes found in endodontic infections. Liu et al. (129) found that a kappa-casein peptide significantly inhibited planktonic growth of *E. faecalis* in both planktonic and biofilm cultures. The glycosylated form of the peptide effectively inhibited the *E. faecalis* biofilm formation and may therefore have potential to promote the efficacy of traditional root canal disinfecting agents. Another study used confocal microscopy to test the antibiofilm activity of four synthetic lipopeptides against *E. faecalis* biofilm, showing that the lipopeptide had antibiofilm effect against *E. faecalis* (130). Lee et al. (131) evaluated the antibiofilm efficacy of human β-defensin-3 peptide against multispecies endodontic biofilms. The peptide exhibited higher bactericidal activity on the 3-week-old biofilm than calcium hydroxide and 2% CHX solution. A recently introduced peptide 1018 effectively inhibited oral plaque biofilm growth in the presence and absence of saliva (132). Moreover, peptide 1018 showed adjunctive antibiofilm effect when using together with 2% CHX, resulting in over 50% biofilm killing (132). A more recent study compared a novel D-enantiomeric peptide (DJK-5) with peptide 1018 on single- and multi-species oral biofilms and reported higher effectiveness against biofilms by DJK-5 than peptide 1018 (133).

Despite the promising antibiofilm effect from the peptides, limitations also exist in their therapeutic utility. The antibiofilm power of the peptides can be significantly reduced in the presence of biological fluids compared to their performance in non-physiological conditions (134). In addition, antibiofilm peptides are expensive and difficult to manufacture in large quantities due to the complex processes of isolation, extraction and purification (135).

2.2.3 Challenges and difficulties in irrigation

Anatomical complexity

The root canal system is inherently complex with branches, anastomosis, and irregular structures (136). The anatomical complexity of the root canal system enables bacteria to hide and multiply and prevents irrigants from adequately reaching those specific areas containing debris and bacteria (98). The anatomical spaces of the confining geometry such as accessory canals, isthmuses and dentinal tubules were areas with difficulty of solution exchange.

In a root canal system that is close-ended, the vapor lock phenomenon can occur when there is air entrapment at the end of the close-ended channel (137). The use of an apical patency file may decrease the presence of air bubbles in the middle and coronal portions of a root canal, but the air bubbles are usually difficult to eliminate (138). Another possible solution can be manual agitation with a gutta-percha cone to facilitate the irrigant to the apical third (139). Moreover, the use of apical negative pressure irrigation such as the EndoVac system can effectively mitigate the challenge by apical vapor lock (139). A more recent scanning electron microscopic study showed that GW was able to clean the entire uninstrumented root canal system of permanent premolars with no debris left (140).

Microbial factors

Smear layers are created on root canal walls touched by endodontic instruments. The smear layer is composed of organic material including necrotic pulp tissues, embedded bacteria, predentin as well as inorganic material from the mineralized dentin (141). Inadequate irrigation is likely to result in the incomplete removal of the smear layer in the root canal and leave microorganisms behind. The residual bacteria in the root canal system may be potential risk for further infection. A relatively new premixed and ready to use irrigant, QMiX, showed killing efficacy equal to that of high-concentration NaOCl with the ability to remove smear layer (19, 142). Moreover, any remaining microbes or necrotic tissue can also jeopardize the integrity of the sealing by root filling (143).

Biofilm resistance due to EPS formation and the presence of persisters is another microbial factor as discussed above in section 2.1.4.

Chemical factors

Sodium hypochlorite is the most important irrigating solution for root canal treatment and should be used throughout the instrumentation (143). The recommended final irrigation protocol for disinfection and smear layer removal is NaOCl followed by EDTA or citric acid (144). However, clinicians may alternate the use of the solutions and often use NaOCl as the final rinse after EDTA. Decalcifying solutions such as EDTA or citric acid followed by NaOCl can cause erosion of the root canal dentin (143). When EDTA is used in the final rinse, the hydroxyapatite (HA) is quickly dissolved close to the main root canal, exposing the collagen fibers. If NaOCl is used again at this stage, it can directly dissolve the collagen and cause considerable destruction of the collagen backbone of surface dentin. Sodium hypochlorite used as a final irrigant solution after demineralization agents caused marked erosion of peritubular and intertubular dentin in a SEM study (145). A more recent study using energy dispersive X-ray spectroscopy showed significant

calcium and phosphorus loss deep into dentin after 5 min of EDTA followed by 5 min of NaOCl treatment in the final irrigation (146). A previous study has shown that long term exposure of 1 – 2 hours to high concentrations of NaOCl can also lead to a significant reduction in the flexural strength and elastic modus of dentin (147). Thus, long term NaOCl irrigation after demineralization agents should be avoided.

Although NaOCl kills bacteria quite effectively, it can cause severe facial pain, immediate swelling, ecchymosis and other serious complications if it accidentally gain access to the periapical area or adjacent structures, resulting in a NaOCl accident (148). Chlorhexidine digluconate can also be cytotoxic to human cells (149). However, it does not cause such severe reactions as NaOCl if extruded through the apical foramen. Moreover, CHX cannot be used with NaOCl at the same time because of the formation of parachloroaniline, which may be carcinogenic (150). Hence, a thorough saline rinse is recommended between these two irrigants.

2.3 Current biofilm models for the evaluation of endodontic disinfection

2.3.1 Open-culture model

Oral biofilms are designed to grow in standardized *in vitro* conditions to obtain predictable structure and behavior (151). Open-culture biofilm models use different substrates (e.g. glass, polycarbonate, HA, dentin) to grow monocultures and mixed cultures in aerobic and anaerobic environments. Kristich et al. (152) cultured *E. faecalis* biofilm on the bottom of 96-well plates to study the influence of a specific cell surface protein on *E. faecalis* biofilm formation. Kishen et al. (153) used fluorescence microscopy to study the adherence of *E. faecalis* on dentin. An open-culture biofilm model was also used to evaluate the interaction between *E. faecalis* biofilm and immune cells (154). Multispecies plaque biofilm open-culture model has been used in a series of

studies for testing the efficacy of different antimicrobial agents (e.g. CHX (155, 156), NaOCl (18), antibiofilm peptides (132, 133, 157)) on biofilms in different circumstances (biofilm age, nutrition, length of treatment time etc.) using confocal laser scanning microscopy (CLSM).

The advantage of an open-culture model is that the substrate can usually provide a static base for biofilm growth. By controlling the time, nutrients and oxygen, the biofilm sample can be stably formed for the further evaluation. Colony forming unit counting can be done for the planktonic bacterial cells separated from the biofilm, and the proportion of killed bacterial cells can also be calculated with biovolume measured in the CLSM.

2.3.2 Dentin canal biofilm model

The involvement of dentin in the biofilm models is designed to further mimic root canal infections in the clinic. The survival of the bacteria *in vivo* can be attributed to their invasion of the dentinal tubules where they can be protected from disinfectants. Previous studies have shown that dentin can affect the antibacterial effectiveness of endodontic disinfectants (158, 159). Different dentin infection models have been developed for the evaluation of dentin disinfection in endodontics. Earlier studies used culture methods to study bacterial invasion of dentinal tubules (160, 161). The infection of tubules was uneven with regard to the number of infected tubules and the depth of penetration. A more recent study introduced a new method that can obtain a heavy and evenly distributed infection of dentin tubules using centrifugation (19). Multiple follow-up studies have applied this new dentin infection model to analyze the antimicrobial effect of various disinfecting solutions (96) and root canal sealers (162, 163) on both monospecies and multispecies biofilms (104). Lin et al. (164) designed and used a novel, standardized biofilm model in extracted teeth by culturing biofilm in an artificial apical groove to quantify the efficacy of different endodontic instruments in biofilm removal in hard-to-reach areas. The teeth were split before biofilm

inoculation, reassembled for biofilm incubation and cleaning, and finally split again for SEM observation and measurement of the cleaning (164). Different NiTi instruments and irrigation methods have been evaluated so far using this model (165). Despite its closer-to-clinic experimental design, the dentin infection model also has limitations including a time-consuming sample preparation process, a smaller area of detection compared to open-culture model, and higher technical requirement.

2.4 Outlook

Most of the open-culture and dentin biofilm models so far were designed for static biofilm growth. It would be innovative to mimic the dynamic biofilm formation using pre-fabricated flow cell systems monitored with equipment such as a charge-coupled-device camera or a live-cell imaging CLSM in the future research.

Most of the current biofilm studies are designed for short term analysis *in vitro*. However, there is a lack of long-term clinical studies. Obtaining evidence-based data will be a remarkable advancement enabling the identification of the most effective antibiofilm agents and appropriate circumstances for their uses.

Chapter 3: Aim and Hypotheses

3.1 Aim

Since bacteria are the main cause of apical periodontitis, elimination of the bacteria in the infected root canal system is the primary goal of root canal treatment. Despite various debridement techniques and irrigation solutions that have been developed and used over the decades, the eradication of all microorganisms remains a challenge in root canal treatment. More advanced techniques and more effective irrigation solutions are being developed to obtain more complete biofilm dissolution and killing of biofilm microbes. Even though biofilm can be highly pathogenic, biofilm structure can be vulnerable to different treatment strategies and compounds used. So far, however, there is no widely accepted model for testing the efficacy and dynamics of biofilm dissolution by endodontic irrigating solutions. Therefore, it is important to develop a standardized model and protocol to evaluate the efficacy in biofilm removal by the various disinfecting solutions or their combinations used in endodontic clinical practice.

The present study aims to establish a standardized biofilm dissolution model which allows non-invasive evaluation of the effect of different endodontic irrigation solutions on oral multispecies biofilm. This model will be used to examine the dynamic effects of some commonly used irrigants on established multispecies biofilms.

3.2 Hypotheses

The null hypotheses of the present study are that 1) treatment of plaque biofilm with NaOCl and CHX does not reduce the biovolume of the biofilm; 2) treatment of plaque biofilm with CHX does not inhibit the biofilm growth on HA disks compared to untreated controls.

Chapter 4: Materials and Methods

4.1 Biofilm culturing

Sterile HA disks (9.65 mm diameter × 1.52 mm thickness; Clarkson Chromatography Products, Williamsport, PA) were used as the plaque biofilm culturing substrate. Biofilms were grown on the HA disks using a previously established model (156). The HA disks were coated with bovine dermal type I collagen (10 mg/mL collagen in 0.012 N HCl in water) (Cohesion, Palo Alto, CA) by overnight incubation at 4 ºC in 24-well tissue culture plates (Corning Inc., NY) containing 2 mL of the collagen solution per disk.

Subgingival plaque was collected from an adult volunteer and suspended in brain-heart infusion broth (BHI; Becton Dickinson, Sparks, MD) by pipetting. The bacterial suspension was adjusted to optical density (OD) = 0.10, which was measured in a 96-well microtiter plate (150 μl, 405 nm) by a microplate reader (Model 3350; Bio-Rad Laboratories, Richmond, CA). The collagen coated HA disks were placed in the 24-well tissue culture plates, each well containing 1.8 ml of sterile BHI and 0.2 ml of dispersed plaque suspension. The disks were incubated in the BHI-plaque suspension under anaerobic conditions (AnaeroGen, OXOID, Hampshire, UK) at 37 ºC for 3 days and 3 weeks. Fresh medium was changed once a week for the 3-week-old biofilm samples.

4.2 Dynamic analysis of biofilm dissolution

After 3 days and 3 weeks of anaerobic incubation in BHI broth, a total of 32 biofilm samples from each of the 3-day and 3-week-old biofilm groups were used for the dynamic analysis of biofilm dissolution. The biofilm specimens were gently rinsed in 0.85% physiological saline for 15 seconds to remove the culture broth. LIVE/DEAD Bac-Light Bacterial Viability kit L-7012 (Molecular Probes, Eugene, OR), containing two component dyes (SYTO 9 and propidium iodide

in a 1:1 mixture), was used for staining the biofilm following the manufacturer's instructions. The bottom of the HA disks was then dried on paper (Kimwipes, Irving, Texas). The young (3-day-old) and old (3-week-old) biofilms were exposed to the sterile water control, 2% or 6% NaOCl (EMD Chemicals Inc, Darmstadt, Germany) or 2% CHX (Sigma Chemical Corp., St Louis, MO) by gently adding 100 μL of medicament solution on top of the biofilm. Each group contained 8 samples. Then the biofilm sample was transferred to a glass bottom petri dish (35 mm diameter petri dish with 14 mm microwell; MatTek corp., Ashland, MA, USA) for the live-cell imaging confocal laser scanning microscopy (LC-CLSM) (FV10i-LIV, Olympus, ON, Canada) (Fig. 1) analysis.

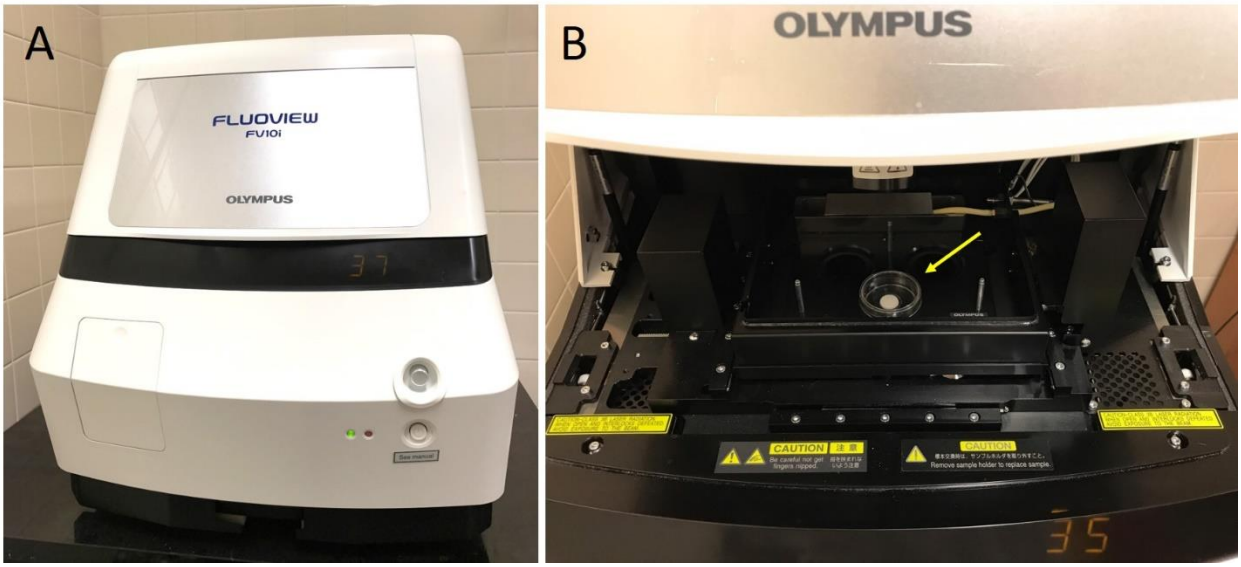

Figure 1. Live-cell imaging confocal laser scanning microscopy (FV10i-LIV, Olympus, ON, Canada). (A) The outside view of the FV10i-LIV confocal system with inside temperature controlled at 37 ºC; (B) The inside view of the built-in box of confocal system showing a biofilm sample on HA disk in the glass bottom petri dish (yellow arrow) for scanning.

The excitation/emission maxima for the dyes were 480/500 nm for SYTO 9 and 490/635 nm for propidium iodide. Fluorescence from stained cell was viewed using the LC-CLSM (FV10i-LIV,

Olympus, Canada) equipped with 4 laser diodes (405 nm, 473 nm, 559 nm, 635 nm) at a resolution of 512 × 512 pixels and a scanning area of 500 μm × 500 μm using a 10 × lens. Simultaneous dual-channel imaging was used to display green and red fluorescence. A random area of biofilm on each disk was scanned. A stack of slices in 2-μm step-size Z axis was captured from the top to the bottom of the biofilm. The time between medicament placement and the start of confocal scanning was controlled within 2 minutes. The total length of live-cell imaging scanning was set for 30 minutes with repetitious scanning of the whole thickness of the biofilm with scanning cycles of 2 minutes.

Confocal images in the 30-minute time lapse were then quantitated and analyzed using the Imaris 7.2 software (Bitplane Inc., St. Paul, MN). The software reconstructed the 2-dimentional intensity of fluorescence of all scanned layers into a 3-dimentional volume stack at each cycle of scanning. The red (dead cells) and green (viable cells) fluorescence signals were separated by color threshold and the biovolume covered by each segmented color was calculated. The total biovolume of the biofilms was sum of the volumes of green and red fluorescence.

The proportions of dissolved and killed biofilm were calculated as follows: Dissolved biofilm% = (Biofilm volume in sterile water group - biofilm volume in treatment group) × 100% / Biofilm volume in sterile water group. Killed bacterial cells in residual biofilm% = (Biofilm volume of red fluorescence / The total biofilm volume of green and red fluorescence) × 100%. Dissolved and killed residual bacteria% = Dissolved biofilm% + (1- Dissolved biofilm%) * Killed bacterial cells in residual biofilm%.

