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The Antibacterial Effects of Silver Nitrate on Oral Biofilm

by

Monika Alcorn

A thesis

submitted in partial fulfillment

of the requirements for the degree of

Master of Science in the Department of Dental Hygiene

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Committee Approval

To the Graduate Faculty:

The members of the committee appointed to examine the thesis of MONICA ALCORN find it satisfactory and recommend that it be accepted.

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February 6, 2014

Monika Alcorn
Stop 8048

RE: Your application dated 1/20/2014 regarding study number 4035: Antibacterial Effects of Silver Nitrate

Dear Ms. Alcorn:

Thank you for your response to requests from a prior review of your application for the new study listed above. Your study is eligible for expedited review under FDA and DHHS (OHRP) designation.

This is to confirm that your application is now fully approved. The protocol is approved through 2/6/2015.

You are granted permission to conduct your study as most recently described effective immediately. The study is subject to continuing review on or before 2/6/2015, unless closed before that date.

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Sincerely,

Ralph Baergen, PhD, MPH, CIP
Human Subjects Chair

Dedication

I would like dedicate this study to my mother, Ursula Steuer.

Acknowledgments

The authors would like to thank Dr. Steve Duffin and Dr. LiuningYu for their encouragement, mentorship, and guidance. Thank you to Marcus Duffin for his time and effort in creating the blinded ampules for the study, and also we would like to thank Dr. Martina Ralle and Megan Duffy of OHSU's Biochemistry and Molecular Biology Lab for their mass spectrometry analysis of the salivary samples.

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ABBREVIATIONS

GI	Gingival Index
MQHPI	Modified Quigley Hein Plaque Index
OHO	Oral Health Outreach
OHSU	Oregon Health & Science University
PI	Principal Investigator

Abstract

This double-blinded, randomized, study was designed to determine if a onetime application of silver nitrate decreased plaque accumulation and gingival inflammation in volunteers during a two-week period in which no oral hygiene was performed. Thirty volunteers, assigned to either a control (saline) or experimental (silver nitrate) group, received the control or experimental application at baseline. Participants refrained from oral hygiene procedures during the two-week study period. Gingival inflammation and plaque accumulation were assessed using the Gingival Index (GI) and Modified Quigley Hein Plaque Index (MQHPI) at baseline, week one, and week two. Data were analyzed with repeated measures ANOVA. Salivary samples were collected and analyzed via mass spectrometry to quantify the presence of silver ions. Results suggest that silver nitrate may have the ability to prevent a worsening of gingival inflammation. Additional studies are needed to determine the effect of silver nitrate on periodontal disease parameters and specific bacterial species.

CHAPTER 1

Introduction

Pathogenic bacteria have played an active role in causing disease and death in humans, indiscriminately harming all levels of society, resulting in plagues and pandemic illness (Donlan & Costerton, 2002; Health Media Lab, 2004; Stenseth et al., 2008). In spite of a progressive increase in lifespan, humans have always battled, and will continue to wage war on these smallest of enemies. Long before the advent of modern medicine and science, humans had few weapons at their disposal to combat disease-causing bacteria. However, thousands of years ago it was observed that silver provided a means to keep potable water safe from contamination. Through many centuries of empirical surveillance people began to exploit silver's antibacterial properties (Russell & Hugo, 1994). In recent human history, and with the creation of antibiotics, the medicinal value of silver as an antimicrobial has waned.

The discovery and use of antibiotics within the last century has revolutionized successful treatment of disease caused by pathogenic bacteria. Only recently it has been demonstrated that bacteria have adapted and become increasingly resistant to these wonder drugs (Rai, Deshmukh, Ingle, & Gade, 2012). As a result, researchers are

showing a renewed interest in the antimicrobial properties of silver (Chopra, 2007; Maillard & Denyer, 2006). Modern science has since provided a better understanding of silver's potential as an antimicrobial, as well as its mode of action. Silver may be an old weapon in the war on pathogens, but it is still an important one.

