# IN VITRO EFFECT OF SODIUM HYPOCHLORITE ON *STREPTOCOCCUS MUTANS* MONO-SPECIES BIOFILM.

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A Thesis
Submitted to
the Temple University Graduate Board

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in Partial Fulfillment
of the Requirements for the Degree
MASTER OF SCIENCE

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December, 2017

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## ABSTRACT

*Objectives:* Bacterial biofilms are ubiquitous, found in a variety of sites within the human body, and are associated with the pathogenesis of dental caries. They are formed when free-floating microorganisms attach to a surface. One such organism, *Streptococcus mutans*, has adhesins allowing its attachment to tooth surfaces. *S. mutans* is associated with all forms of human dental caries. *S. mutans* can rapidly metabolize dietary sugars to acid, locally creating a low pH on tooth surfaces, where it can optimally grow and become more competitive in dental plaque biofilms, in contrast to acid-sensitive bacterial species associated with sound non-carious tooth surfaces.

Commensal and natural biofilm aggregates contain multiple microbial species that are believed to co-exist, interact, and form families with high bacteria and niche diversity. In contrast, most biofilms that are chronically infectious tend to have low bacterial diversity with sovereign mono-species such as *S. mutans*. Hence, it is advantageous to study mono-species biofilms of dental caries-associated bacteria.

Although dental biofilms cannot be completely eliminated, their pathogenicity can be lessened through effective oral hygiene measures. Continuous and regular disruption of dental biofilms is imperative for prevention and management of oral infectious diseases. Mechanical methods, most notably tooth brushing and flossing, are required to regularly and effectively disrupt dental plaque biofilms in the human oral cavity. Antiseptics, such as mouth rinses, can also help control dental plaque biofilms, and may gain access to oral sites inaccessible by mechanical methods.

Among available antiseptics, sodium hypochlorite is a particularly potent agent

against bacteria, fungi, and viruses. Sodium hypochlorite occurs naturally within phagocytic cells (neutrophils, monocytes, and macrophages) participating in the human innate immune response to microbial infection. Several randomized controlled clinical trials support the efficacy of rinsing twice weekly with 0.25 % sodium hypochlorite to improve periodontal health. To date, no studies have been reported concerning the effect of sodium hypochlorite rinsing on dental caries incidence or progression. Several in vitro studies have addressed the effect of dilute sodium hypochlorite on various oral bacteria species. However, there are no literature reports addressing the effect of sodium hypochlorite on bacteria attached to solid surfaces in biofilms. Hence, the objective of this study is to compare the in vitro antimicrobial effect of sodium hypochlorite to another antiseptic agent, chlorhexidine, on the acidigenic and aciduric bacterial species, *S. mutans* and *Lactobacillus casei*, both in a free-form (planktonic) state, and as a biofilm attached to two hard surfaces (glass and hydroxyapatite).

*Methods:* The effect of sodium hypochlorite on *S. mutans* and *L. casei* was determined by finding the minimum inhibitory concentration (MIC) by broth dilution assays. The MIC was considered to be the lowest concentration of the agent that prevented bacterial growth, resulting in a clear test tube in a broth dilution assay. Experiments with sodium hypochlorite were repeated twice against each bacterial species, and compared to chlorhexidine in similar assays. The effect of a 20-second exposure of MIC concentration (1.0%) of sodium hypochlorite on the growth of *S. mutans* planktonic cells was measured. Finally, the effect of a 20-second exposure of 1% hypochlorite on *S. mutans* single-species biofilms was assayed using sterile microplates and the MBEC Biofilm Inoculator (Innovatech).

*Results*: Values for inhibition of growth of planktonic cells were as follows: sodium hypochlorite (MIC = 0.1%; MBC = 0.1%); chlorhexidine (MIC = 0.0015%; MBC = 0.0025%). A 20-second exposure to either solution at the MBC inhibited growth of planktonic cells in refreshed media. Cells adherent to glass or hydroxyapatite pegs were growth-inhibited, but not detached, in refreshed media by a 20-second exposure to 0.2% and 0.4% sodium hypochlorite, and 0.01% and 0.12 % chlorhexidine, but not by more dilute solutions.

*Conclusions:* Chlorhexidine is more potent than sodium hypochlorite, but dilute solutions of both antimicrobial agents inhibited growth of *S. mutans* mono-species biofilm cells without detachment of cells.