4.3 SEM examination

The morphology of the biofilm bacteria after disinfection and dissolution were observed by SEM. Two additional 3-day and 3-week-old biofilm samples were subjected to 100 µl droplet of sterile water, 2% NaOCl, 6% NaOCl and 2% CHX each using the same protocol as mentioned above for 10 minutes followed by rinsing in saline for 1 minute. Samples were then prefixed with 2.5% glutaraldehyde for 10 minutes and further fixation in 1% osmium tetroxide for 1 hour. The specimens were then subjected to increasing concentrations of ethanol (50%, 70%, 80%, and 100%) for dehydration. The dehydrated specimens were dried using a critical point drier (Samdri-795; Tousimis Research Corporation, Rockville, MD), sputter-coated with iridium (Leica EM MED020 Coating System, Tokyo, Japan), and examined by SEM (Helios Nanolab 650, FEI, Eindhoven, the Netherlands) on two randomly selected areas on each sample using low (1000 ×) and high (8000 ×) magnifications at an accelerating voltage of 3 kV.

4.4 Colony forming unit test

4.4.1 CFU for dispersed biofilm

The 3-day and 3-week-old biofilms on HA disk surfaces were scraped off into sterile water using a plastic loop, followed by pipetting and vortexing. The suspension was adjusted to an OD_{405} of 0.25 corresponding to 1.13×10^{8} CFU mL^{-1} as determined by serial tenfold dilutions and culturing on blood agar plates for colony forming units (CFU) counts. A 100 µl sample of the plaque suspension was added to 400 µl of sterile water, 2% NaOCl, 6% NaOCl or 2% CHX for 30 seconds, 2, 4 and 10 minutes. At each of the indicated times of exposure, 100 µl of bacterial solution was added to 900 µl BHI medium and diluted serially in 10-fold steps. The first two tubes in the dilution series contained inactivator (3% Tween 80 and 0.3% a-lecithin) (Sigma-Aldrich, St Louis, MO, USA) for CHX group and 0.5% sodium- thiosulfate (Fisher Scientific, Ottawa, ON, Canada) for

NaOCl groups to reduce the carry-over effect. Twenty µl from the dilution tubes was spotted onto blood agar plates (BHI agar with 5% heparinized sheep's blood; Difco, Detroit, MI, USA). The blood agar plates were cultured anaerobically at 37 °C for 48 hours, and the colony forming unit (CFU) count was calculated. The number of CFU was generated as follows: (Number of bacterial colonies in one 20 µl droplet)×50×5×10 $^{\text{number of tenfold dilutions-1}}$. The percentage of killed bacteria was determined as the difference of the percentage of living bacterial cells in the initial inoculum and after exposure to the irrigation solutions.

4.4.2 CFU for intact biofilm

The percentage of killed plaque biofilm bacteria after exposure of the biofilm without dispersion to different medicaments was also calculated in an additional experiment. The 3-day and 3-week-old biofilms on HA disks were exposed to sterile water, 2% and 6% NaOCl and 2% CHX by dropping 100 µl of medicament solution on top of the biofilm for 10 minutes. The NaOCl treated samples were rinsed in 1 ml of 0.5% sodium-thiosulfate solution, and the CHX treated samples were rinsed in 1 ml of CHX inactivator for 1 minute respectively. All the samples were rinsed in 1 ml sterile water for 1 minute. Then the biofilms were scraped off to 1 ml sterile water and the OD_{405} value was adjusted to 0.25. A 100 µl sample of the plaque suspension was added to 400 µl of sterile water. A 100 µl of the bacterial solution was added to 900 µl BHI medium and diluted serially in 10-fold steps. Twenty µl from the dilution tubes was spotted onto blood agar plates. The blood agar plates were cultured anaerobically at 37 °C for 48 hours. The percentage of killed bacteria was calculated as stated above. All the CFU experiments were performed in triplicate.

4.5 Biofilm inhibition

Sixteen new HA disks were coated with bovine dermal type I collagen as described in section 2.1 above. Following collagen coating the HA disks were immersed in sterile water, 2% CHX, 0.2% CHX or 0.02% CHX in 2 ml solution for 3 minutes with four samples each in 24-well plates. The HA disks were washed in sterile water for 1 minute. Fresh plaque was collected using the same protocol mentioned in section 2.1. One hundred and fifty microliters of BHI-plaque suspension with the addition of 50 μl LIVE/DEAD BacLight viability stain was filled onto the coverslip of the glass bottom petri dish (MatTek corp., Ashland, MA). The CHX coated HA disks were put on the glass bottom (CHX coated surface facing the plaque suspension) and the petri dish was immediately subjected to LC-CLSM scanning at 37℃. A random area on the HA disk was selected with 512 × 512 pixel using a 10 × lens. A stack of 40 slices in 2 μm step sizes was captured in the area of interest. A total scanning time of 12 hours with a full scan cycle of every 20 minutes was applied. Confocal images in the 12-hour time lapse were quantitated and analyzed using Imaris 7.2 software (Bitplane Inc., St. Paul, MN) as mentioned above. The total biovolume in the CHX and sterile water control groups at different time points was determined.

4.6 Statistical analysis

Statistical analysis was performed with the use of SPSS 16.0 (SPSS Inc., Chicago, IL) for Windows. Means and standard deviations of the biofilm biovolume and the proportions of dead cell volume were calculated respectively. The normality of distribution was ensured by the Kolmogorov–Smirnov test and the homogeneity of variance was determined using Levene's test.

The sample size was determined based on the results of pilot study using G-Power 3.1 software (available from University of Düsseldorf, Germany; http://www.gpower.hhu.de/en.html). A priori

power analysis with the test family of F test (Analysis of variance, ANOVA) was applied resulting in the power of 0.92 and a required minimum sample size of 4 for each group.

Two-way repeated measures ANOVA was applied to determine the significance of the differences in biovolume and proportion of dissolved and killed bacteria, considering treatment as main effect and treatment time as the repeated measure. Two-way ANOVA was applied to determine the significance of differences in the CFU counts. Post hoc multiple comparisons were used to isolate and compare the significant results using Tukey test at a 5% significance level.

Chapter 5: Results

5.1 Dynamic biofilm dissolution

5.1.1 Three-day-old biofilm dissolution

The dynamic biofilm dissolution process for 3-day-old biofilm is shown in Figures 2-6. No statistically significant ($P>0.05$) biofilm biovolume variation was observed in the sterile water group (Fig. 2) or the 2% CHX group (Fig. 5) during the 32-minute exposure period (Table 1; Fig. 6). Both 2% and 6% NaOCl (Figs. 3 and 4) significantly ($P<0.001$) reduced the biovolume of 3-day-old biofilm in 32 minutes (Fig. 6). Six percent NaOCl dissolved biofilm faster than 2% NaOCl (24% dissolved) by dissolving over 70% of the biofilm in the first 2 minutes (Table 1). The proportion of dissolved biofilm increased significantly with time ($P<0.001$) and only 6% of the biofilm was left in the 6% NaOCl group, whereas 37% of the biofilm remained in the 2% NaOCl group at the end of the exposure.

With no antibacterial treatment (sterile water group), 5% to 6% of the bacteria were dead (Table 2). Forty-one percent of the undissolved biofilm bacteria were killed during the first 2 minutes of 2% NaOCl treatment, and the percentage of killed bacteria gradually increased to 72% ($P<0.001$) in 32 minutes (Table 2; Fig. 7). In comparison, 6% NaOCl resulted in a significantly higher proportion of killed biofilm bacteria than in the 2% NaOCl group during treatment (Fig. 7). Interestingly, the percentage of killed bacteria in the remaining biofilm from the 6% NaOCl group increased to a peak of 95% at 6 minutes followed by a drop down to less than 80% in 30 minutes ($P<0.001$) (Table 2). The amount of killed bacteria steadily increased in the 2% CHX group from 33% after the 2-minute treatment, up to 86% after the 32-minute treatment (Table 2). No

statistically significant difference was found in the killing of the bacteria between the 2% NaOCl and 2% CHX solutions ($P>0.05$).

When combining dissolution and killing of bacteria together, 6% NaOCl showed the highest antimicrobial effect with 94% bacterial reduction in 2 minutes and 99% bacterial reduction in 32 minutes ($P<0.001$) (Table 3). The total bacterial reduction percentage increased with time for 2% NaOCl and 2% CHX. Two percent NaOCl resulted in significantly higher bacterial reduction percentage than 2% CHX in 32 minutes ($P<0.001$) (Table 3).

Table 1. Proportion of Dissolved 3-day-old Plaque Biofilm during Exposure to Endodontic Disinfecting Agents

Time (min)	2% NaOCl[a]	6% NaOCl[b]	2% CHX[c]
2	0.24±0.05	0.70±0.05	0.07±0.10
4	0.26±0.11	0.73±0.05	0.13±0.14
6	0.38±0.08	0.76±0.04	0.09±0.15
8	0.37±0.06	0.79±0.04	0.05±0.14
10	0.48±0.08	0.79±0.04	0.17±0.07
12	0.55±0.11	0.82±0.03	0.08±0.13
14	0.55±0.10	0.85±0.03	0.06±0.15
16	0.54±0.07	0.86±0.02	-0.03±0.15
18	0.52±0.08	0.88±0.02	-0.11±0.13
20	0.55±0.09	0.91±0.02	-0.02±0.13
22	0.55±0.08	0.90±0.02	0.06±0.14
24	0.54±0.08	0.91±0.02	0.07±0.12
26	0.61±0.09	0.92±0.02	0.07±0.10
28	0.63±0.08	0.93±0.02	-0.09±0.12
30	0.62±0.09	0.93±0.03	-0.09±0.12
32	0.63±0.08	0.94±0.03	-0.10±0.09

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

Table 2. Proportion of Killed Biofilm Microbial Cell Biovolume in the Residual 3-day-old Biofilm during Exposure to Endodontic Disinfecting Agents

Time (min)	Sterile water[a]	2% NaOCl[b]	6% NaOCl[c]	2% CHX[b]
2	0.05±0.01	0.41±0.02	0.80±0.05	0.33±0.02
4	0.05±0.02	0.42±0.03	0.94±0.03	0.36±0.02
6	0.05±0.01	0.46±0.04	0.95±0.02	0.49±0.02
8	0.06±0.02	0.47±0.04	0.91±0.02	0.43±0.03
10	0.06±0.02	0.54±0.04	0.87±0.03	0.48±0.04
12	0.05±0.01	0.58±0.05	0.83±0.04	0.57±0.02
14	0.05±0.01	0.56±0.04	0.78±0.07	0.57±0.04
16	0.05±0.02	0.58±0.03	0.78±0.10	0.58±0.03
18	0.05±0.01	0.59±0.03	0.82±0.03	0.61±0.02
20	0.05±0.01	0.61±0.03	0.80±0.05	0.69±0.02
22	0.05±0.01	0.61±0.04	0.79±0.04	0.69±0.02
24	0.05±0.02	0.61±0.03	0.79±0.07	0.75±0.03
26	0.05±0.02	0.70±0.04	0.75±0.04	0.75±0.03
28	0.05±0.02	0.72±0.04	0.78±0.07	0.79±0.02
30	0.05±0.01	0.69±0.03	0.76±0.08	0.80±0.02
32	0.05±0.01	0.71±0.04	0.82±0.05	0.86±0.01

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

Table 3. Proportion of Dissolved and Killed Residual Bacteria in the 3-day-old Biofilm during Exposure to Endodontic Disinfecting Agents

Time (min)	2% NaOCl[a]	6% NaOCl[b]	2% CHX[c]
2	0.55±0.04	0.94±0.02	0.38±0.07
4	0.58±0.06	0.98±0.01	0.44±0.09
6	0.66±0.05	0.99±0.01	0.53±0.09
8	0.67±0.04	0.98±0.01	0.46±0.10
10	0.76±0.04	0.97±0.01	0.57±0.06
12	0.81±0.04	0.97±0.01	0.60±0.07
14	0.80±0.04	0.97±0.01	0.59±0.07
16	0.81±0.04	0.97±0.02	0.57±0.07
18	0.80±0.04	0.98±0.00	0.57±0.07
20	0.82±0.04	0.98±0.00	0.69±0.05
22	0.82±0.04	0.98±0.00	0.71±0.04
24	0.82±0.04	0.98±0.01	0.77±0.04
26	0.88±0.02	0.98±0.00	0.76±0.04
28	0.90±0.02	0.98±0.00	0.77±0.03
30	0.88±0.03	0.98±0.01	0.78±0.02
32	0.90±0.02	0.99±0.00	0.84±0.01

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

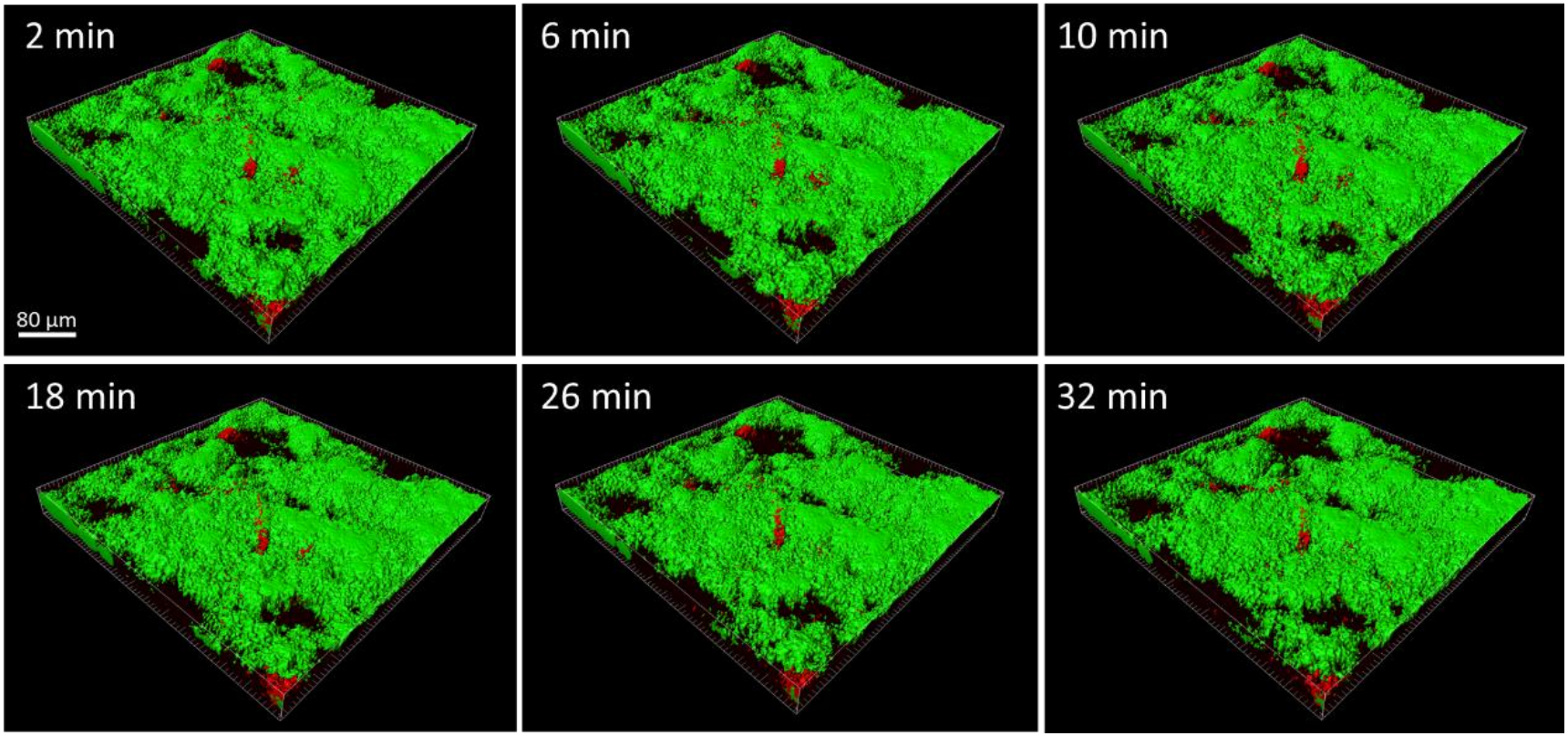

Figure 2. LC-CLSM images of killing of biofilm microbes and dynamic dissolution of 3-day-old biofilm during 32-minute exposure to sterile water. (Green: live bacteria; red: killed bacteria; black: no bacteria)

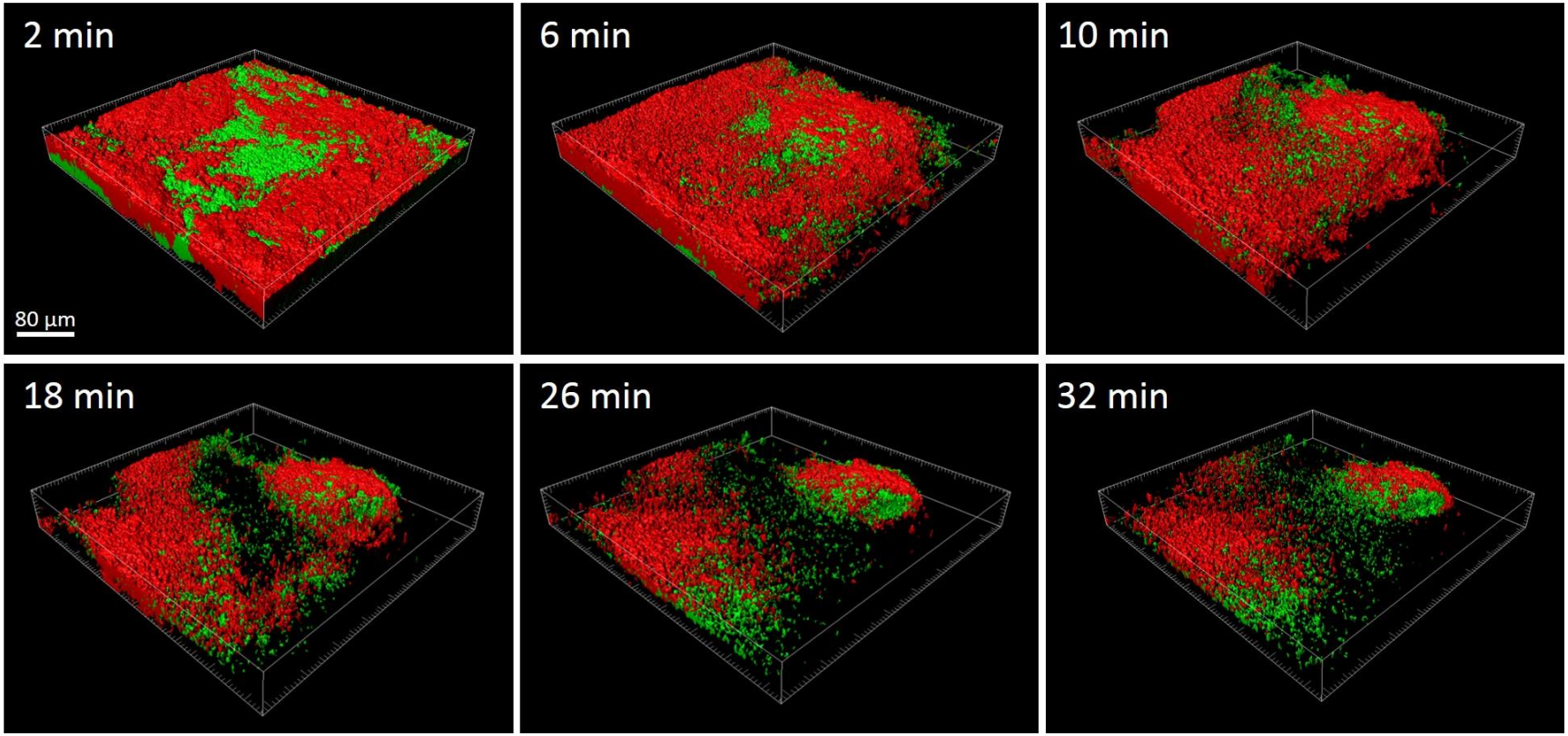

Figure 3. LC-CLSM images of killing of biofilm microbes and dynamic dissolution of 3-day-old biofilm during 32-minute exposure to 2% NaOCl.