Problem Statement

Humans experience a myriad of disease processes, from the acute to the chronic. While the etiologies of these ailments may vary immensely and are not exclusively caused by pathogenic bacteria, bacteria are still a frequent causative agent in acute, chronic, and opportunistic infections (Socransky & Haffajee, 2002; Wilson, 1996). Whenever disease-causing bacteria are permitted to gain a foot-hold in a susceptible host, a microscopic battle ensues. When that foot-hold takes the form of a biofilm, the bacteria gain a substantial advantage for survival. Two well-known and wide-spread diseases that result from biofilm and pathogenic changes in the oral environment are dental caries and periodontal disease (Belstrom et al., 2014).

Bacteria thrive in a stable and protected environment, such as in a biofilm. Oral biofilm, commonly referred to as dental plaque, can be quite complex in structure and harbor many microbes of varying pathogenicity. As the biofilm ages, thickens, and moves subgingivally, the existing species become more diverse, more pathogenic, and increasingly anaerobic. Therefore, more studies which specifically address the susceptibility of pathogens residing in multispecies biofilms are needed (ten Cate, 2006; Wilson, 1996).

Although these pathogens cause oral diseases, such as dental caries and periodontal disease, studies have shown that their impact can go beyond that of the oral cavity, going on to affect the overall systemic health of patients if the disease process is not halted (Armitage, 2010). The infective process is comprised of six components: a pathogen(s), a reservoir (such as biofilm), a means of leaving the reservoir, a means of transmission, a portal to enter a host or other organ systems, and a susceptible host (Infection Control for Nursing Students, n.d.). If any one of these six components is removed, the disease process is effectively halted.

In the case of dental caries and periodontal disease the most direct approach has been to remove the reservoir of pathogens, namely the biofilm. The removal of biofilm requires its physical disruption via a mechanical approach such as debridement. Adjunctive use of antibiotics is frequently utilized to further reduce the subgingival populations of pathogenic bacteria. Unfortunately, there is evidence of a substantial increase of antibiotic resistant oral pathogens, making antibiotics ineffective as adjunctive therapy (Spacciapoli, Buxton, Rothstein, & Friden, 2001). As a result, the scientific and medical community is once again interested in exploring the antibacterial benefits of silver as a means to combat oral disease.

Establishing efficacy, minimal concentration levels, delivery, and safety are all critical questions that need to be addressed before widespread use of silver can be implemented into routine, clinical application. This requires more controlled, *in vivo* studies (Wilson, 1996) that reveal the effect of silver in reducing oral plaque and restoring a healthy bacterial composition to the oral cavity. There have been some *in*

vitro and *in vivo* studies that have demonstrated the efficacy of silver in arresting active caries as well as preventing new carious lesions (Rosenblatt, Stamford, & Niederman, 2009; Tan, 2006; Tan, Lo, Dyson, Luo, & Corbet, 2010) . Silver nitrate ($AgNO_3$) has historically been used to arrest active caries and is currently used on patients in Oregon.

Purpose

The purpose of this study was to determine if the silver ions in silver nitrate, when applied *in vivo*, had the ability to decrease oral biofilm accumulation and gingival inflammation in healthy volunteers during a two week period in which no oral hygiene homecare was performed.

Significance of the Study

All humans develop oral biofilm. It begins to form moments after it is removed and will continue to accumulate, diversify, and become increasingly complex until it is again disrupted. While oral biofilm is a universal human condition, so too are the dental caries and periodontal disease which result from this bacterial community. There are the additional increased risks for abscesses, endodontic infections, and implantitis (Allaker, 2010). Left untreated, these infections can progress from a localized condition to one that has systemic consequences. Conditions such as diabetes, pneumonia, stroke, and heart disease have all been associated with periodontal disease (Dewhirst et al., 2010; Louhelainen et al., 2014). This association does not come as a surprise since the oral cavity is the portal to the rest of the body.