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# CHAPTER 1

## INTRODUCTION

Dental plaque has been defined as the soft concentrated mass which develops on tooth surfaces one to two days after refraining from tooth brushing (Dawes et al. 1963). It is made up of many types of bacteria embedded in a matrix of proteins, glycoproteins, and polysaccharides. The bacteria in dental plaque are characterized by their potential for forming organic acids from fermentable carbohydrates, and by their ability to synthesize extracellular polysaccharides (Guggenheim 1970). Without exception, dental plaque has been associated with dental caries, a process where localized and progressive breakdown of teeth is initiated by demineralization of its outer surfaces (Keyes 1968). Dental plaque has long been recognized as a microbial biofilm, and many of the pioneering concepts of biofilm development, progression, and inhibition in biologic systems originated in dental caries research (Costerton 1978)

Microbial biofilms contribute to the progression of the multifactorial disease known as dental caries (Costerton et al. 1999). Dental biofilms, bound to hard tissue, exhibit an altered phenotypic response to their environment as compared to their cell-free or planktonic counterparts (Costerton et al. 1987). Biofilm organization confers a range of properties, one of which may be a reduced susceptibility to antimicrobial killing (Wilson 1997). In contrast to individual bacteria, a microbial biofilm is a complex and communal arrangement of bacteria. Bacterial biofilms are ubiquitous in nature, and are potentially found in a variety of sites within the human body. As applied to dentistry, bacterial biofilms are found in water lines of dental units, on tooth surfaces, on dental prostheses, and on oral mucous membranes. Biofilms in the form of supragingival and

subgingival plaque are considered as etiologic agents in dental caries and periodontal diseases (van Houte 1994).

The primary requisite for any group of microbes to flourish in the human mouth is their ability to adhere to the tooth surfaces (Socransky & Haffajee 2000). Biofilms are formed when free-floating microorganisms attach to a surface. Mutans streptococci, a group which includes *Streptococcus mutans* and other closely-related species (Selwitz et al. 2007), possess adhesins allowing attachment to hard surfaces. A major surface protein produced by *S. mutans* belongs to the adhesion family of proteins called SpaP (or P1), which mediates attachment of the bacterial species to hard surfaces (Bowen et al. 1991).

Streptococci represent the majority of supragingival bacteria in a healthy human oral cavity (Aas et al. 2005). Within the complex oral microbiome, only a small group of bacteria (mostly streptococci and *Actinomyces* species) can adhere in low numbers to uncoated apatite tooth surfaces, and even fewer to pellicle-coated tooth surfaces via adhesion/receptors and/or charge interactions. *S. mutans* is not always the most abundant organism within the initial colonizing community of bacteria on tooth surfaces. However, it can orchestrate the development of cariogenic biofilms through exoenzymes, such as glucosyltransferases, which are constituents of tooth surface pellicle and also bind to bacterial cell surfaces (Selwitz et al. 2007).

*S. mutans* and *Lactobacillus* species are amongst the family of microorganisms collectively designated as lactic acid bacteria. Lactic acid bacteria characteristically ferment sugars through a glycolytic pathway to form pyruvate, which is then converted to lactate (lactic acid) (Selwitz et al. 2007). Mutans streptococci are associated with all

forms of dental caries, and lactobacilli are efficient producers of lactic acid and tolerate low pH environments (Selwitz et al. 2007). However, lactobacilli are poor colonizers of smooth tooth surfaces, and probably do not initiate dental caries at these sites. Most likely lactobacilli are secondary colonizers of established dental caries lesions, where their aciduric properties allow them to out-compete other oral microorganisms (Selwitz et al. 2007). Acid production by lactobacilli will then exacerbate pre-existing dental caries lesions and facilitate their extension into tooth dentin (Selwitz et al. 2007).

Clinical studies have shown that dental caries is associated with increases in dental plaque proportions of acidogenic and aciduric bacteria, especially mutans streptococci and lactobacilli, which are capable of demineralizing enamel (Marsh 2006). These bacteria can rapidly metabolize dietary sugars to acid, creating locally a low pH environment on tooth surfaces. Since they grow and metabolize optimally at low pH values, they become more competitive in the oral microbial ecosystem, in contrast to most bacterial species associated with sound non-carious tooth surfaces and sensitive to acidic environmental conditions (Selwitz et al. 2007).