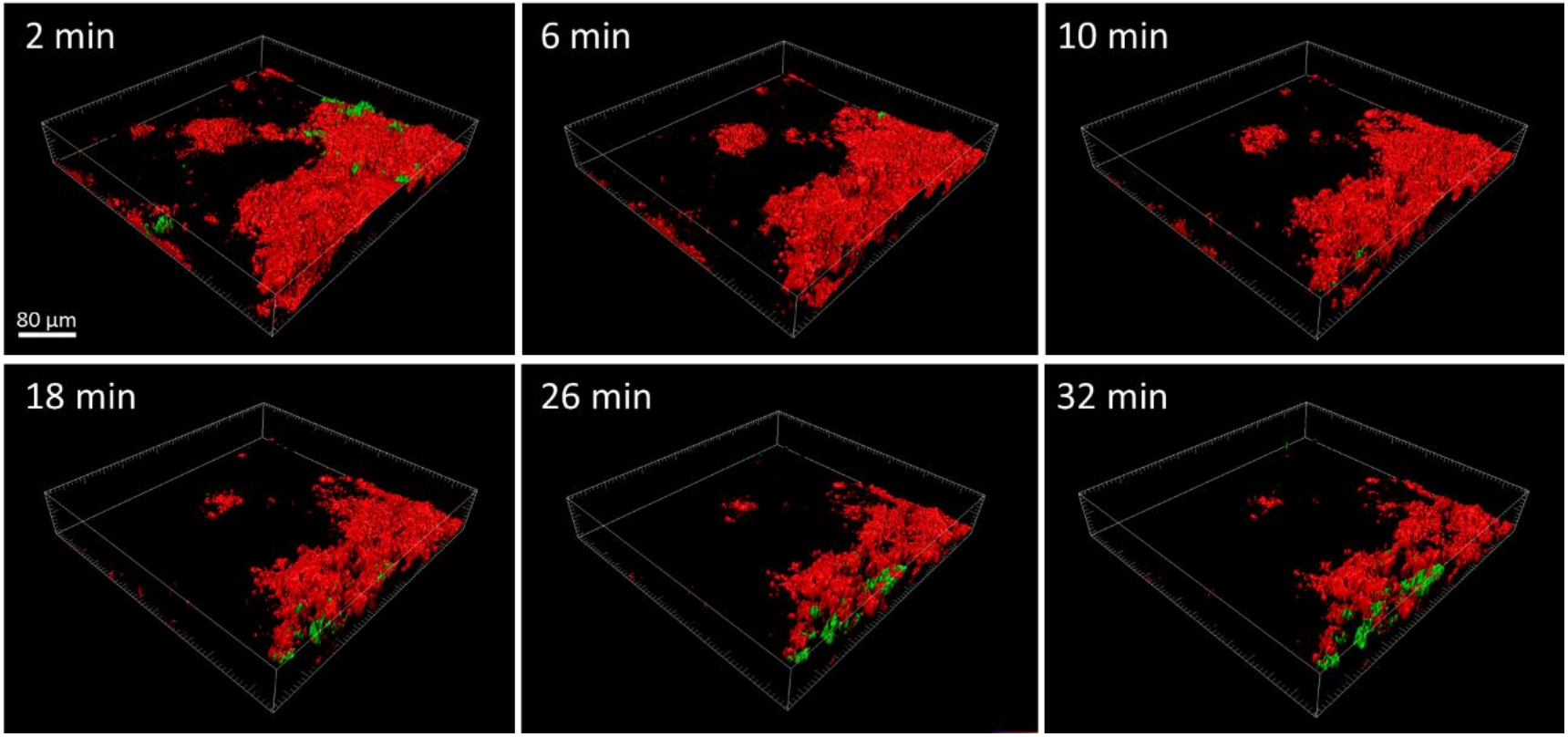

Figure 4. LC-CLSM images of killing of biofilm microbes and dynamic dissolution of 3-day-old biofilm during 32-minute exposure to 6% NaOCl.

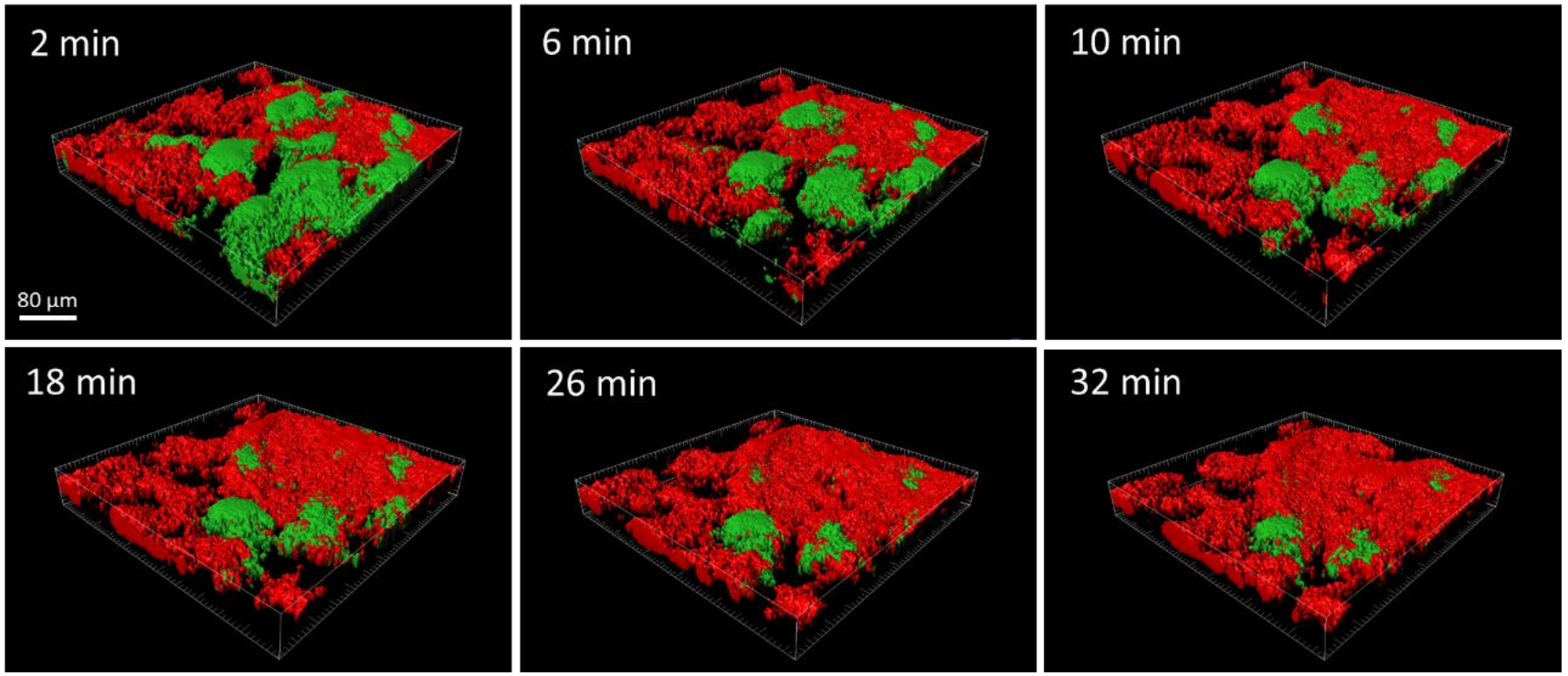

Figure 5. LC-CLSM images of killing of biofilm microbes and dynamic dissolution of 3-day-old biofilm during 32-minute exposure to 2% CHX.

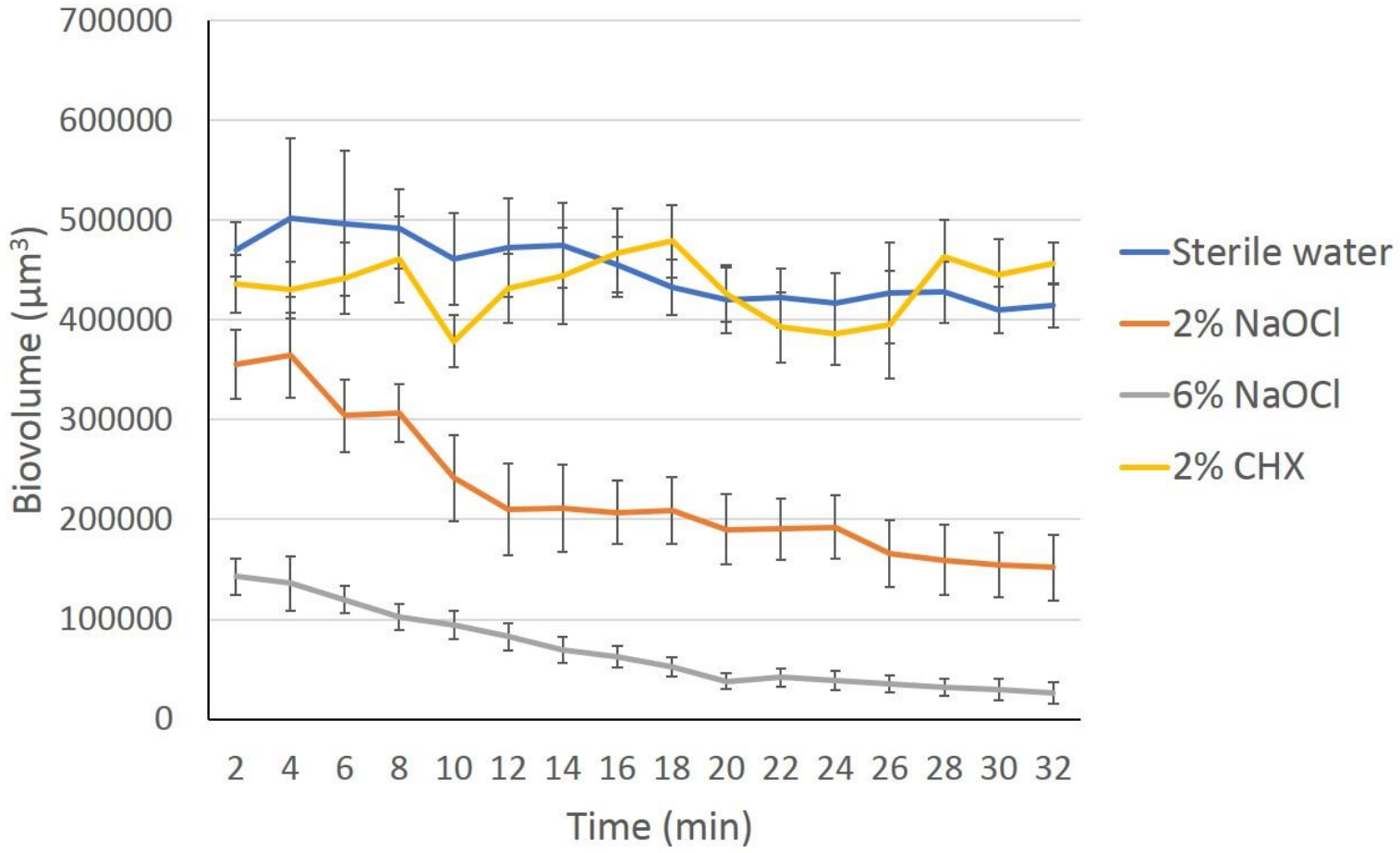

Figure 6. Biovolume of 3-day-old biofilm during exposure to different endodontic disinfecting agents for 32 minutes. Repeated measures ANOVA showed significant biovolume reduction in 32 minutes for 2% and 6% NaOCl groups ($P<0.05$), but no significant changes in biovolume was found between sterile water and CHX groups in 32 minutes ($P>0.05$).

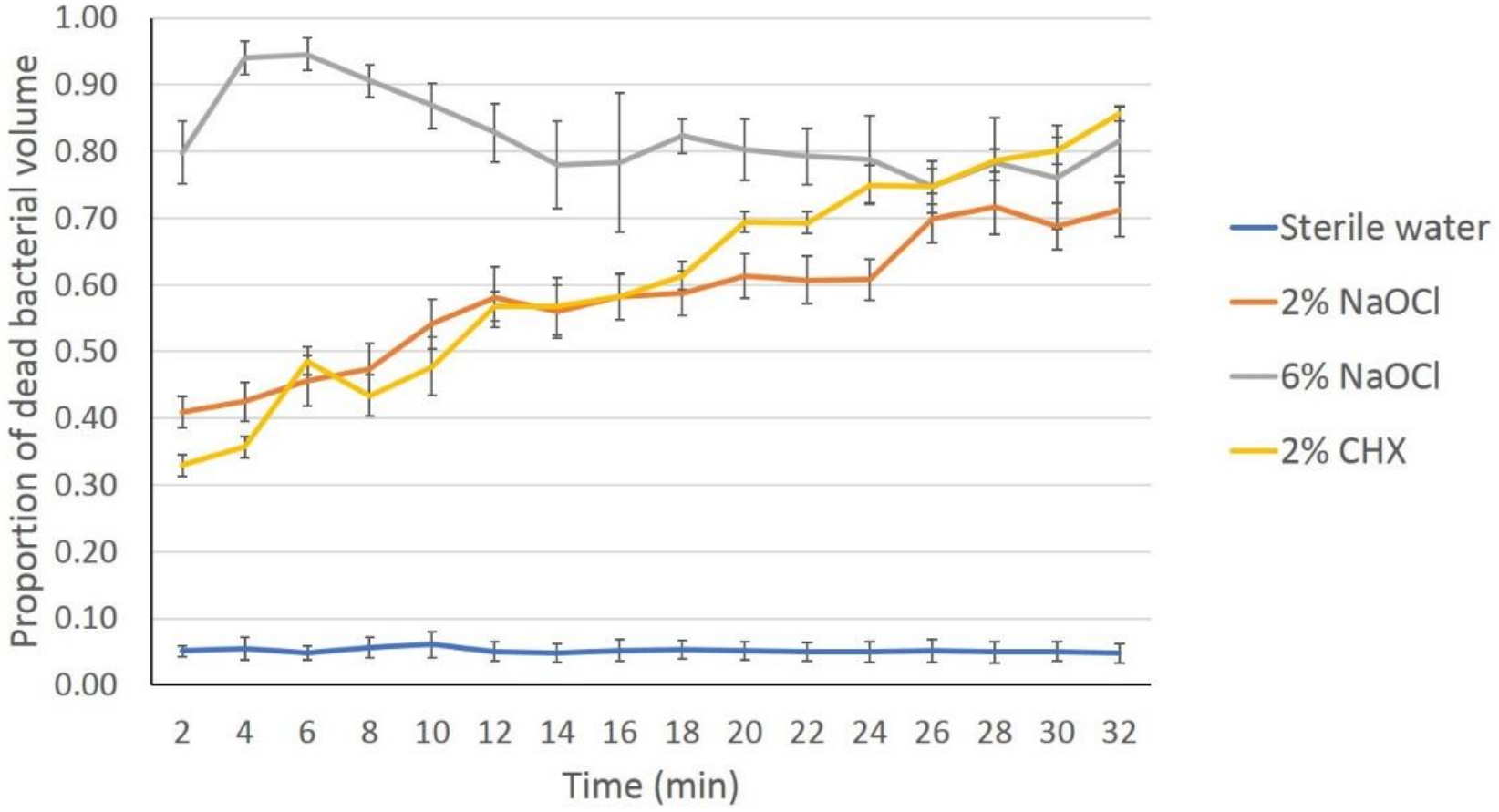

Figure 7. The proportion of dead biofilm microbial cells in the residual 3-day-old biofilm during exposure to endodontic disinfecting agents for 32 minutes. Repeated measures ANOVA showed significant increase in the killing of residual bacteria in biofilm in 32 minutes for 2% CHX and 2% NaOCl groups ($P<0.05$). Six percent NaOCl reached the highest killing in 6 minutes ($P<0.001$). No significant change in proportion of dead biofilm bacterial cells was found in sterile water group ($P>0.05$).