The respiratory and digestive system must first be accessed via the mouth and pharynx, while the circulatory system is easily accessed by the highly vascular nature of

the mouth (Dewhirst et al., 2010). Gingivitis and periodontal disease cause ulcerations of the epithelial tissues and within the sulcus. These wounds allow oral pathogens to gain entry into the blood supply and deeper connective tissues (Armingohar, Jorgensen, Kristoffersen, Abersha-Belay, & Olsen, 2014; Nibali, Henderson, Sadiq, & Donos, 2014). Once the epithelial barrier of the mouth is breached, bacteria, such as *Treponema denticola, Aggregatibacter actinomycetemcomitans,* and *Porphyromonas gingivalis,* can find their way into the systemic circulatory system. This scenario can promote an inflammatory response within the blood vessels, increasing the risk for atherosclerosis because of irritation to the endothelial cells. As a result, oral pathogens have been found in arterial plaque (Armingohar et al., 2014; Nibaldi et al., 2014). Research is also investigating an association of periodontal pathogens with Alzheimer's disease and rheumatoid arthritis (Nibaldi et al., 2014).

The most effective way to stop these sequelae is routine and thorough dental hygiene. This requires a knowledge base, a minimal armamentarium, and a certain degree of dedication. As is true for any disease, prevention is the most effective means to combat pathology and ill health (Maciosek, Coffield, Flottemesch, Edwards, & Solberg, 2010). However, the ease with which this process is achieved is dependent on the individual. For the young, the mentally and physically challenged, and the institutionalized elderly, this level of home care may be unachievable.

Frequently, acute infections may require the use of antibiotics, yet their use also comes with risk. The risk of an allergic reaction increases with repeated exposures as well as the possibility of the pathogens becoming resistant to the medicament.

Additionally, antibiotics may kill beneficial bacteria, upsetting the microbial balance of the digestive system (Marsh, Head, & Devine, 2014). Silver ions may have the potential of replacing antibiotic treatment and preventing their need by breaking the chain of infection.

Biofilm, once removed, must start reforming from the beginning. Repopulation begins with the early colonizers. These microbes set the stage for the later colonizers, those that are increasingly pathogenic (Allaker, 2010). Stopping the progression of dental biofilm at its inception may be an effective way to promote health, thwart the disease process, and minimize the misery of oral disease.

This study intends to investigate the ability of silver ions to fill this medical and dental need. The potential of minimizing caries and periodontitis, especially for those that are most at risk, such as vulnerable populations, is worth pursuing and investigating further. This goal is consistent with the American Dental Hygienists' Association's research goal of investigating emerging research and science that could potentially reduce the risk of oral disease in susceptible populations (ADHA, 2007).

Research Questions

The following questions guided the conduct of this study.

1) Do the antimicrobial properties of silver ions, as provided by silver nitrate, inhibit the adhesion and development of oral biofilm on the posterior teeth of healthy adults when compared to that of a control group, as determined by the Modified Quigley-Hein Plaque Index, at baseline, at one week, and two weeks?

2) Do the antimicrobial properties of silver ions, as provided by silver nitrate, inhibit gingival inflammation between the experimental and control groups, as measured by the Gingival Index, at baseline, at one week, and two weeks?

3) What are the levels of silver ions, as provided by silver nitrate, in the experimental group as measured by mass spectrometry, at baseline, at one week, and at two weeks?

Hypothesis of the Study

Accordingly, the null hypotheses (H_o) were:

1) Silver nitrate does not affect plaque formation. There is no statistical difference in the quantity of plaque between the silver nitrate experimental group and control group, as measured by the Modified Quigley Hein Index at baseline, at one week, and two weeks.

2) Silver nitrate does not inhibit gingival inflammation. There is no statistical difference in gingival inflammation between the silver nitrate experimental group and control group, as measured by the Gingival Index at baseline, one week, and two weeks.

3) Silver ions are not detectable at a statistically significant level in the oral cavity of the experimental group at one week and two weeks, as measured by the mass spectrometry of salivary samples.

Definitions

The following terms are provided with definitions to aid the reader in understanding words or phrases that may be unfamiliar in this paper (Farlex, 2013).