An ecological plaque hypothesis has been proposed to explain the relationship between the resident oral microbiota and dental disease (Marsh 2003). Briefly, a substantial change in local environmental conditions can alter the competitiveness of plaque bacteria leading to the enrichment of organisms most suited to the new environment. In other words, the commensal microbial biofilm becomes more like a mono-species biofilm. Commensal and natural biofilm aggregates contain multiple species that are believed to co-exist, interact, and form families with a high level of bacterial and niche diversity. In contrast, most biofilms associated with chronically

infectious conditions tend to have low bacterial diversity with sovereign mono-species (Costerton et al. 1999). Hence, it is advantageous to study mono-species biofilms of dental caries-associated bacteria. Likewise, it is important to control biofilm formation and development to control dental caries.

The pathogenicity of dental plaque biofilms is enhanced by the fact that in biofilm form, the component bacteria have increased resistance to antibiotics and other chemotherapeutic agents, and are less able to be phagocytized by host inflammatory immune cells (Wilson 1996). Biofilms protect bacteria living within their structures, and thereby provide an advantage over free-floating (planktonic) bacterial populations. The slimy extracellular matrix produced by biofilm bacteria encloses the microbial community and protects it from the surrounding environment, including attacks from chemotherapeutic agents. Chemotherapeutic agents have difficulty penetrating the polysaccharide matrix to reach and affect underlying microorganisms (Gilbert et al. 1997). Thus, the matrix helps to protect bacteria deep within biofilms from antibiotics and antiseptics, increasing the likelihood of their survival (Wilson 1996). Further, the extracellular matrix keeps bacterial species banded together, so they are not flushed away by the flow of saliva and gingival crevicular fluid. Therefore, control of dental plaque biofilms is a major objective of dental professionals and critical to the maintenance of optimal oral health.

Although dental biofilms cannot be completely eliminated, their pathogenicity can be lessened through effective oral hygiene measures. Continuous and regular disruption of dental plaque biofilms is imperative for prevention and management of oral infectious diseases. Mechanical methods, including tooth brushing, interdental cleaning, and

professional scaling procedures, are required on a regular basis to effectively disrupt and remove dental plaque biofilms. Antiseptics, such as mouth rinses, can help control dental plaque biofilms, but must be formulated so as to be able to penetrate the dental plaque biofilm matrix and gain access to pathogenic bacteria. Bacteria from biofilms on mucosal and tooth surfaces are shed constantly into saliva and transferred to other areas of the mouth. Since oral mucosa, which represents about 80% of the oral cavity surface (Mager et al. 2003) can serve as a reservoir for pathogenic bacteria that potentially can be transferred to tooth surfaces, supplementing mechanical plaque control methods with liquid antiseptics may also play an important role in reducing reservoirs of microbial pathogens that are unaffected by brushing and flossing directed solely at tooth surfaces.

Among antiseptics, sodium hypochlorite is a particularly potent agent against bacteria, fungi, and viruses (Rich & Slots 2015). It occurs naturally in phagocytic cells (neutrophils, monocytes, and macrophages) participating in the human innate immune response to infection (Rich & Slots 2015). Sodium hypochlorite has been used in dentistry for more than a century. The American Dental Association has designated dilute sodium hypochlorite solutions as a "mild antiseptic mouthrinse" with no contraindications (Rich & Slots 2015).

Several randomized controlled clinical trials support the efficacy of rinsing twice weekly with 0.25 % sodium hypochlorite to improve periodontal health. Galvan et al. (2014) reported that such a regimen produced marked decreases in dental plaque and bleeding on probing. In another study, compared to a water rinse, the diluted bleach rinsing group demonstrated a statistically significant 48.1% reduction in Plaque Index scores, a statistically significant 52.4% reduction in Gingival Index scores, and a

statistically significant 39.1% reduction in the percentage of periodontal sites that bled on probing (DiNardeo et al. 2012). These marked differences between a water rinse versus a diluted sodium hypochlorite rinse are of such a magnitude as to be clinically relevant in dental patient care. To date, no studies have been reported concerning the effect of rinsing with sodium hypochlorite on dental caries incidence or progression.