5.1.2 Three-week-old biofilm dissolution

The 3-week-old biofilm had significantly higher biovolume than the 3-day-old biofilm ($P<0.01$). No significant change was observed in the 3-week-old biofilm during the 32-minute exposure to sterile water (Figs. 8 and 12). Similar to the effect of disinfecting solutions on 3-day-old biofilm, 2% and 6% NaOCl significantly dissolved the 3-week-old biofilm (Figs. 9 and 10) while 2% CHX had no dissolution effect (Figs. 11 and 12). During the first 10 minutes of treatment, significantly lower proportions of 3-week-old biofilm volume was dissolved by 6% NaOCl than the proportion for the 3-day-old biofilm (Tables 1 and 4) ($P<0.05$). After 32 minutes of treatment, 86% of the 3-week-old biofilm was dissolved by 6% NaOCl compared to 94% dissolved biofilm for the 3-day-old group (Tables 1 and 4). No significant difference was found in the reduction of biovolume by the 2% NaOCl solution for the 3-day and 3-week-old biofilms ($P>0.05$).

Only 4-5% of the bacteria were dead in the 3-week-old biofilm exposed to sterile water (Table 5). Two percent NaOCl killed 28% of the residual 3-week-old biofilm in 2 minutes and 38% in 8 minutes (Table 5), these results were both significantly lower than those obtained for the dissolution of the 3-day-old biofilm (Table 2). The proportion of killed bacteria increased to 75% in 32 minutes with no statistically significant difference ($P>0.05$) in the percentage of killed bacteria at the same time interval for the 3-day-old biofilm (71%). Six percent NaOCl resulted in a similar pattern of dissolution for the 3-week-old and 3-day-old biofilms, reaching the highest killing effect of 97% in 6 minutes (Figs. 7 and 13; Tables 2 and 5). Two percent CHX showed increasing killing effect in 32 minutes for the 3-week-old biofilm. However, 2% CHX resulted in a lower percentage of killed bacteria (73%) in 3-week-old biofilm samples, than in 3-day-old biofilm (86%) ($P<0.001$) (Tables 2 and 5).

The proportion of dissolved and killed residual bacteria in the 3-week-old biofilm showed no significant difference ($P>0.05$) compared to the 3-day-old biofilm for the 2% and 6% NaOCl groups (Tables 3 and 6). For the 2% CHX group, a significantly smaller proportion of dissolved and killed residual bacteria was found in the 3-week-old biofilm samples than in the 3-day-old biofilm samples ($P<0.01$). Two percent NaOCl resulted in almost the same level of bacterial reduction (94%) as that found in the 6% NaOCl group (95%) in 32 minutes (Table 6). Two percent CHX showed an increase in dissolved and killed residual bacteria in 32 minutes (Table 6).

Table 4. Proportion of Dissolved 3-week-old Plaque Biofilm during Exposure to Endodontic Disinfecting Agents

Time (min)	2% NaOCl[a]	6% NaOCl[b]	2% CHX[c]
2	0.24±0.06	0.54±0.06	0.07±0.03
4	0.23±0.07	0.58±0.07	0.04±0.05
6	0.37±0.07	0.65±0.06	0.09±0.05
8	0.45±0.06	0.68±0.05	0.05±0.05
10	0.53±0.05	0.71±0.05	0.15±0.09
12	0.58±0.06	0.76±0.04	0.04±0.06
14	0.58±0.06	0.82±0.03	0.06±0.15
16	0.57±0.07	0.83±0.03	-0.03±0.09
18	0.56±0.07	0.85±0.02	-0.08±0.12
20	0.63±0.06	0.86±0.02	0.02±0.08
22	0.61±0.06	0.83±0.03	0.09±0.10
24	0.61±0.06	0.82±0.03	-0.04±0.14
26	0.70±0.06	0.84±0.03	0.17±0.13
28	0.72±0.07	0.86±0.02	0.13±0.09
30	0.76±0.06	0.85±0.03	0.16±0.12
32	0.75±0.06	0.86±0.03	0.07±0.09

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

Table 5. Proportion of Killed Biofilm Microbial Cell Biovolume in the Residual 3-week-old Biofilm during Exposure to Endodontic Disinfecting Agents

Time (min)	Sterile water[a]	2% NaOCl[b]	6% NaOCl[c]	2% CHX[b]
2	0.05±0.01	0.28±0.04	0.85±0.02	0.20±0.01
4	0.04±0.01	0.29±0.04	0.95±0.01	0.27±0.02
6	0.04±0.01	0.33±0.05	0.97±0.01	0.45±0.02
8	0.04±0.01	0.38±0.04	0.94±0.01	0.40±0.02
10	0.04±0.01	0.48±0.07	0.93±0.01	0.40±0.04
12	0.04±0.02	0.51±0.11	0.96±0.01	0.54±0.02
14	0.05±0.01	0.48±0.11	0.89±0.02	0.52±0.06
16	0.04±0.02	0.52±0.12	0.84±0.03	0.56±0.02
18	0.04±0.02	0.53±0.12	0.79±0.03	0.57±0.02
20	0.04±0.01	0.55±0.12	0.78±0.06	0.66±0.02
22	0.04±0.02	0.56±0.12	0.74±0.10	0.66±0.02
24	0.04±0.02	0.56±0.12	0.72±0.07	0.58±0.07
26	0.04±0.02	0.71±0.04	0.69±0.09	0.69±0.06
28	0.04±0.02	0.73±0.08	0.76±0.06	0.71±0.02
30	0.04±0.02	0.65±0.07	0.65±0.08	0.65±0.07
32	0.04±0.02	0.75±0.04	0.64±0.10	0.73±0.12

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

Table 6. **Proportion of Dissolved and Killed Residual Bacteria in the 3-week-old Biofilm during Exposure to Endodontic Disinfecting Agents**

Time (min)	2% NaOCl[a]	6% NaOCl[b]	2% CHX[c]
2	0.45±0.05	0.93±0.00	0.26±0.02
4	0.46±0.06	0.98±0.00	0.30±0.03
6	0.58±0.05	0.99±0.00	0.50±0.03
8	0.66±0.04	0.98±0.00	0.43±0.03
10	0.76±0.05	0.98±0.00	0.49±0.08
12	0.79±0.06	0.99±0.00	0.56±0.03
14	0.78±0.07	0.98±0.00	0.55±0.04
16	0.79±0.07	0.97±0.00	0.55±0.05
18	0.79±0.07	0.97±0.00	0.53±0.08
20	0.83±0.06	0.97±0.01	0.67±0.03
22	0.83±0.06	0.96±0.02	0.69±0.04
24	0.83±0.06	0.95±0.02	0.57±0.05
26	0.91±0.01	0.95±0.02	0.75±0.03
28	0.92±0.03	0.97±0.01	0.75±0.03
30	0.91±0.03	0.95±0.02	0.70±0.10
32	0.94±0.02	0.95±0.02	0.75±0.03

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

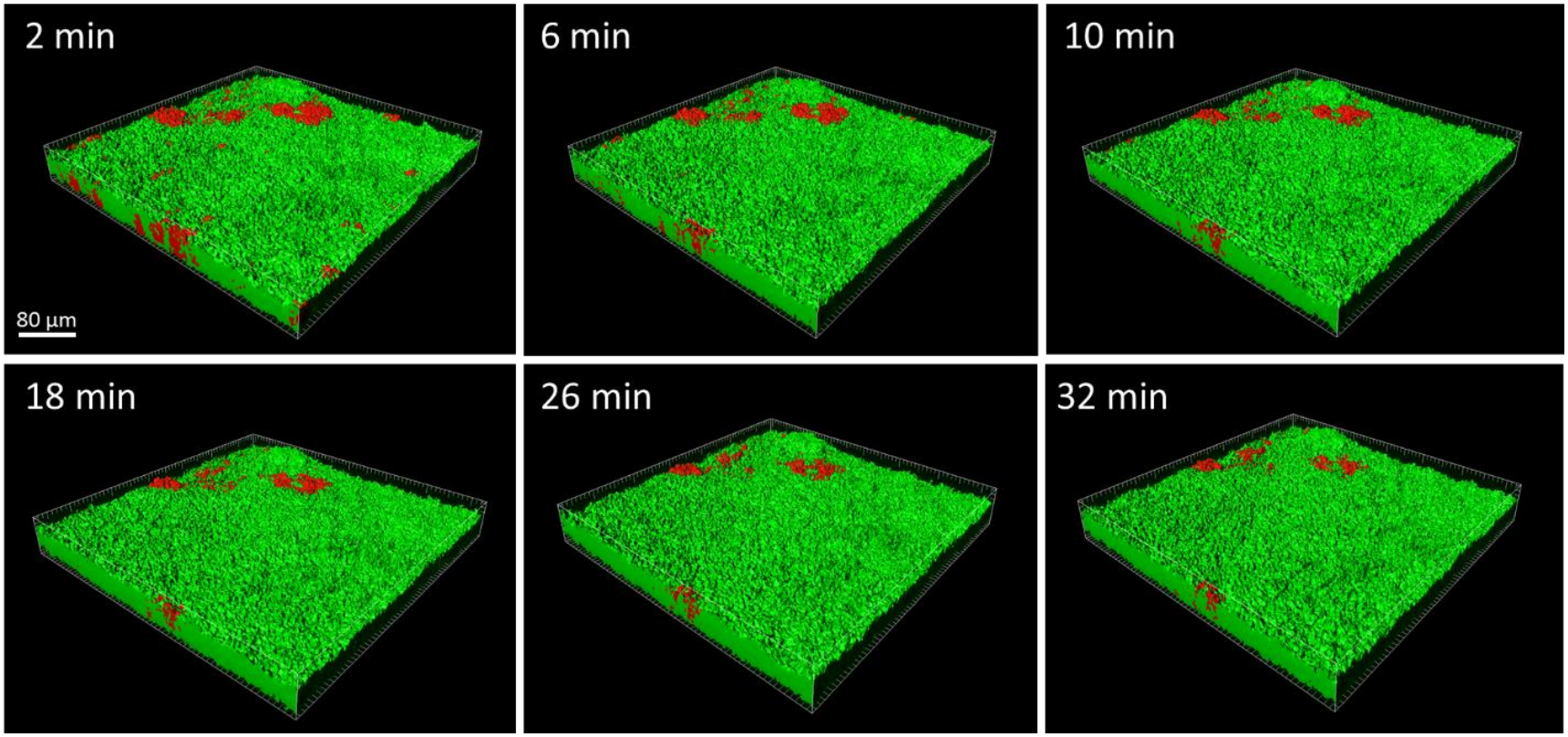

Figure 8. LC-CLSM images of killing of biofilm microbes and dynamic dissolution of 3-week-old biofilm during 32-minute exposure to sterile water.

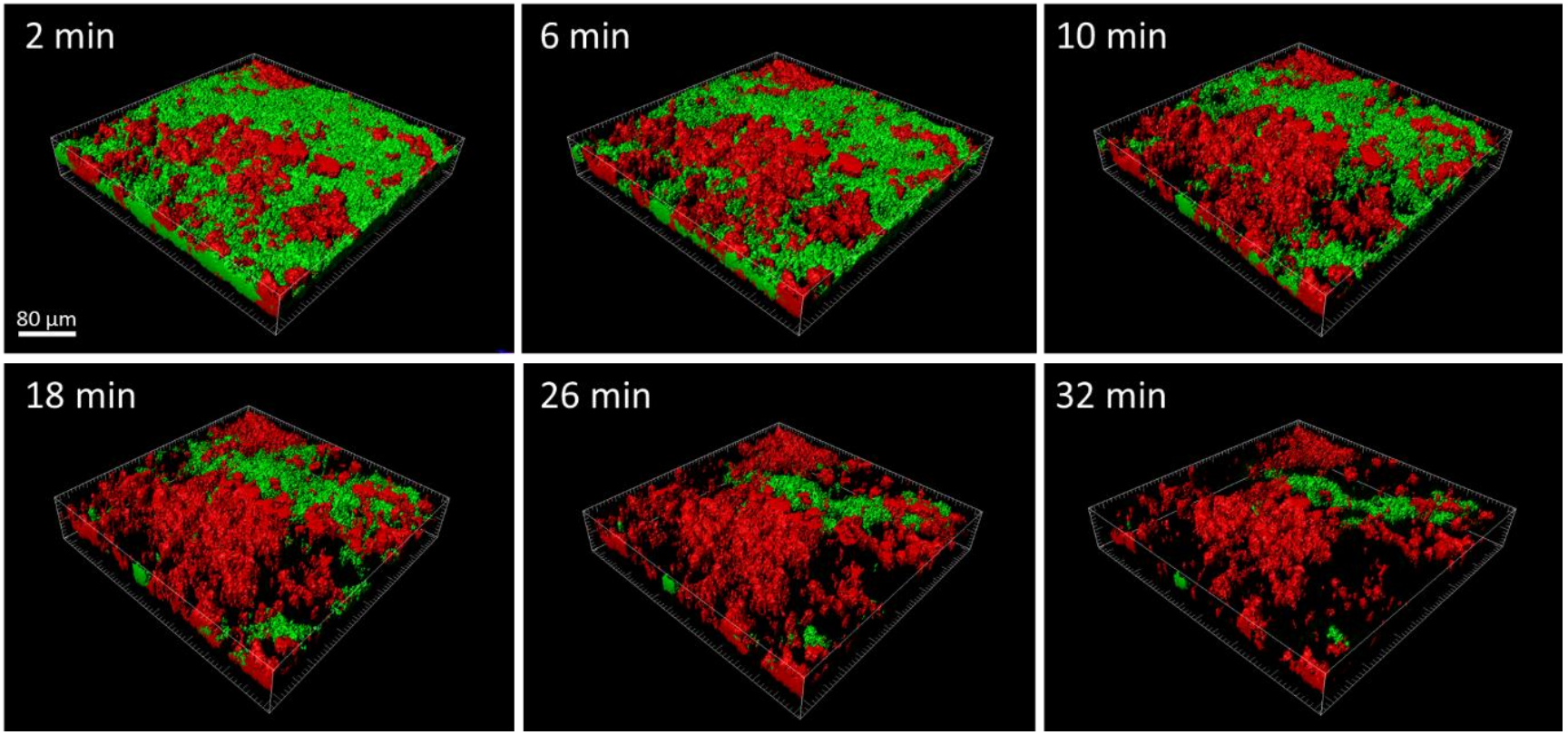

Figure 9. LC-CLSM images of killing of biofilm microbes and dynamic dissolution of 3-week-old biofilm during 32-minute exposure to 2% NaOCl.

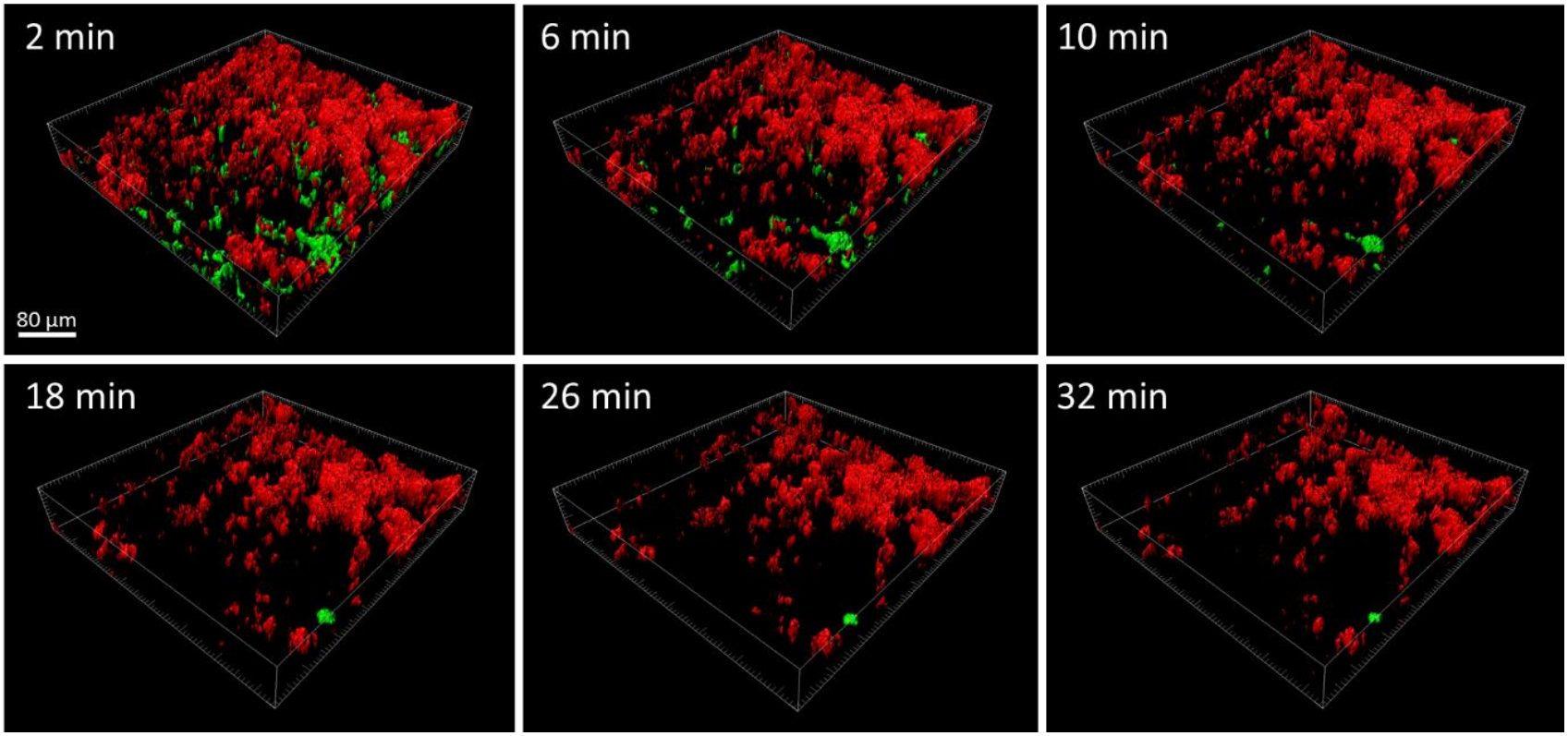

Figure 10. LC-CLSM images of killing of biofilm microbes and dynamic dissolution of 3-week-old biofilm during 32-minute exposure to 6% NaOCl.