Operational:

Acquired pellicle: a membrane that is composed of organic substances which quickly forms over newly brushed dental surfaces; principally composed of salivary proteins. This layer provides a means of attachment for early microbial colonizers in the development of biofilm.

Adhesion: the uniting of two structures which are usually separate.

Bacteriophage: a virus that is capable of infecting and lysing bacterial cells.

Biofilm: consists of microbes adhering to a surface via the production of sticky secretions.

Coaggregation: the adherence of different bacteria in a planktonic state.

Conjugation: the process by which one bacterial cell will temporarily link to another bacterial cell for the purpose of transferring genetic information.

Gingival inflammation: a localized inflammatory response to an irritant or injury to the gingival tissues within the mouth, characterized by edema, heat, redness, pain, and loss of function.

Gingival Index: A score from zero to three is assigned to four locations around each selected tooth (buccal, lingual, mesial, and distal). These scores are (Rebelo & de Queiroz, 2011, p. 42; Tolle, 2010):

- Score 0: gingiva of normal texture and color, no bleeding;

- Score 1: mild inflammation: slight change in color and slight edema but no bleeding on probing;
- Score 2: moderate inflammation: redness, edema and glazing, bleeding on probing; and
- Score 3: severe inflammation: marked redness and edema, ulceration with tendency to spontaneous bleeding.

Juxtaposition: refers to microbes being in close proximity to one another.

Mass spectrometry (mass spectroscopy): a lab technique that vaporizes a specimen or sample into a gas for the detection and quantification of specific ions, in this case silver ions (Helmenstine, 2014).

Modified Quigley Hein Index: The MQHPI has a scoring system from zero to five. It is used to assess plaque accumulation on the buccal and lingual surfaces of all non- restored teeth, except third molars (Malmö University, n. d.). Scoring for the surfaces is as follows, (Malmö University, n. d., table 1):

- Score 0: no plaque;
- Score 1: separate flecks of plaque at the gingival margin;
- Score 2: a continuous band of plaque, measuring up to one mm, at the cervical, gingival margin;
- Score 3: a continuous band of plaque wider than one mm but less than one-third of the tooth's crown;

- Score 4: plaque covers at least one-third but less than two-thirds of the tooth's crown; and
- Score 5: plaque covers two-thirds or more of the tooth's crown.

Nanoparticles: particles that range in size from 1 to 100 nanometers, at least in one of their dimensions.

Planktonic: bacteria that are free floating in the oral cavity and not attached to any biofilm.

Plaque accumulation: the build-up of an adhering layer of bacteria, food, and salivary products on dental surfaces that occurs between oral hygiene procedures.

Phenotype: refers to the visible traits of a microbe or organism that are influenced by the local environment and expressed genes.

Plasmid: foreign DNA that is circular in form, separate and distinct from the primary DNA of the bacterial cell. Plasmids contain information regarding antibiotic resistance and are transferrable between bacterial cells.

Posterior teeth: the premolars (8 total, 2 in each quadrant) and molars (12 total, 3 in each quadrant) in an adult dentition.

Quorum sensing: the ability of bacteria residing in a biofilm to communicate with each other via molecules and chemicals that increases their ability to coordinate behavior and survive as a community.

Silver nitrate: ($AgNO_3$), a compound that has been used as a local antiseptic and antimicrobial.

Transduction: the process by which a bacterial cell can transfer genetics from itself to another bacterial cell via a plasmid or bacteriophage.

Transformation: the transformation of a cell after it has received DNA from another bacterial cell or virus.

Conceptual:

Dental caries: the demineralization of teeth that results in tooth decay, loss of minerals from the enamel and dentin of teeth.

Implantitis: inflammation of the hard and soft tissues surrounding a dental implant.

In vitro: pertaining to an artificial environment, such as in a lab setting.

In vivo: within a living organism or body.

Periodontal disease: an inflammatory disease which causes destruction of the periodontium (alveolar bone, cementum, periodontal ligament, and gingiva), primarily of bacterial etiology, may be acute, chronic, generalized, or localized.