Several in vitro studies have addressed the effect of dilute sodium hypochlorite on oral bacterial species. Heling et al. (2001) found that solutions of 0.3% sodium hypochlorite were bactericidal to common endodontic pathogens. Another study found dilute bleach (0.5%) to be an effective endodontic irrrigant against *S. mutans*, *Porphyromonas gingivalis*, *Parvimonas micra* (formerly *Peptostreptococcus micros*), and *Prevotella intermedia* (Yesilsoy et al. 1995). Chlorhexidine (0.01%), cetylpyridinium chloride (0.01%), povidone iodine (10%) and sodium hypochlorite (0.5%) are effective at inhibiting the growth of *S. mutans*, *Streptococcus sanguinis* and *Lactobacillus acidophilus* (Evans et al. 2014). However, there are no literature reports addressing the effect of sodium hypochlorite on bacteria attached to solid surfaces, outside of one that focused on detachment of bacteria from dentures (Salles et al. 2015). Hence, the objective of this study is to compare the in vitro antimicrobial effect of dilute sodium hypochlorite to chlorhexidine gluconate on the acidigenic and aciduric organisms *S. mutans* and *L. casei* in a free-form (planktonic) state, and as a biofilm attached to two hard surfaces (glass and hydroxyapatite).

# CHAPTER 2

# MATERIALS AND METHODS

## Bacterial Cultivation and Preparation of Cells

*S. mutans* (ATCC strain 25175), and *L. casei* (ATCC strain 7469) were purchased from the American Type Culture Collection (Manassas, Virginia, USA). The purchased freeze dried cells were reconstituted in brain heart infusion broth (BHI or LB Broth, Fisher Scientific Company, Hampton, New Hampshire, USA) and then streaked onto agar plates. Colonies were examined for purity, single colonies transferred to broth, and grown to log phase, at which time they were used experimentally. All streptococci were inoculated in or onto BHI broth or agar, whereas lactobacilli were inoculated in or onto lactobacillus broth or agar. The concentration of log phase cells that were used was between $10^8$-$10^{10}$ colony forming units (CFU)/ml as determined by comparison to a #1 McFarland standard.

## Preparation of Antimicrobials

Sodium hypochlorite (Sultan Healthcare Company, Hackensack, New Jersey, USA) was purchased from Henry Schein Corporation (Melville, New York, USA). Chlorhexidine digluconate was purchased as a 20% solution from Sigma Chemical Company (St. Louis, Missouri, USA). Dilutions of the antimicrobial agents were made in sterile saline (chlorhexidine final pH = 7.2; sodium hypochlorite final pH = 11.0).

## Determination of MIC by Broth Dilution Assay

For planktonic cell bacterial growth studies, 100 µl of serial dilutions were used. To this, a fixed culture of bacteria (100 µl bacterial suspension, at a cell density equal to a 0.5 McFarland standard, optical density (OD) at 600 nm = 1.5 (equal to $10^8$ CFU/ml))

and 2.8 ml of culture media was added to obtain a final volume of 3.0 ml. Broth alone served as a negative control and the bacterial suspension (100 µl) added to culture media (2.9 ml) was used as a positive control. The pH of the buffered broth medium was 7.2, and the added antimicrobial agents did not change the pH. The minimum inhibitory concentration (MIC) was considered to be the lowest concentration of antimicrobial that prevented bacterial growth, i.e., a clear test tube. Experiments were repeated twice for each bacterial species. Growth was measured as turbidity on a spectrophotometer at 600 nm. All tubes, broths, agars, and pipet tips were sterilized prior to use by autoclaving at 121°C for 20 minutes.

Effect of Time of Exposure of Antimicrobial to Planktonic Cells

*S. mutans* and *L. casei* cells from 12 hours of growth were suspended in broth and adjusted to a 0.5 McFarland standard turbidity (OD 1.5 at 600 nm). Aliquots (100 ul) were suspended in 100 µl broth (control) or broth containing 1% sodium hypochlorite for 20, 40, or 60 seconds. After exposure for the designated time, the *S. mutans* cells were immediately centrifuged at 10,000 rpm, washed once in sterile broth, and re-suspended in 2.9 ml sterile broth for 24 hours at 37°C. Growth was monitored visually and on a spectrophotometer at 600 nm.

Effect of 20-Second Exposure of Antimicrobials on Single Species Biofilm Vitality on Hydroxyapatite or Glass

The effect of a 20-second exposure to antimicrobials on *S. mutans* single species biofilm vitality was measured similar to the method of Welch et al. (2012). The assay on hydroxyapatite employed sterile microplates and the MBEC Biofilm Inoculator (Innovatech Corporation, Edmonton, Alberta, Canada). Biofilm cells attached to sterile

glass test tubes were assayed in a similar manner. All experiments were conducted in a laminar control hood (Environmental Air Control, Inc., Hagerstown, Maryland, USA). The surface of the hood was disinfected with sodium hypochlorite wipes prior to conducting the experiments.