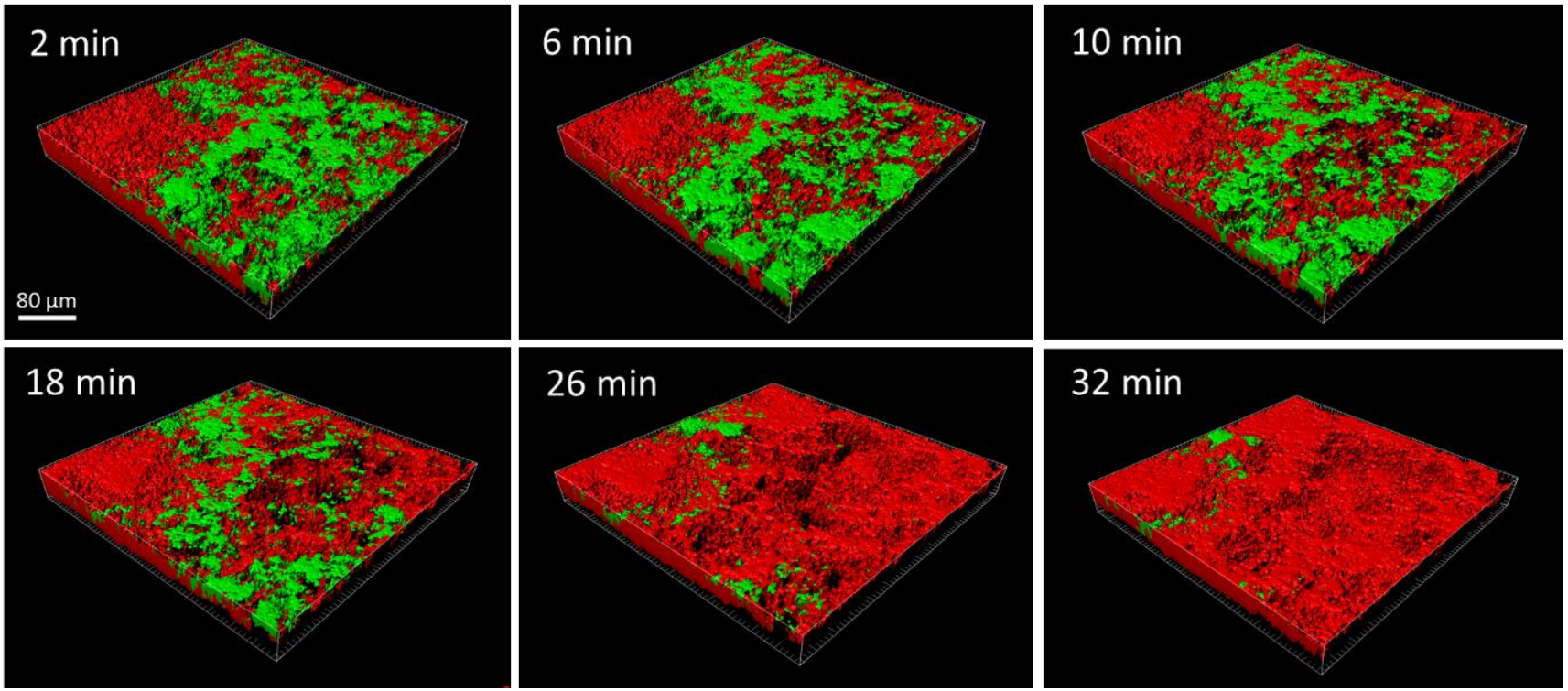

Figure 11. LC-CLSM images of killing of biofilm microbes and dynamic dissolution of 3-week-old biofilm during 32-minute exposure to 2% CHX.

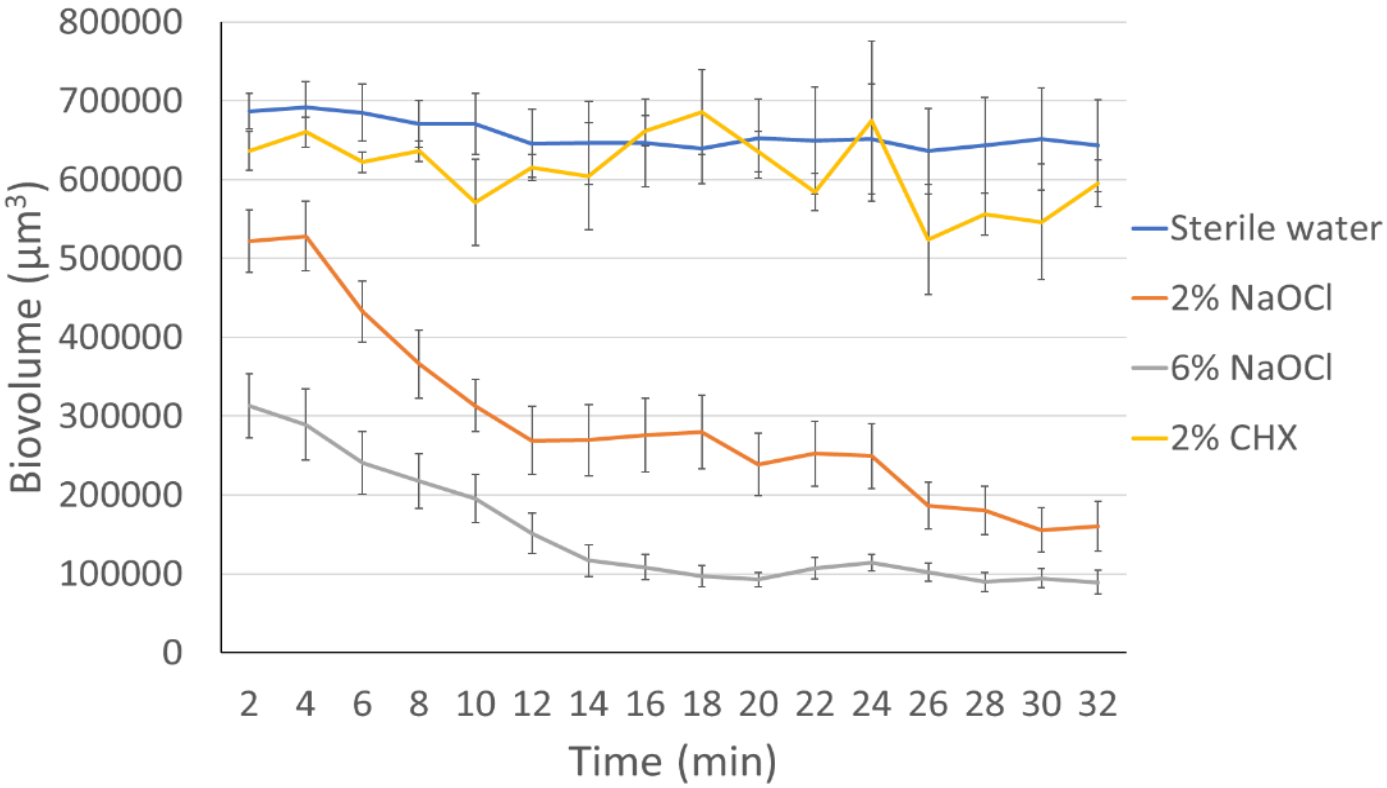

Figure 12. Biovolume of 3-week-old biofilm during exposure to different endodontic disinfecting agents for 32 minutes. Repeated measures ANOVA showed significant biovolume reduction in 32 minutes for 2% and 6% NaOCl groups ($P<0.05$), but no significant changes in biovolume was found between sterile water and CHX groups in 32 minutes ($P>0.05$).

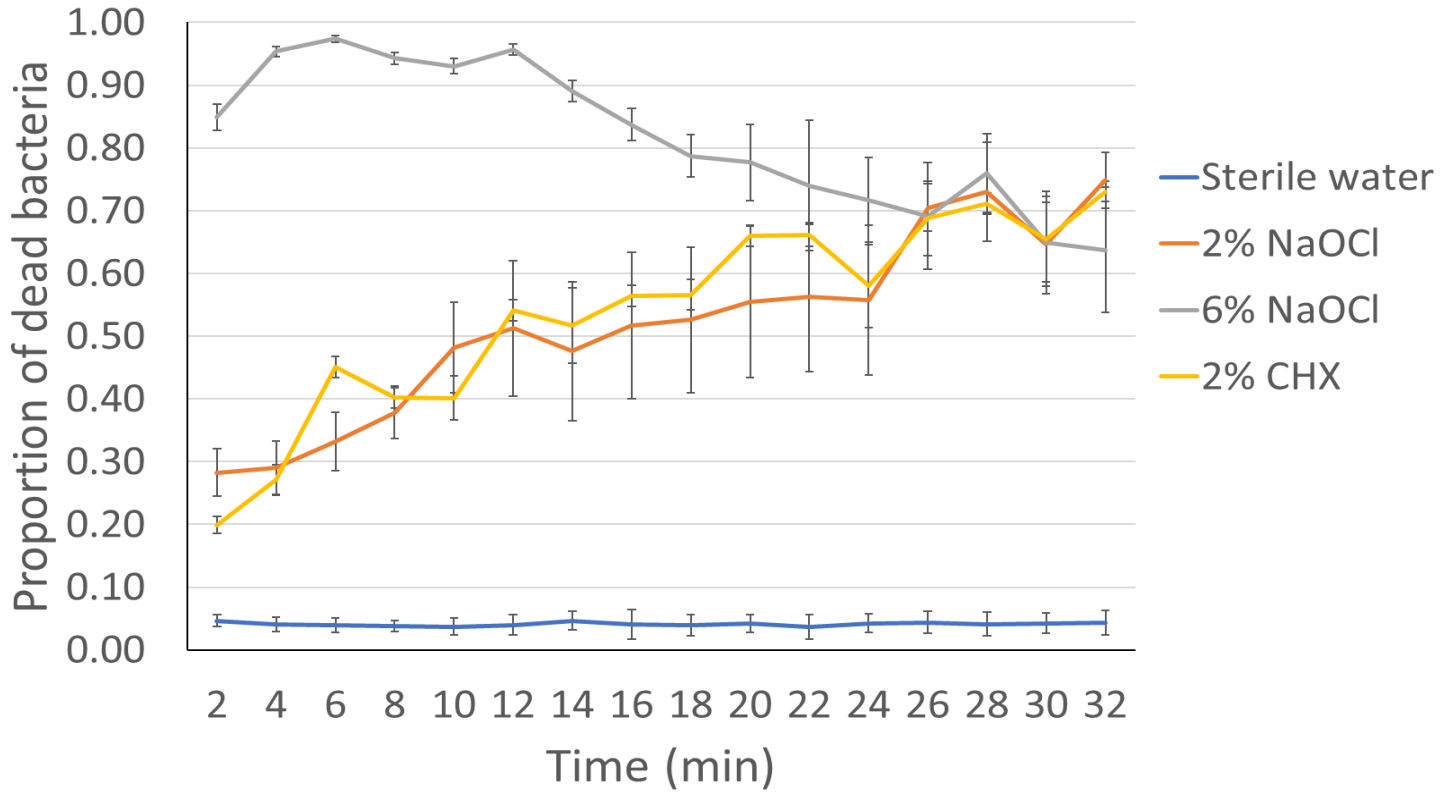

Figure 13. The proportion of dead biofilm microbial cells in the residual 3-week-old biofilm during exposure to endodontic disinfecting agents for 32 minutes. Repeated measures ANOVA showed significant increase in the killing of residual bacteria in biofilm in 32 minutes for 2% CHX and 2% NaOCl groups ($P<0.05$). Six percent NaOCl reached the highest killing in 6 minutes ($P<0.001$). No significant change in proportion of dead biofilm bacterial cells was found in sterile water group ($P>0.05$).

5.2 Effect on biofilm structure

The morphology of the 3-day and 3-week-old plaque biofilms after treatment (10 minutes) by disinfecting solutions was examined by SEM. The sterile water group showed a well-organized network structure with cocci, rods, and filaments within the biofilms (Figs. 14 and 15A and B). Two percent NaOCl partially dissolved the 3-day-old biofilms and exposed the HA disk surface under the biofilm, as shown in the low magnification SEM micrograph in Figure 14C. Disrupted biofilm bacterial cells and matrix could be observed under high magnification (Fig. 14D). Almost no original biofilm structure was shown after treatment by 6% NaOCl for 10 minutes (Fig. 14E). High magnification SEM showed disperse bacterial cells on the HA disk surfaces with intracellular lysis deposition (Fig. 14F). An intact multispecies biofilm layer was shown after a 10-minute treatment by 2% CHX (Fig. 14G). Cell lysis induced by CHX showed fine particles released on the bacterial cell surfaces (Fig. 14H).

More cocci and rod-shaped bacteria than filaments were shown in the 3-week-old biofilms. The dissolution of biofilm by 2% NaOCl resulted in gaps and cracks within the 3-week-old biofilm layer (Fig. 15C) and bacterial cell rupture (Fig. 15D). Six percent NaOCl dissolved most of the 3-week-old biofilm and exposed HA disk surface (Fig. 15E). Dispersed bacterial cells lost their cellular integrity after a 10-minute treatment with 6% NaOCl (Fig. 15F). Two percent CHX resulted in a similar cell lysis effect for both the 3-week-old biofilm (Fig. 15G and H) and the 3-day-old biofilm.

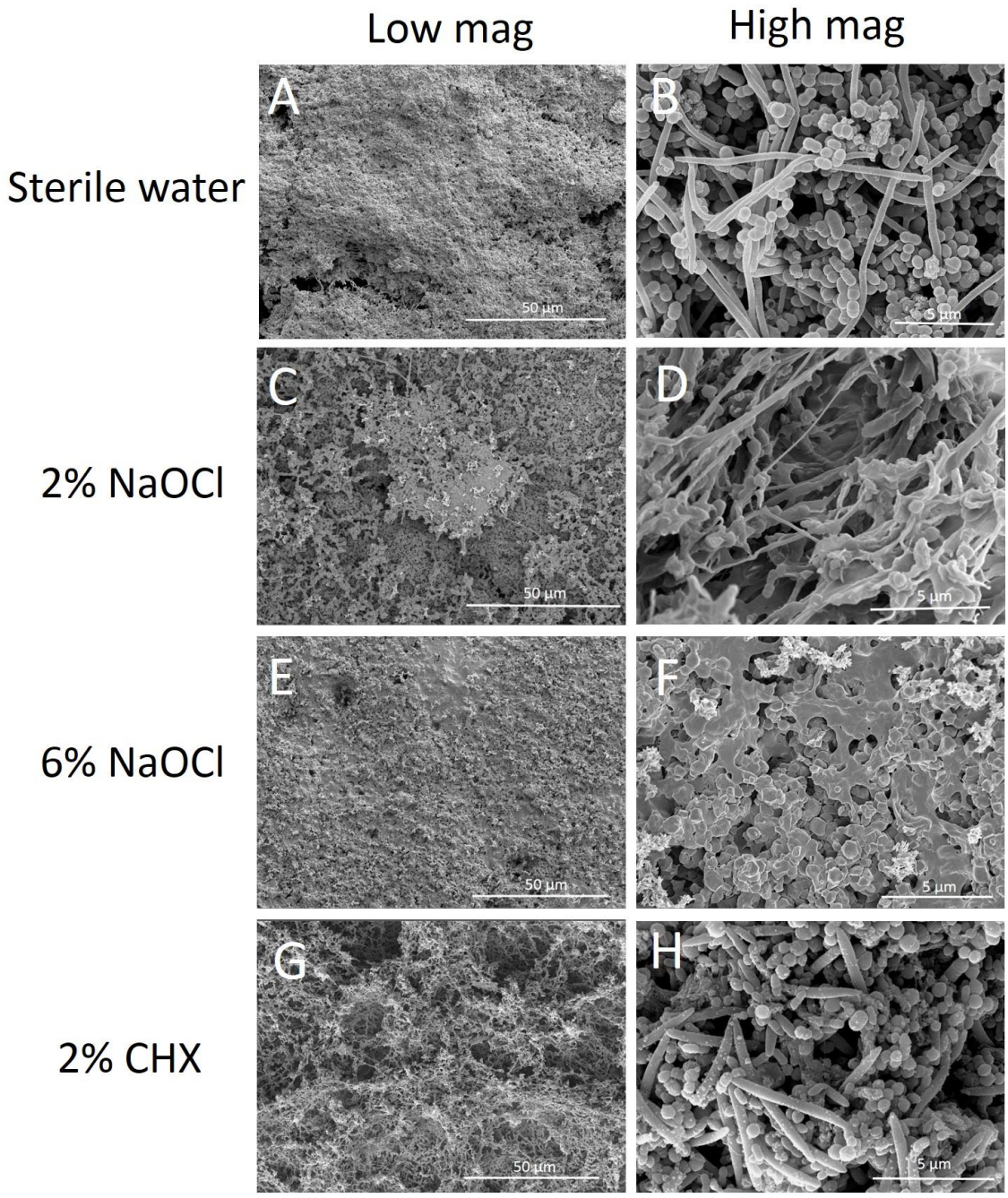

Figure 14. SEM micrographs showing dissolution and bacteria of 3-day-old plaque biofilms exposed to different disinfecting agents for 10 minutes. (A, B) 3-day-old biofilm exposure to sterile water in low (A) and high (B) magnifications. (C, D) 3-day-old biofilm exposure to 2% NaOCl in low (C) and high (D) magnifications. (E, F) 3-day-old biofilm exposure to 6% NaOCl in low (E) and high (F) magnifications. (G, H) 3-day-old biofilm exposure to 2% CHX in low (G) and high (H) magnifications.