*Reference Dose: "*an estimate of daily exposure to the entire population (including sensitive subgroups) that is unlikely to be associated with an appreciable risk of deleterious effects during a lifetime" (Fung & Bowen, 1996, p. 120).

Oral pathogens:

The oral cavity is populated by many microbes, many of them benign. However, the following list, while not exhaustive, is comprised of pathogens associated with oral diseases (Darby & Walsh, 2010; Donlan & Costerton, 2002; Louhelainen et al., 2014; Mei, Li, Chu, Lo & Samaranayake, 2013; Perez-Chaparro et al., 2014; Quirynen, Teughels & van Steenberghe, 2003; Tanner et al., 2011; Ventura et al., 2009):

Actinomycetes gerensceriae,

Actinomycetes naeslundii,

Aggregatibacter actinomycetemcomitans,

Bifidobacterium dentium,

Campylobacter gracilis,

Campylobacter rectus,

Campylobacter showae,

Dialister pneumosintes,

Eubacterium nodatum,

Fusobacterium nucleatum,

Fusobacterium periodonticum,

Fusobacterium polymorphum,

Lactobacilli acidophilus,

Lactobacilli rhamnosus,

Parvimonas micra,

Peptostreptococcus micros,

Porphyromonas gingivalis,

Prevotella intermedia,

Prevotella nigrescens,

Scardovia wiggsiae,

Staphylococcus aureus,

Staphylococcus epidermidis,

Streptococcus anginosus,

Streptococcus cristatus,

Streptococcus constellatus,

Streptococcus gordonii,

Streptococcus mitis,

Streptococcus mutans,

Streptococcus oralis,

Streptococcus sanguinis,

Streptococcus sobrinus,

Tannerella forsythia,

Treponema denticola,

Treponema socranskii, and

Veillonella parvula.

CHAPTER 2

Oral Biofilm

Oral pathogens can be likened to enemy combatants. Their presence and level of virulence can become a challenge to one's immune system and overall health. Success in battling these organisms is dependent on one's ability to understand them. Therefore, having knowledge of their weaknesses, as well as their strengths, is critical. It permits formulating a strategy that shifts the odds in favor of the host; to the detriment of the pathogens.

Scientific research has provided an increasing comprehension of the adaptability, resiliency, and interdependence of oral pathogens. A basic knowledge and appreciation of biofilms is a necessary prerequisite to understanding the goal of this study. Therefore, the central purpose, physiology, and disease implications will be discussed. The following is a review of pertinent scientific studies which will provide a foundation from which a potential therapeutic tool may be developed, one which can decrease oral disease incidence and prevalence.

The most frequently used search engine was Google Scholar. Key terms included: oral biofilm, oral plaque, oral microbes, oral pathogens, silver nitrate, silver ions, metallic ions, anti-microbial agents, and silver toxicity.

Relationship to Human Disease

The Nobel Prize winning geneticist, Joshua Lederberg, created the term microbiome "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" (Dewhirst et al., 2010, p. 5002). It is with this understanding, therefore, that the oral microbes may also be referred to as the oral microbiome and are also residents of oral biofilm. They act as a community, with collaborative, organized relationships that are mutually beneficial nutritionally, metabolically, genetically, and environmentally (Marsh, 2005). Therefore, the disease potential of a bacterial community must be considered as a whole, rather than as an assortment of individual species simply cohabitating (Jakubovics, 2010; Marsh et al., 2014).

The host benefits in several ways when the oral cavity is inhabited by benign, commensal bacteria. Pathogenic bacteria have a more difficult time becoming established when the mouth is already populated by healthy bacteria. This healthy microbiome aids the host's immune defenses while also minimizing inflammatory responses (Marsh et al., 2014). However, these benefits can be lost when the healthy bacterial community and oral environment is altered in such a way as to favor disease causing bacteria, a condition referred to as dysbiosis (Nibali et al., 2014).