For hydroxyapatite-coated surfaces, experiments were conducted as follows: to each well in a row of 12 wells of a sterile plastic microdish was added 150 µl BHI media and 20 µl *S. mutans* bacteria ($10^8$ CFU/ml). A row of wells did not receive bacteria and served as negative control. Hydroxyapatite pegs on the MBEC Biofilm Inoculator were then inserted into the microdish wells, and incubated with rotation for 24 hours at 37°C. After 24 hours, the pegs were rinsed thoroughly with distilled water, and then the rows of pegs with adherent *S. mutans* cells were inserted for 20 seconds into another sterile plastic microdish containing a test antimicrobial agent or broth alone (positive control). Antimicrobial agents studied were 0.1%, 0.2% and 0.4% sodium hypochlorite, and 0.0025%, 0.005%, 0.01%, and 0.12% chlorhexidine. After the pegs were lifted out of the test solution, they were rinsed again with sterile saline and added to a third sterile plastic microdish containing refreshed media. After incubation for 24 hours at 37°C, growth in the wells was visualized and measured spectrophotometrically at 600 nm.

For glass-coated surfaces, experiments were conducted as follows: to 2 ml sterile broth was added 100 µl *S. mutans* cells, which were allowed to grow for 12 hours at 37°C. Planktonic cells were then decanted and the tubes rinsed with sterile saline. Adherent *S. mutans* cells were exposed to sodium hypochlorite (at concentrations of 0.9%, 0.5%, 0.25%, and 0.125%) or sterile saline (control) for 20 seconds, rinsed again,

and then incubated with refreshed media for 24 hours at 37°C. Growth was visualized and monitored by measuring turbidity on a spectrophotometer.

For estimation of the quantity of *S. mutans* cells attached to hydroxyapatite pegs, each row of 12 pegs was dipped in and stained with crystal violet (Sigma-Aldrich Corporation, St. Louis, Missouri, USA) for 15 minutes. Crystal violet is a dye that is specifically taken up by gram positive bacteria. The pegs were then thoroughly rinsed and allowed to dry overnight. The positive control row received *S. mutans* cells but no antimicrobial, and the negative control row received broth but no *S. mutans* cells. Stained biofilm was removed from each hydroxyapatite peg by immersing the pegs into 100 µl of 30% acetic acid for 5 minutes. The eluent (100 µl) was then added to 900 µl distilled water and the absorbance measured on a spectrophotometer at 500 nm. The quantity of *S. mutans* cells adherent to glass tubes were estimated in a similar fashion, i.e., addition of crystal violet, rinsing, and eluting with 30% acetic acid.

# CHAPTER 3

# RESULTS

## Determination of MIC by Broth Dilution Assay

The MIC of sodium hypochlorite for *S. mutans* was determined to be 0.1% (Figure 1).

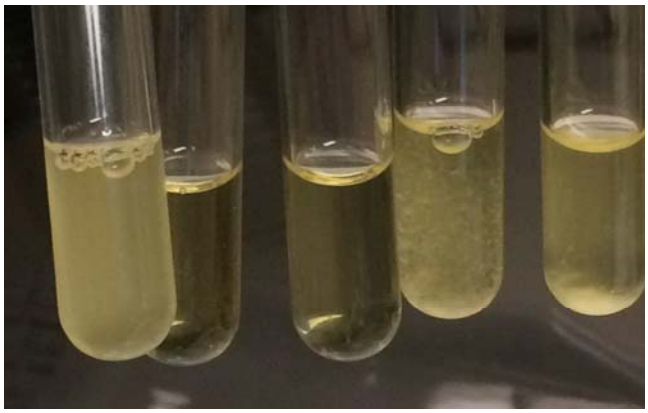

Figure 1. MIC of sodium hypochlorite against *S. mutans* determined by a broth dilution method. *S. mutans* inoculated tubes from left: untreated control, and those exposed to 0.2%, 0.1 %, 0.05% and 0.025% sodium hypochlorite, respectively (all tubes incubated for 24 hours).

Subculture of the 0.1% MIC tube on BHI agar showed no growth indicating that the MBC = 0.1% was identical to the MIC = 0.1%.