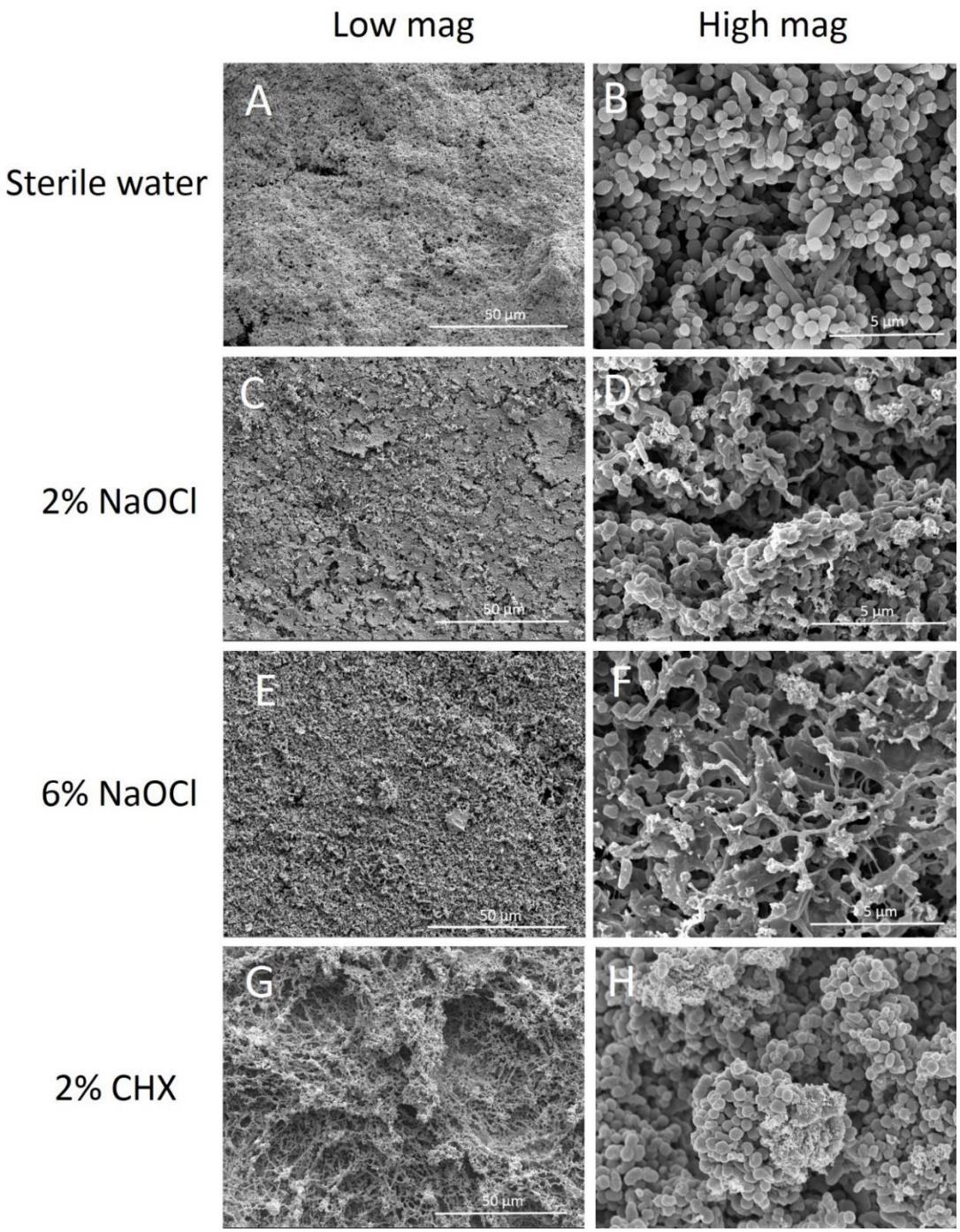

Figure 15. SEM micrographs showing dissolution and bacteria of 3-week-old plaque biofilms exposed to different disinfecting agents for 10 minutes. (A, B) 3-week-old biofilm exposure to sterile water in low (A) and high (B) magnifications. (C, D) 3-week-old biofilm exposure to 2% NaOCl in low (C) and high (D) magnifications. (E, F) 3-week-old biofilm exposure to 6% NaOCl in low (E) and high (F) magnifications. (G, H) 3-week-old biofilm exposure to 2% CHX in low (G) and high (H) magnifications.

5.3 Colony forming unit test

All the disinfecting solutions eliminated plaque biofilm bacteria in the planktonic cultures in 10 minutes (Table 7). The 3-day-old biofilm bacteria in the planktonic cultures were killed completely by 2% NaOCl and 2% CHX in 4 minutes. However, the 3-week-old biofilm bacteria were more resilient. For the 3-week-old biofilms, 2% NaOCl and 2% CHX killed 99.82% and 99.50% of the bacteria in 4 minutes, respectively (Table 7). Six percent NaOCl killed biofilm microbes faster than 2% NaOCl by eliminating 3-day-old biofilm bacteria in 2 minutes ($P<0.01$) and 3-week-old biofilm bacteria in 4 minutes ($P<0.01$). Over 99% of the bacteria were killed in 30 seconds by 6% NaOCl (Table 7).

For the undisrupted plaque in biofilm cultures exposed to disinfecting solutions for 10 minutes, only 6% NaOCl was able to eliminate the biofilm bacteria (Table 8). Two percent NaOCl and 2% CHX showed a 97%-99% killing effect on 3-day-old biofilm and a 94%-97% killing effect on 3-week-old biofilm. Six percent NaOCl showed the strongest antibiofilm activity, while 3-week-old biofilm was more resistant to disinfecting solutions than 3-day-old biofilm ($P<0.01$) (Table 8).

Table 7. Percentage (% ± Standard Deviation) of Killed Plaque Biofilm Bacteria in Planktonic Culture after Exposure to Different Medicaments

Time (min)		30 s[e]	2 min[f]	4 min[g]	10 min[h]
2% NaOCl	3-day[a]	94.62±1.84	99.77±0.13	100.00±0.00	100.00±0.00
	3-week[a]	95.73±1.57	98.81±0.42	99.82±0.06	100.00±0.00
6% NaOCl	3-day[b]	99.71±0.11	100.00±0.00	100.00±0.00	100.00±0.00
	3-week[b]	99.54±0.18	99.96±0.02	100.00±0.00	100.00±0.00
2% CHX	3-day[c]	92.57±2.18	99.25±0.18	100.00±0.00	100.00±0.00
	3-week[d]	90.99±1.84	94.37±2.42	99.50±0.11	100.00±0.00

Different superscript letters indicate statistically differences between groups ($P < 0.05$). Two-way ANOVA was applied for statistical analysis.

Table 8. Percentage (% ± Standard Deviation) of Killed Plaque Biofilm Bacteria, Measured by Culturing, after Exposure of the Biofilm to Different Medicaments for 10 Minutes

	3-day[a]	3-week[b]
2% NaOCl[c]	97.44±1.13	94.33±1.48
6% NaOCl[d]	100.00±0.00	99.76±0.12
2% CHX[e]	98.10±1.07	96.82±1.74

Different superscript letters indicate statistically differences between groups ($P < 0.05$). Two-way ANOVA was applied for statistical analysis.

5.4 Biofilm inhibition

The biovolume of plaque bacteria remained stable on the HA disks coated with 2% and 0.2% CHX during the 12-hour (720 minutes) culturing period ($P>0.05$) (Figs. 17, 18, and 20). The biovolume of the plaque biofilm started to increase significantly ($P<0.001$) at 4 hours in the sterile water control group (Figs. 16 and 20). Plaque on the sterile water coated HA disks reached a biovolume level that was 53 times higher in 12 hours than the biovolume at the zero hour (Fig. 20). The plaque biovolume on the 0.02% CHX coated HA disk did not increase in the first 8 hours (480 minutes) ($P>0.05$) (Fig. 20). However, a dramatic increase in biovolume ($P<0.001$) was observed from 8 to 11 hours (660 minutes) for the 0.02% CHX group followed by a stabilization at 12 hours (Figs. 19 and 20).

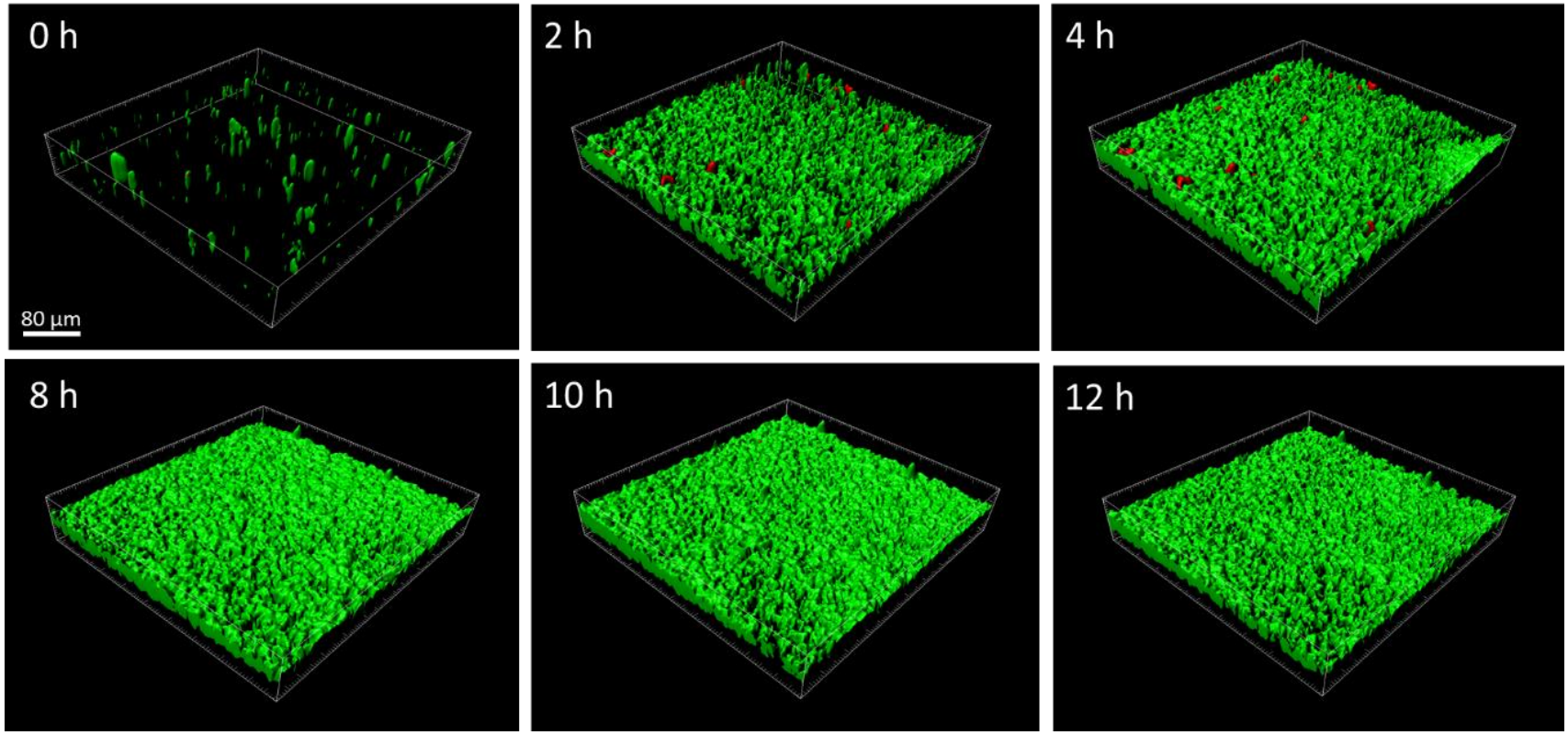

Figure 16. LC-CLSM images of biofilm development on HA disks pretreated with sterile water during the first 12 hours of biofilm incubation.

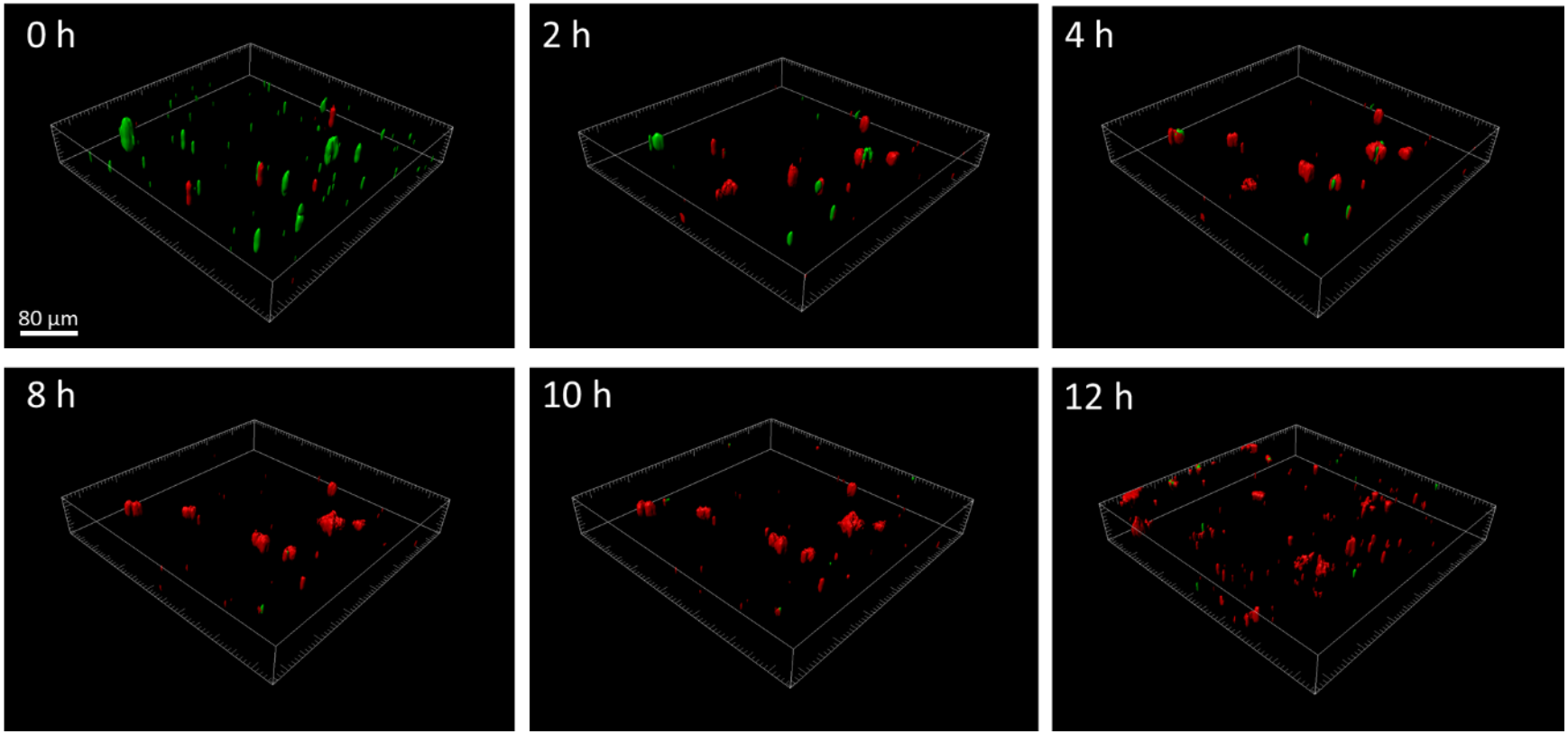

Figure 17. LC-CLSM images of biofilm development on HA disks pretreated with 2% CHX during the first 12 hours of biofilm incubation.