The initial colonizers within oral biofilm are typically compatible with health, acting as a barrier against infection (Avila, Ojcius, & Yilmaz, 2009; Marsh, 2003). However, dysbiosis will occur if the healthy oral microbiome is disrupted; providing an opportunity for pathogenic species to become oral residents (Avila et al., 2009; Marsh, 2003; Marsh et al., 2014; Nibali et al., 2014). The bacterial profile of an unhealthy mouth is unlike that of a previously healthy mouth (Belstrom et al., 2014). These biofilm-residing pathogens are responsible for some of the most common diseases known to humans: dental caries and periodontal disease (Al-Ahmad et al., 2007; Baehni & Takeuchi, 2003; Bradshaw, Marsh, Allison, & Schilling, 1996; Donlan & Costerton, 2002; Filoche, Zhu, & Wu, 2004; Kuramitsu, He, Lux, Anderson, & Shi, 2007; Lamont et al., 2002; Trombelli & Tatakis, 2003; Wilson, 2001).

The level of complexity, formation, and virulence can vary with the individual, yet in spite of this, biofilm can contain hundreds of different species, most of which cannot be cultured in a laboratory (Belstrom et al., 2014; Diaz et al., 2006; Foster & Kolenbrander, 2004; Marsh, 2003; Marsh, 2005; Palmer, Kazmerzak, Hansen, & Kolenbrander, 2001; ten Cate, 2006; Wilson, 2001). While hundreds of different species reside in the oral cavity, nine different phyla are typically represented. Alphabetically they are: *Actinobacteria, Bacteroidetes, Deferribacteres, Firmicutes, Fusobacteria, Proteobacteria,* and *Spirochaetes,* as well as two other phyla that could not be cultivated (Zijing et al., 2010). Another study listed the following microbes as being the most commonly found in a healthy mouth, alphabetically, they are: *Actinomyces, Capnocytophaga, Eikenella, Eubacteria, Fusobacterium, Haemophilis, Lactobacterium, Leptotrichia, Nisseria, Peptostreptococcus, Porphromonas, Prevotella,*

Propionibacterium, *Staphylococcus*, *Streptococcus*, *Treponema*, and *Veillonella* (Avila et al., 2009).

The oral cavity forms a natural entry point to the body. The digestive and respiratory systems are accessed by the oral cavity, and because of its highly vascular nature, the circulatory system can be breached as well. Disease-causing microbes are capable of more than just dental caries and periodontal disease. They are also responsible for endodontic infections, abscesses, implantitis, alveolar osteitis (Allaker, 2010; Dewhirst et al., 2010), and endocarditis (Avila et al., 2009). Periodontal pathogens have also been implicated in causing tonsillitis and sinusitis (Quirynen, Teughels, & van Steenberghe, 2003). This latter situation comes as no surprise since the bacterial composition detected in saliva is similar to the bacteria found on the tongue, oropharynx, and tonsils (Belstrom et al., 2014). The tongue, in particular, is capable of harboring a large population of bacteria, especially those that are pathogenic and cause halitosis (Suzuki, Yoshida, & Nakano, 2005).

Due to the ability of pathogens to gain access to the systemic system via the oral cavity they are also credited for contributing to the risk of cardiovascular disease, stroke, diabetes, and pneumonia (Avila et al., 2009; Dewhirst et al., 2010; Louhelainen et al., 2014; Suzuki et al., 2005, Trombelli & Tatakis, 2003). Infections that originate in the mouth may spread to the brain, lungs, and liver (Avila et al., 2009).

Dental caries. Approximately 95% of the global population has experienced the disease of dental caries (O'Connor et al., 2006). The bacterial residents in a healthy mouth are different from those found in a diseased mouth (Belstrom et al., 2014; Filoche,

Wong, & Sissons, 2010). It is the lack of pathogens that typically defines a healthy state. In the case of dental caries, the teeth are subjected to acidogenic forces which lead to demineralization and destruction of enamel and dentin (Bowden & Hamilton, 1998; O'Connor et al., 2006; Ventura et al., 2009).