Similar results were found for *L. casei*, as MIC = 0.125% and MBC = 0.125% for sodium hypochlorite (Table 1). For chlorhexidine, the MIC for *S. mutans* was determined to be 0.0025% (Table 1). Subculture of dilutions of chlorhexidine tubes indicated that the MBC was slightly higher at 0.005% (Table 1).

Table 1. Summary of effects of test antimicrobial agents.

| Cells | Sodium hypochlorite | Chlorhexidine |
|---|---|---|
| *S. mutans* planktonic | MIC = 0.1%<br>MBC = 0.1%<br>Killed by 20-second exposure to 0.1% (turbidity $OD_{600}$ = 0.00). | MIC = 0.0025%<br>MBC = 0.005% |
| *L. casei* planktonic | MIC = 0.125%<br>MBC = 0.125% | Not determined |
| *S. mutans* hydroxyapatite biofilm | Killed by 20-second exposure to 0.2% ($OD_{600}$= 0.00).<br>No effect of 20-second exposure to < 0.2% (turbidity $OD_{600}$=0.834). | Killed by 20-second exposure to 0.01% ($OD_{600}$= 0.00).<br>No effect of 20-second exposure to < 0.01% (turbidity $OD_{600}$=0.955). |
| *S. mutans* glass biofilm | Killed by 20-second exposure to 0.5% ($OD_{600}$ = 0.00).<br>No effect of 20-second exposure to < 0.5% (turbidity $OD_{600}$=0.687). | Not determined |

Effect of Time of Exposure of Antimicrobial to Planktonic Cells

A 20-, 40- or 60-second exposure of 0.1% sodium hypochlorite was bactericidal to both *S. mutans* (Figure 2) and *L .casei* cells (Table 1).

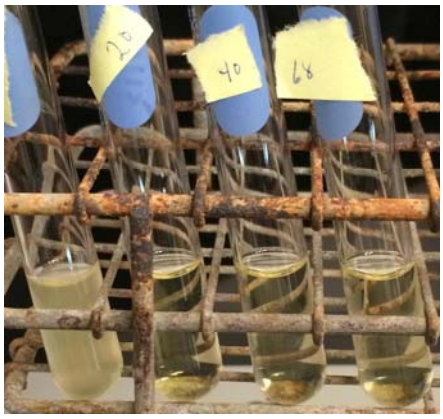


Figure 2. Effect of 20-second exposure of 0.1% sodium hypochloride on *S. mutans.* *S. mutans* cells after 12 hours growth were pelleted, suspended in 0.1% sodium hypochlorite for 0-, 20-, 40-, or 60-seconds (tubes left to right, respectively), then re-pelleted, risnsed with cold sterile saline, and inoculated into refreshed media. A 20-second exposure time was bactericidal to the *S. mutans* cells.

Effect of 20-Second Exposure of Antimicrobials on Single Species Biofilm Vitality on Hydroxyapatite or Glass

Single species biofilms of *S. mutans* on hydroxyapatite pegs were killed by a 20-second exposure to 0.2% sodium hypochlorite and 0.01% chlorhexidine, but not by more dilute solutions of either antimicrobial agent (Figure 3).

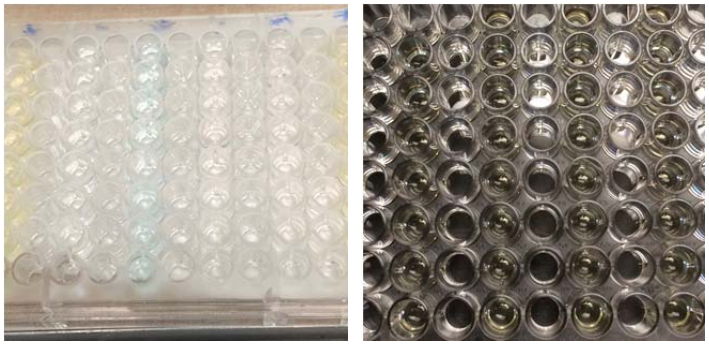

Figure 3. Effect of 20-second exposure of antimicrobials on *S. mutans* hydroxyapatite biofilms. Hydroxyapatite pegs coated with *S. mutans* biofilms were inserted into the columns of wells (above left) for 20 seconds (wells from left: untreated negative control, 0.01% chlorhexidine, 0.12% chlorhexidine, 0.2% sodium hypochlorite, 0.4% sodium hypochlorite. After rinsing, the pegs were placed into growth media for 24 hours. No growth was observed in the wells (above right), indicating that the microbial dip killed the adherent *S. mutans* cells.