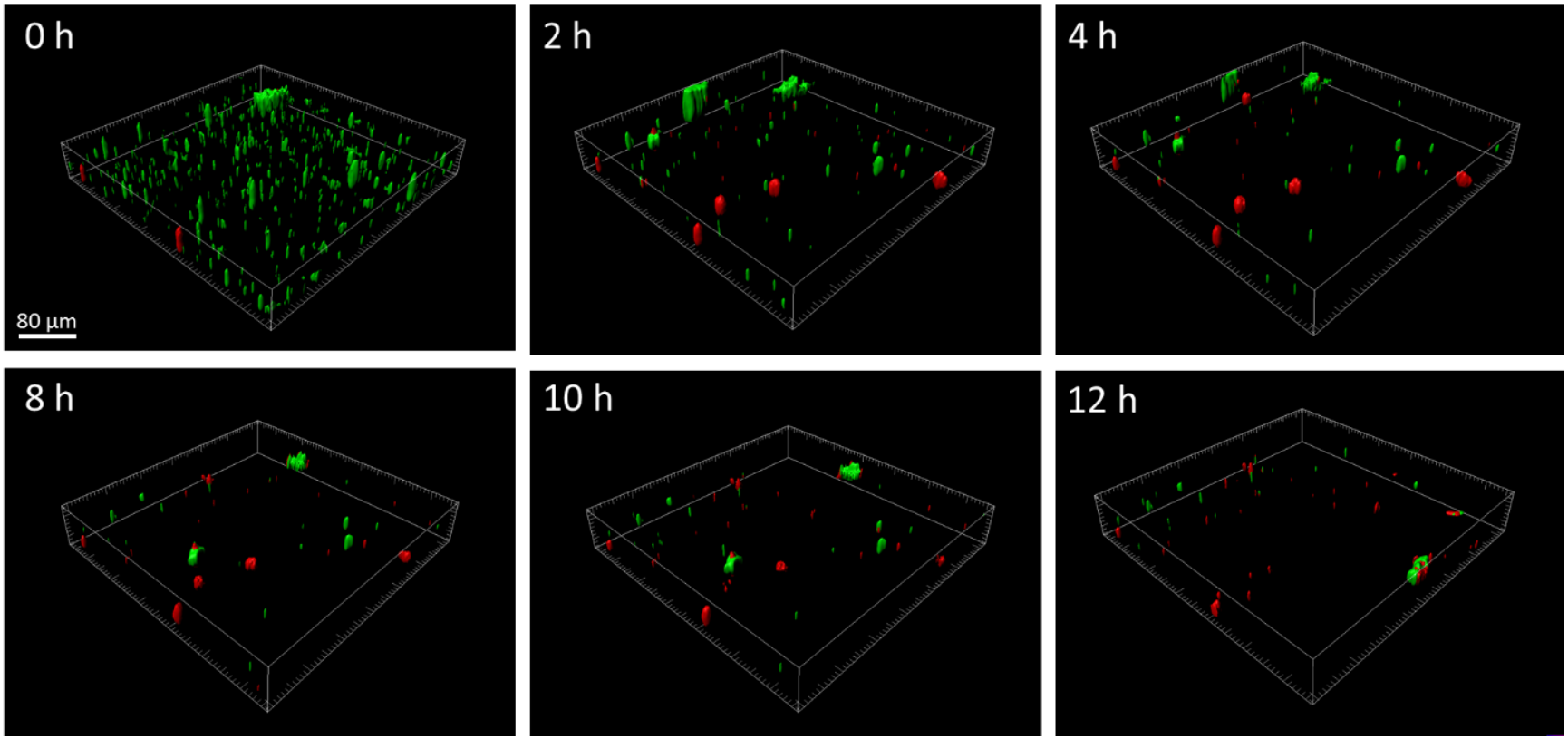

Figure 18. LC-CLSM images of biofilm development on HA disks pretreated with 0.2% CHX during the first 12 hours of biofilm incubation.

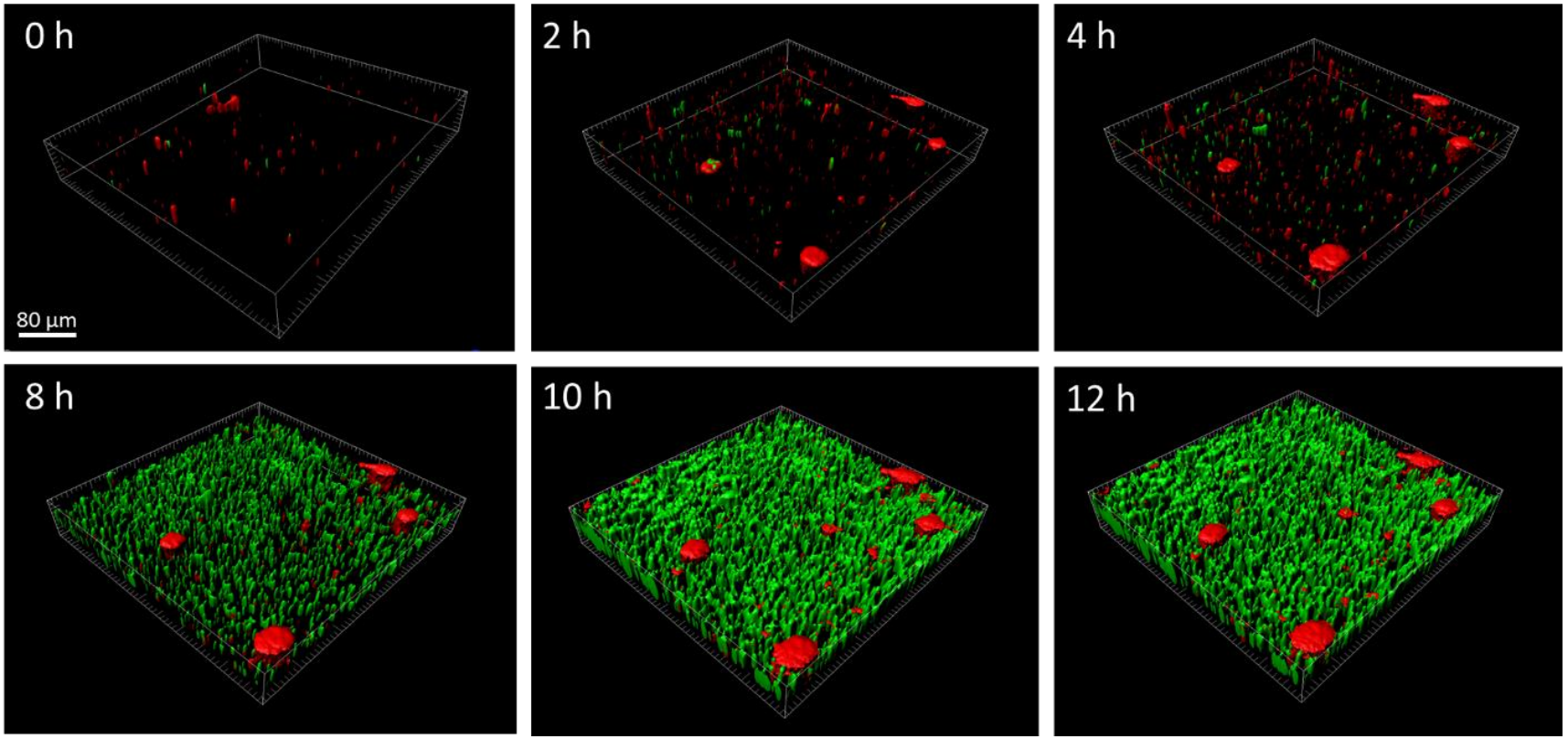

Figure 19. LC-CLSM images of biofilm development on HA disks pretreated with 0.02% CHX during the first 12 hours of biofilm incubation.

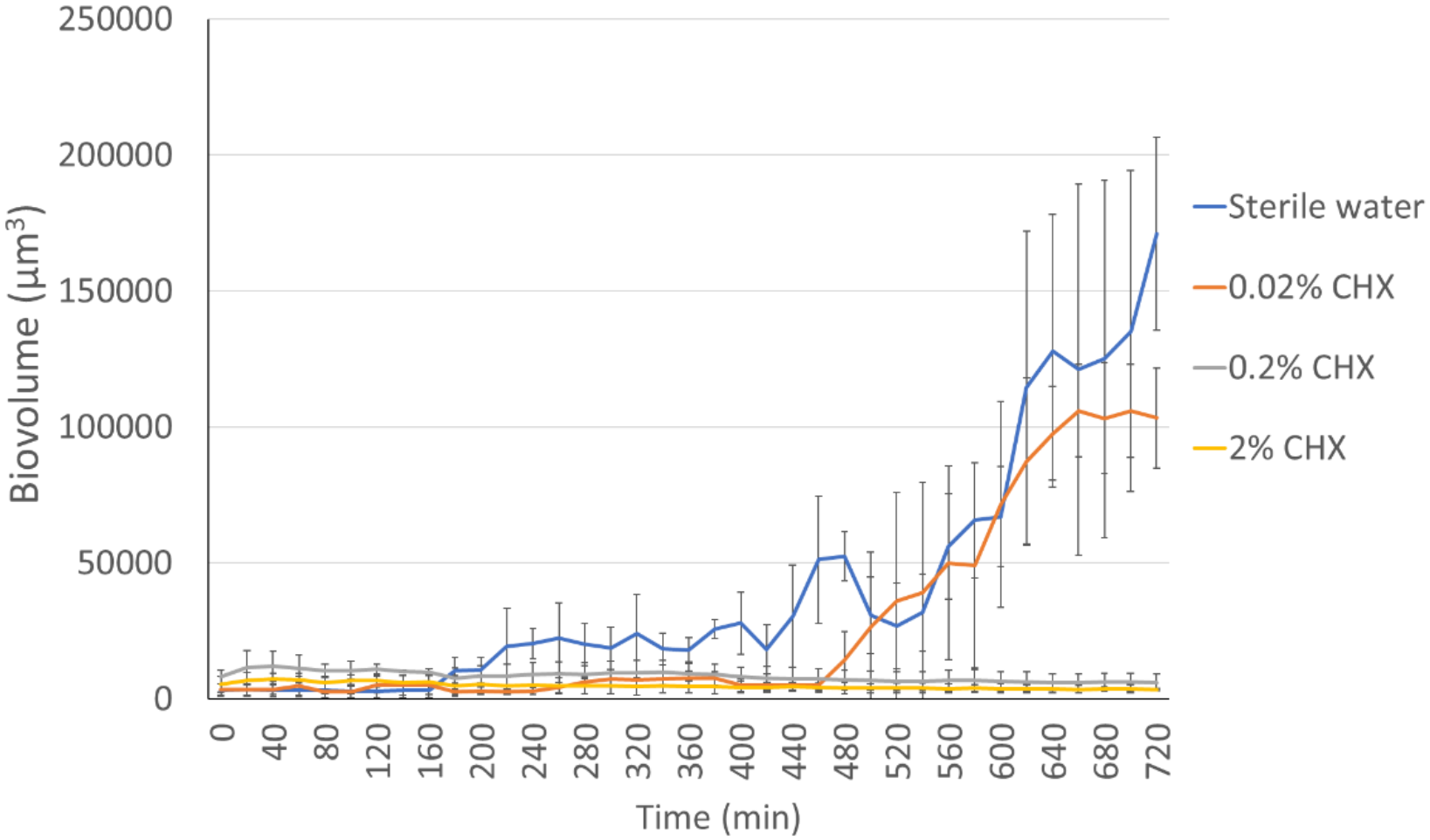

Figure 20. Plaque biofilm growth on different concentrations (2%, 0.2% and 0.02%) of CHX coated HA disk for 12 hours (720 minutes).

Chapter 6: Discussion

In endodontic treatment of root canal infection, dissolution of biofilms by disinfecting agents is crucial because a significant area of the root canal system is inaccessible to mechanical instrumentation. Tissues from a variety of sources including bovine pulp (15, 166), human pulp (167), bovine muscle tissue (90), porcine muscle tissue (168), rabbit liver (169), rat connective tissue (170), and pig palatal mucosa (171) have been used in previous studies for the determination of the dissolving ability of different clinical irrigants. The percentage of weight loss of the tissue after treatment has been used as a major index for the assessment of the dissolving ability of the irrigants. However, none of these previous studies have evaluated the weight loss of actual biofilm after endodontic disinfection. Biofilm dissolution is a dynamic process, but most of the previous investigations have focused on the fixed time period of tissue dissolution (14-16). One of the major challenges is that biofilm is extremely light weight, samples are usually analyzed in micrograms (172), thus it is difficult to be guarantee the accuracy of the balance. The moisture within the biofilm might be another factor that influences the accurate measurement of biofilm loss. Dehydration may disrupt the structural motifs and spatial distributions of the biofilm (70). Moreover, there is a lack of a standardized model to monitor the dynamics of biofilm disruption. Therefore, assessments other than weight measurement, along with more advanced measuring techniques may need to be developed to determine the biofilm dissolution effect.

Multispecies biofilm models formed from subgingival plaque have been used in previous studies to test the effect of endodontic antimicrobial agents (155, 156). Fluorescence from the biofilm after viability staining was captured by CLSM and the biovolume of the live and dead bacteria was used to calculate the proportion of killed biofilm bacteria. This advantage allows for analysis of the biomass of biofilm in a non-invasive approach in the absence of dehydration or sputter-coating of

the sample. A more recent study used CLSM and acridine orange stain to analyze the oral biofilm dissolution on bovine dentin by using CHX and different concentrations of NaOCl (173). Sodium hypochlorite was found to be more effective at dissolving biofilm than 2% CHX, which was consistent with the results in the present study. However, a two-dimensional analysis was performed and fixed treatment time intervals were applied in this dentin infection model. In the present study, an in-depth three-dimensional biofilm structure was obtained through live-cell imaging CLSM combined with LIVE/DEAD fluorescent labeling. A series (stacks) of optical sections of the biofilm were collected in real time to examine the antimicrobial effect of the disinfecting solutions without involvement of impact from other external confounding factors.

The BacLight LIVE/DEAD viability stain was applied at the beginning of the experiment and left in the biofilm for the 32-minute period for the dynamic dissolution/inhibition treatment in the present study. The LIVE/DEAD viability kit contains SYTO 9 green-fluorescent stain and the propidium iodide red-fluorescent stain. The SYTO 9 stain labels all bacteria with intact and with damaged membranes (174). In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present (175). Thus, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. Sterile water control groups in the present study showed that there was no significant change in biovolume in the 32-minute treatment and the percentage of dead bacteria was within 6% (Figs. 6, 7, 12, and 13), indicating that the viability stain did not induce biofilm dissolution or killing effects. Moreover, in the biofilm inhibition test, the sterile water control group showed normal biofilm growth in 12 hours (Fig. 20), indicating the viability stain and sterile water did not inhibit biofilms growing in BHI medium.

The dynamic live-cell imaging CLSM showed strong biofilm dissolution and killing effects by NaOCl. The mechanism of action of the NaOCl is mainly due to its oxidizing effect and high pH (176). Hypochlorous acid (HOCl) and hypochlorite ions (OCl^-) from NaOCl lead to amino acid degradation and hydrolysis. Hypochlorous acid uses porins on the bacterial cell membrane as channels to evade the bacterial defenses and cause more damage to internal structures by disrupting the proper operation of DNA and proteins (11). The high pH of NaOCl interferes with the cytoplasmic membrane integrity with an irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism, and phospholipid degradation in lepidic peroxidation (177). The SEM results in the present study showed significant disruption of biofilm after NaOCl treatment (Figs. 14 and 15).

The dissolving capability of NaOCl relies on its concentration and contact time. A previous study analyzing 2-dimensional CLSM images showed that 5.25% NaOCl dissolved a significantly higher amount (97%) of 3-day-old oral plaque biofilm than 2.5% NaOCl (39.5% dissolved) in 5 minutes, but there was no difference between them in 30 minutes (173). This result is partially consistent with the present study by showing that higher concentration NaOCl has a higher biofilm dissolving ability. However, the percentages of dissolved biofilm were not exactly the same, and the current study showed that 2% NaOCl dissolved a significantly lower amount of biofilm than 6% NaOCl in 32 minutes. These differences could be due to the different experimental settings (e.g. different biofilm substrates and different volumes of NaOCl applied to the biofilm) and different methods of data analysis. It is interesting to observe that biofilms in the present study were not 100% dissolved or killed in the 32-minute long exposure to disinfecting agents. When combining the dissolving and killing effect, 6% NaOCl reached a 94% bacterial reduction level as quickly as 2 minutes but did still not reach 100% in 32 minutes (Tables 3 and 6). Two percent NaOCl took

longer time to reach 90% bacterial reduction but also did not achieve 100% reduction (Tables 3 and 6). One possibility is that the limited amount of disinfecting solution (100 μl) did not completely dissolve the biofilm containing a protective shield of EPS. The EPS encapsulates bacteria within the biofilm and protects them from antimicrobial agents (73). Another possible explanation for the biofilm tolerance is the existence of persister cells which represent dormant or slow growing bacteria in a population that resist the action of antimicrobial agents (107). Those highly resistant persister bacterial cells may dominate the population of the survived biofilm after a 32-minute treatment and may also partially explain the reason that bacteria could not be eliminated in the root canal system.