Although the short exposure killed *S. mutans* cells bound to hydroxyapatite, the bacterial cells were not detached from the pegs, as evidenced by the intensity of crystal violet stain seen on treated pegs (Figure 4).

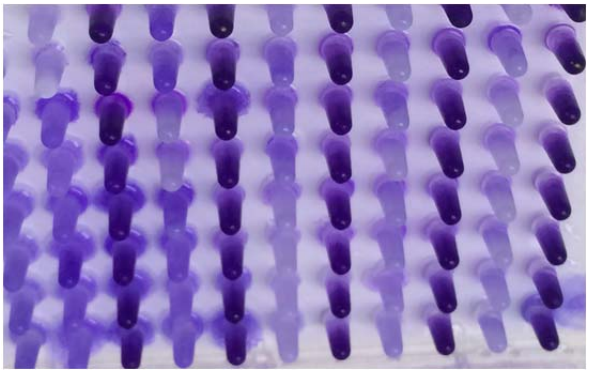

Figure 4. Staining of *S. mutans* mono-species biofilm after immersion in antimicrobials for 20-seconds. Hydroxyapatite pegs were immersed in wells

and *S. mutans* was allowed to grow and adhere for 24 hours. All pegs were rinsed and the immersed in antimicrobials for 20 seconds (treated pegs from left: untreated negative control, 0.01% chlorhexidine, 0.12% chlorhexidine, 0.02% sodium hypochlorite, 0.04% sodium hypochlorite). After another rinse, the pegs were stained with crystal violet. Absorbance values for eluents from the pegs were as follows: untreated negative control = 0.00; 0.01% chlorhexidine = 0.647; 0.12% chlorhexidine = 0.688; 0.1% sodium hypochlorite = 0.624; 0.2% sodium hypochlorite = 0.639; untreated positive control = 0.692).

A slightly higher concentration (0.5%) of sodium hypochlorite was required to kill *S. mutans* bound to glass (Table 1).

# CHAPTER 4

# DISCUSSION

Control of dental plaque biofilms is a major objective of dental professionals and critical to the maintenance of oral health. Dentists strive to accomplish such control by encouraging regular dental visits by patients for professional prophylaxis and re-enforcement of daily oral hygiene procedures. Mechanical removal of tooth biofilms can be accomplished by proper tooth brushing, and use of interdental devices such as floss or interproximal brushes. In addition, plaque removal can be aided by rinsing with oral mouth rinses (Fine 1988).

As a mouth rinse, dilute sodium hypochlorite (0.25%) has been shown to be clinically effective reducing dental plaque and gingival inflammation (Galvan et al. 2014). To date, no in vivo studies have been reported on the efficacy of using diluted bleach for preventing or arresting dental caries. A first step towards such an approach is to study in vitro the effect of sodium hypochlorite on bacteria associated with dental caries, including the effect of short term exposure to this antimicrobial on both planktonic and attached cells. The present study confirms previous in vitro investigations in regard to MIC and MBC values for sodium hypochlorite and chlorhexidine. For example, the finding for the MIC for sodium hypochlorite against *S. mutans* (MIC = 0.1%) is similar to that found by Heling et al. (2001) of 0.3%. Similarly, the MIC value for chlorhexidine against *S. mutans* (0.00125%) in the present study is close to the MIC value (0.0025%) reported by Evans et al. (2015). Minor differences in MIC and MBC values between studies may be expected due to methodological differences in strains of bacteria evaluated, concentrations of cells used in assays, and other experimental conditions.

Further, bacteria of the same species may not exhibit the same phenotype (Rogers 1977). However, the overall MIC and MBC values appear to be similar from study to study.

A unique aspect of the present study was to assess the effect of time-of-exposure of MBC concentrations of the test antimicrobials on both planktonic cells of *S. mutans* and cells of the organism attached to hydroxyapatite. This was done to mimic the time period of rinse exposure in the oral cavity that likely occurs in vivo. The data indicate that a 20-second rinse of 0.1% sodium hypochlorite was bactericidal to planktonic cells, with slightly higher concentrations lethal to cells attached to hydroxyapatite (0.2%) or to glass (0.4%). A similar pattern was observed with chlorhexidine, such that a 20-second exposure to the MBC concentration (0.005%) was lethal to planktonic *S. mutans* cells, while slightly higher concentrations were needed during a 20-second exposure to be effective against cells attached to hydroxyapatite (0.01%), or to glass (0.05%). The finding that both antimicrobials were lethal to planktonic *S. mutans* cells is significant because free-floating microorganisms in saliva are a source for re-colonization of cleaned tooth surfaces.

The present study data also indicated that a 20-second exposure of either test antimicrobial agent did not detach cells from hydroxyapatite pegs or glass. This lends credence to the in vivo practice of combining antiseptic mouth rinse usage with mechanical debridement procedures to rid teeth of attached biofilms, whether they are composed of vital or non-vital microorganisms. It is interesting that a short burst of the oxidizing agent sodium hypochlorite has penetrating power into a mono-species biofilm of *S. mutans* formed over 24 hours in vitro, yet does not detach the cells from hard, non-shedding surface. This finding agrees with the recent report of Juczyk et al. (2016), in

which the authors show that while chlorhexidine but not sodium hypochlorite prevents initial biofilm formation, sodium hypochlorite was able to kill bacteria in established biofilms and disrupt biofilm matrix, especially for older (4-day) biofilms. One explanation these findings may be that the small molecular size of sodium hypochlorite may penetrate between bacterial cells and affect bacterial cell proteins.

Dentists recognize the difficulty that patients have in maintaining good levels of plaque control with tooth brushing alone. As a result, added therapeutic value may be found for regular use of an antimicrobial rinse that is both effective against dental plaque biofilms and inexpensive in cost. As the dental caries process is site-specific in its location on tooth surfaces, rinses may access tooth sites that are poorly impacted by mechanical plaque removal procedures. Additionally, the therapeutic dose of an antimicrobial can be altered to gain more potency over brief in vivo oral exposure time periods. Since the etiology of dental caries is largely bacterial in nature, it is a disease that can be managed using principles employed in long-term chronic infections. Thus, antimicrobial mouth rinses may be used to interrupt the chain of infection of mutans streptococci colonization of tooth surfaces.

Dilute sodium hypochlorite offers some advantages over other mouth rinse formulations in that it is not only bactericidal, but offers a high pH (11.0). It is known that dental biofilms are stable in composition until there is a shift to low pH (Marsh 2006). The recovery from low pH usually is attributed to the buffering effects of saliva, but the marked alkalinity of sodium hypochlorite provides a rapid burst at a high pH. The mechanism of action of sodium hypochlorite, based on its high pH, causes perforation of the phospholipid cytoplasmic membrane of bacteria and inhibits metabolic

enzymes (Estrela 2002). Another advantage of sodium hypochlorite is its over-the-counter availability and affordability compared to other commercial mouth rinses. An appropriate dilution of regular household bleach for use as an oral rinse is made by adding a teaspoon of to 1 cup (8 ounces) of drinking water (Rich & Slots 2015). At this dilution, household bleach is cheap, safe, effective, and easy to use. This additional home care technique may help reduce caries by reducing cariogenic microbial species like *S. mutans*, and alkalize the oral cavity. Thus, rinsing with diluted sodium hypochlorite may be particularly applicable to low-income households and underprivileged population groups (Rich & Slots 2015). It would appear from the in vitro data here reported, that diluted sodium hypochlorite possesses sufficient antibacterial potential warranting clinical studies related to its potential to prevent or arrest dental caries.

# CHAPTER 5

# CONCLUSIONS

Values for inhibition of growth of planktonic cells were as follows: sodium hypochlorite (MIC = 0.1%, MBC = 0.1%), chlorhexidine (MIC = 0.0015%, MBC = 0.003%). A 20-second exposure to either solution at their MBC inhibited growth of planktonic cells in refreshed media. Cells adherent to glass or hydroxyapatite pegs were growth-inhibited, but not detached, in refreshed media by a 20-second exposure to 0.2% and 0.4% sodium hypochlorite, and to 0.01% and 0.12 % chlorhexidine, but not to more dilute solutions. Thus, for this in vitro study, it was found that chlorhexidine is more potent than sodium hypochlorite against the test microorganisms, but dilute solutions of both antimicrobial agents inhibited growth of *S. mutans* mono-species biofilm cells without inducing detachment of the cells.

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