When combining the dissolving and killing effects together, 2% NaOCl showed stronger disinfecting effects than 2% CHX (Tables 3 and 6), whereas no difference was found between them when comparing the killing of residual biofilm microbes (Tables 2 and 5). This is probably due to the lack of a biofilm dissolution effect of CHX, which is consistent with previous studies (70, 173). Although the SEM images showed different surface profiles for CHX and sterile water treated biofilm, the biovolume of the biofilm did not change. The mechanism underlying this fact might be due to ionic interactions between the negatively charged EPS matrix and the positive CHX, causing immediate collapse of the superficial matrix polysaccharides (107). The EPS matrix tends to hinder the diffusion of CHX into the deeper layers of biofilms (66). Hope et al. (70) used time-lapse CLSM to evaluate the dynamic killing effect of CHX in a 15-minute treatment cycle and found the contraction of biofilm thickness in a time series. The present study did not find significant reduction of biovolume in the CHX group with the 32-minute treatment, which is probably due to the different amount of treatment solution and different magnification of CLSM used compared to the previous study. Around 86% and 73% of the 3-day and 3-week-old biofilm

bacteria were killed in 32 minutes, respectively, and biofilms treated with both 2% CHX and 2% NaOCl showed similar susceptibility (Tables 2 and 5). The killing mechanism of CHX is due to its interaction with the negatively charged bacterial cell surface and its translocation to the cytoplasmic membrane where it damages the membrane barrier leading to cell death (178).

Clinically, CHX is considered to be advantageous as a treatment prior to obturation due to its substantivity in the root canal while remaining antimicrobially effective to the microbes (108). Rosenthal et al. (108) reported that CHX remained in the root canal dentin in antimicrobially effective amounts for up to 12 weeks. White et al. (105) found antimicrobial activity was present (agar diffusion test) in 2% CHX treated teeth throughout a 72-hour testing period and in 0.12% CHX for 6 to 24 hours. The present study used different concentrations of CHX to first coat the HA disks and then monitored the plaque biofilm growth under LC-CLSM for 12 hours. The coating by 2% and 0.2% CHX inhibited biofilm growth, while the 0.02% CHX group showed delayed growth (Fig. 20). This result probably indicated that CHX achieved plaque inhibition as a result of an immediate bactericidal action followed by a prolonged bacteriostatic action as a result of adsorption to the HA disk surface (179). However, to achieve such bactericidal and bacteriostatic effects required a high enough CHX concentration.

The present study is the first of its kind to compare the dynamic dissolution and killing effect of endodontic disinfecting agents on young and old biofilms. A recent study showed that the proportion of killed bacteria, by CHX, in mature biofilms was much lower than in young biofilms (66). This was consistent with the killing results obtained in the present study (Tables 2 and 4; Figs. 7 and 13), even though no significant difference in bacterial killing by 2% CHX was found in 4-24 minutes between the 3-day and 3-week-old biofilms. This is probably due to the fact that EPS prevented CHX from penetrating into the biofilm when CHX was initially applied, thus

making mature biofilms more difficult to kill compared with young biofilms. Increased interaction times with CHX changed the hydration, solubility, and structure of the EPS, and CHX was finally able to diffuse into the biofilm, correlating with an increase in dead bacterial cells. Six percent NaOCl removed significantly more 3-day-old biofilm than 3-week-old biofilm, whereas 2% removed a similar volume of young and old biofilms (Tables 1 and 3; Figs. 6 and 12). This result might be explained by a stronger oxidizing effect from 6% NaOCl which was more efficient at dissolving and detaching the 3-day-old biofilm than was the 2% NaOCl.

As the fluorescence of the bacteria is based on cell membrane integrity, with red fluorescence showing bacteria with a damaged cell membrane, the killing effect was further confirmed using traditional CFU culturing tests. A previous study using the CFU test to evaluate the killing effect of NaOCl showed more efficient bacterial elimination in seconds (142), while NaOCl in the present study eliminated planktonic bacteria (suspension of biofilm) in minutes (Table 7). The biofilm bacteria collected in a biofilm suspension were likely to have stronger resistance than planktonic bacteria (132). In biofilm culturing, all the disinfecting solutions could hardly kill all the microbes in 10 minutes (Table 8). The possible mechanisms include physical diffusion barriers in biofilm, altered expression of resistance genes on bacterial surfaces, and the emergence of biofilm-specific phenotypes (107). The 3-week-old mature biofilm showed higher resistance to antimicrobial agents than the 3-day-old biofilm, which was probably because the high content of EPS in the mature biofilm slowed down the killing efficacy (73).

Despite the advantages of using live-cell imaging CLSM to analyze the dynamic dissolution and inhibition of the oral biofilm, some experimental limitations were associated with the method utilized in the present study. There was a 2-minute gap between applying the disinfecting solutions on the biofilm samples and the start of confocal scanning, thus the baseline (0 minute) biovolume

of the biofilm could not be captured. Therefore, this model may not be optimal for the assessment of disinfecting agents that exhibit extremely rapid killing or dissolving properties. Moreover, plaque from only one donor was applied in the present study. However, previous studies have shown that the killing curves of plaque biofilms from different donors by several different disinfecting agents were similar (18, 133). In addition, the variations of the species composition of the plaque biofilm collected from the donor exist because it was not possible to use one-time biofilm collection for all the groups on the same day. Nevertheless, the standard deviations of the biofilm dissolution and killing effects were small in the data shown in the present study (Tables 1-6).

Using this model, various other disinfecting solutions used for biofilm removal or killing can be studied. Future experimental design should include analysis on the formation and dissolution of EPS to further understand the mechanism of biofilm resistance. Infected dentin specimens should also be incorporated in the study design (19), optimally with medium flow to better simulate a clinical situation.

Chapter 7: Conclusions

The experimental model presented in this study using live-cell imaging confocal scanning of multispecies biofilms provided a non-invasive platform to evaluate biofilm dissolution, killing, and inhibition of growth by endodontic disinfecting agents including NaOCl and CHX. The null hypotheses of the present study have been partly rejected. Sodium hypochlorite dissolved biofilm efficiently with the increasing of concentration and time. Chlorohexidine did not significantly reduce the biofilm volume but killed many biofilm bacteria and significantly inhibited biofilm growth on HA disk surface.

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Appendix

Appendix A: Informed consent form, obtaining donor dental plaque

Associate Professor, Division of Endodontics
Ya Shen, DDS, PhD
2199 Wesbrook Mall
Vancouver, BC Canada V6T 1Z3
Tel: [redacted] Fax: [redacted]
E-Mail [redacted]

Participant Information and Consent Form

Dynamics of oral biofilms and mechanical characteristics of instruments

Principal Investigator: Ya Shen DDS PhD
Department: Room 330, Division of Endodontics, Department of Oral Biological & Medical Sciences, the
Institution: University of British Columbia
Contact number: [redacted]

Co-investigator: Markus Haapasalo DDS PhD
Department: Room 310, Division of Endodontics, Department of Oral Biological & Medical Sciences, the
Institution: University of British Columbia
Contact number: [redacted]

For emergency contact number:
[redacted]
For non-emergency contact numbers:
[redacted]

Grant in aid: Start-up funds, Faculty of Dentistry, University of British Columbia, Canada.

1. Introduction:
You are being invited to take part in this research study because we require a study group of adult people who have teeth.

2. Your Participation is Voluntary:
Your participation is entirely voluntary, so it is up to you to decide whether or not to take part in this study. Before you decide, it is important for you to understand what the research involves. This consent form will tell you about the study, why the research is being done, what will happen to you during the study and the possible benefits, risks and discomforts.

If you wish to participate, you will be asked to sign this form. If you do decide to take part in this study, you are still free to withdraw at any time and without providing a reason for your decision.

If you do not wish to participate, you do not have to provide a reason for your decision not to participate.

Please take time to read the following information carefully and to discuss it with your family, friends and/or dentist before you decide.

3. Who is conducting the study
The study is part of the research projects supervised by Dr. Markus Haapasalo and Dr. Ya Shen, faculty of dentistry, UBC.

4. Background Information:
Dental plaque is a biofilm, usually a pale yellow that develops naturally on the teeth. Like any biofilm, dental plaque is formed by colonizing bacteria trying to attach themselves to a smooth surface (of a tooth). Dental plaque can cause dental caries (tooth decay) - the localized destruction of the tissues of the tooth by acid produced from the bacterial degradation of fermentable sugars and periodontal problems such as gingivitis and chronic periodontitis. Further, bacteria from a caries lesion in the tooth crown can access to the root canal space (dental pulp) and cause infection in the root canal as well as in the bone surrounding the tip of the root.

Successful treatment of these diseases (dental caries, gingivitis, chronic periodontitis, root canal and periapical infections) is dependent on elimination of the microorganisms (bacteria). This is being done by brushing teeth with toothbrush and accessory products for dental hygiene and by cleaning the root canal with special instruments and irrigation (flushing) the canal space with solution which have antibacterial activity killing the microorganisms.

5. Purpose: Analysis of biofilms and factors involved in their ability to cause disease and evaluation of existing and development of new, more effective methods for their eradication from the root canal.
Bacteria, organized in biofilm structures, are the etiological factor of the common oral diseases such as caries, periodontal and root canal infections. Knowledge of the biofilm structure is

important to understand its special characteristics as well as to find ways to eliminate it. Therefore, in this study we want to study biofilms in order to:

1) Develop *in vitro* biofilm models with close structural and functional similarity to *in vivo* biofilms and evaluate the effect of environmental factors such as availability and type of nutrients, oxygen, age and source of bacteria to their susceptibility to disinfecting agents.
2) To evaluate existing and develop novel disinfecting agents and/or protocols against oral bacteria for safe and more effective eradication of biofilms.

During regular cleaning of teeth with a tooth brush or a wooden stick you are removing plaque bacteria from your teeth. We would like to use these bacteria in our study in the laboratory.

6. Who can participate in the study?
Any adult person who has teeth.

7. Who should not participate in the study?
There are no medical contraindication (reasons not to participate).

8. What does the study involve?
Subgingival dental plaque will be collected using the tip of wooden stick. Collected samples will be added to culture medium and used for biofilm growth on hydroxyapatite discs. Biofilms of different age will be later used for analysis of factors important for their ability to cause disease and for testing and development of effective disinfection (treatment) methods.
If you agree to take part in this study, the procedures and visits you can expect will include the following:
One visit: Dental plaque will be collected with the tip of wooden stick from molars and premolars (teeth at the back of the mouth). The time required for taking the sample is 2 - 5 minutes.
The samples of dental plaque collected for this study will be used immediately for biofilm growth in the UBC laboratory of Dr. Markus Haapasalo. Unused samples will be destroyed. You may contact Dr. Markus Haapasalo or Dr. Ya Shen in order to ask questions. The results from your dental plaque samples will not be made available to you.

9. What are my responsibilities?
Providing oral plaque by using toothpicks.

10. What are the possible harms and discomforts?
No harm. There are no additional risks or complications of the described sampling procedure.

11. What are the potential benefits of participating?
There is no direct benefit to you as a consequence of this study. Information gained in this study will aid in understanding of the causes and development of dental infections and help us improve our current therapies for these diseases.
12. What are the alternatives to the study treatment?
No alternative treatment is needed.

13.What if new information becomes available that may affect my decision to participate?
If you choose to enter this study and at a later date a more effective treatment becomes available, it will be discussed with you. You will also be advised of any new information that becomes available that may affect your willingness to remain in this study.

14. What happens if I decide to withdraw my consent to participate?
Your participation in this research is entirely voluntary. If you decide not to enter the study, there will be no penalty and your future dental care will not be affected.

15. Can I be asked to leave the study?
If you are not able to follow the requirements of the study or for any other reason, the researcher may withdraw you from the study and will arrange for your care to continue. On receiving new information about the treatment, your research doctor might consider it to be in your best interests to withdraw you from the study without your consent if they judge that it would be better for your health. If you are asked to leave the study, the reasons for this will be explained to you and you will have the opportunity to ask questions about this decision.

16. How will my taking part in this study be kept confidential?
Your confidentiality will be respected. However, research records and health or other source records identifying you may be inspected in the presence of the Investigator or his or her designate by representatives of Health Canada and UBC Clinical Research Ethics Board for the purpose of monitoring the research. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.
You will be assigned a unique study number as a participant in this study. This number will not include any personal information that could identify you (e.g., it will not include your Personal Health Number, SIN, or your initials, etc.). Only this number will be used on any research-related information collected about you during the course of this study, so that your identity will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique study number that is used on your research-related information will not be removed or released without your consent unless required by law.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to ensure that your privacy is respected. You also have the legal right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study doctor.

Reportable diseases
Your personal information or information that could identify you will not be revealed without your express consent unless required by law. If facts become known to the researchers which must be reported by law to public health authorities or legal authorities, then your personal information will be provided to the appropriate agency or authority.

Primary Care Physician(s)/Specialist(s) Notification

Your family physician will be notified of your participation in the study so that your study doctor and your family doctor can provide proper medical care.
Please indicate, by checking the applicable box, whether you want us to notify your primary care physician(s) or specialist(s) of your participation in this study. This is not a consent to release medical information.

Yes, I want the study investigator to advise my primary care physician(s) or specialist(s) of my participation in this study. My primary care physician(s) and/or specialist(s) name(s) is/are: _____

The name of the medical clinic I attend is: _____
Participant Initials: _____

No, I do not want the study investigator to advise my primary care physician(s) or specialist(s) of my participation in this study.
Participant Initials: _____

I do not have a primary care physician or specialist.
Participant Initials: _____

The study investigator is my primary care physician/specialist.
Participant Initials: _____

I understand that if I choose not to advise my primary care physician(s) or specialist(s) of my participation in this study, there may be potential medical consequences which may affect my comprehensive medical care or treatment. I understand that the study investigator may not be responsible for these consequences.
You may wish to discuss the consequences of your decision with the study staff.

Disclosure of Race/Ethnicity
Studies involving humans now routinely collect information on race and ethnic origin as well as other characteristics of individuals because these characteristics may influence how people respond to different medications. Providing information on your race or ethnic origin is voluntary.

17. What happens if something goes wrong?
By signing this form, you do not give up any of your legal rights and you do not release the study doctor, participating institutions, or anyone else from their legal and professional duties. If you become ill or physically injured as a result of participation in this study, medical treatment will be provided at no additional cost to you. The costs of your medical treatment will be paid by your provincial medical plan and/or by the principle investigator**.**

18. What will the study cost me?
All research-related medical care and treatment and any related tests that you will receive during your participation in this study will be provided at no cost to you.

19. Who do I contact if I have questions about the study during my participation?
If you have any questions or desire further information about this study before or during participation, you can contact Dr. Markus Haapasalo and Dr. Ya Shen.

20. Who do I contact if I have any questions or concerns about my rights as a participant?
If you have any concerns or complaints about your rights as a research subject and/or your experiences while participating in this study, contact the Research Participant Complaint Line in the University of British Columbia Office of Research Ethics by e-mail at [redacted] or by phone at [redacted]

21. After the study is finished
You may not be able to receive the study treatment after your participation in the study is completed. There are several possible reasons for this, some of which are:

- The treatment may not turn out to be effective or safe.
- The treatment may not be approved for use in Canada.
- Your caregivers may not feel it is the best option for you.
- You may decide it is too expensive and insurance coverage may not be available.
- The treatment, even if approved in Canada, may not be available free of charge

22. Signatures

Dynamics of oral biofilms and mechanical characteristics of instruments

Participant Consent

My signature on this consent form means:

I have read and understood the information in this consent form.

I have had enough time to think about the information provided.

I have been able to ask for advice if needed.

I have been able to ask questions and have had satisfactory responses to my questions.

I understand that all of the information collected will be kept confidential and that the results will only be used for scientific purposes.

I understand that my participation in this study is voluntary.

I understand that I am completely free at any time to refuse to participate or to withdraw from this study at any time, and that this will not change the quality of care that I receive.

I authorize access to my health records [insert if applicable and samples] as described in this consent form.

I understand that I am not waiving any of my legal rights as a result of signing this consent form.

I understand that there is no guarantee that this study will provide any benefits to me.

The parent(s)/guardian(s)/substitute decision-maker (legally authorized representative) and the investigator are satisfied that the information contained in this consent form was explained to the child/participant to the extent that he/she is able to understand it, that all questions have been answered, and that the child/participant assents to participating in the research.

I will receive a signed copy of this consent form for my own records.

I consent to participate in this study.

Participant's Signature_____

Printed name _____

Date _____

Signature of Person Obtaining Consent _____

Printed name _____

Study Role _____

Date _____

If this consent process has been done in a language other than that on this written form, with the assistance of an interpreter/translator, indicate:

Language: _____

Was the participant assisted during the consent process in one of ways listed below?

Yes

No [Note: For typical situations where the person conducting the consent discussion simply reads the consent with the participant to ensure that informed consent is properly obtained, check "no".]

If yes, please check the relevant box and complete the signature space below:

The consent form was read to the participant, and the person signing below attests that the study was accurately explained to, and apparently understood by the participant.

The person signing below acted as an interpreter/translator for the participant, during the consent process (please check if an interpreter/translator assisted during the consent process).

Signature of Person Assisting in the Consent Discussion_____

Printed Name _____

Date _____

Investigator Signature _____

Printed name _____

Date _____

My signature above signifies that the study has been reviewed with the study participant by me and/or by my delegated staff. My signature may have been added at a later date, as I may not have been present at the time the participant's signature was obtained.