Creation of an acidic oral environment, via simple carbohydrate consumption and lack of preventive care, favors those species that can thrive at low pH levels (Filoche et al., 2010; Kuramitsu et al., 2007; Ventura et al., 2009). Poor oral hygiene, lack of a fluoridated water source, cariogenic food choices, genetics, systemic illness and medications, xerostomia, malocclusion, age, ethnicity, and socioeconomic status of the host, all play a part in the host's susceptibility to disease (Belstrom et al., 2014; Filoche et al., 2010; Gilbert et al., 2014; Marsh et al., 2014).

The caries risk of Western nations is considered to be much higher than that of developing countries. This elevated risk is attributed to the frequent snacking of fermentable carbohydrates (Jakubovics & Kolenbrander, 2010; ten Cate, 2006). The metabolic waste resulting from carbohydrate consumption by bacteria, such as *S. mutans*, is in the form of lactic acid (Gilbert et al., 2014; Jakubovics & Kolenbrander, 2010; Kuramitsu et al, 2007). It is an acid of sufficient strength to dissolve tooth structure (O'Connor et al., 2006).

While the best known cariogenic species is *Streptococcus mutans*, other acidogenic microbes include *Scardovia wiggsiae, Streptococcus cristatus, Streptococcus mitis, Streptococcus anginosus, Streptococcus oralis, Streptococcus sobrinus, Actinomyces gerensceriae, Actinomyces naeslundii, Veillonella parvula, Lactobacilli,*

Bifidobacterium and *Rothia* species, and as well as *Candida albicans* (Filoche et al., 2010; Liljemark & Bloomquist, 1996; Mei, Li, et al., 2013; Tanner et al., 2011). It has been suggested that a more porous biofilm will allow for a more profound reduction in pH when these species are present than that of a more densely constructed biofilm (Liljemark & Bloomquist, 1996).

An example of an opportunistic, cariogenic, aciduric, and acidogenic bacterial species is *Bifidobacterium dentium*. While the genus of *Bifidobacterium* is typically considered to belong to the healthy flora of the gut, *B. dentium* has been found to reside in the mouth. It has been detected in the carious lesions of children and adults as well as root caries in adults (Ventura et al., 2009). *B. dentium* has accounted for as much as 8% of the cultivatable bacteria collected from active caries (Ventura et al., 2009). This species is capable of breaking down and fermenting a wide range of carbohydrates, more so than *S. mutans,* and is able to share this genetic capability with other species (Ventura et al. 2009).

Periodontal disease. While periodontal disease is a common disease in the global human community, it is aggressive periodontitis that is best known for its destructive abilities, represented in 5-20% of the general populace (Quirynen et al., 2003). This scenario requires the presence of aggressive, virulent periodontal pathogens, such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis* (Donlan & Costerton, 2002; Mombelli, 2003; Yilmaz, 2008), and *Tannerella forsythia*, plus a susceptible host that exhibits an exaggerated host response to inflammation (Dorfer, 2003; Li et al., 2004; Marsh & Bradshaw, 1997; Nibali et al., 2014; Quirynen et al., 2003; Socransky & Haffajee, 2002; Trombelli & Tatakis, 2003; Venezia & Shapira, 2003).

Treponema denticola, a virulent periodontal spirochete, is also frequently present in individuals with severe and/or refractory periodontitis (Marttila et al., 2014).

Research has shown the ability of *Porphyromonas gingivalis* to produce proteases. These enzymes alter the host's immune capability to respond (Bowden & Hamilton, 1998) to the pathogen by destroying the cell surface receptors and proteins. Damaged cells may include: neutrophils, macrophages, fibroblasts, endothelial and epithelial cells (Yilmaz, 2008). Epithelial cells play a critical role in immunity due to their ability to recognize and launch an effective defense (Yilmaz, 2008). *Prevotella intermedia* and *Porphyromonas gingivalis* have shown the ability to destroy immune cells produced by the host's humoral immune system (Bowden & Hamilton, 1998). Certain particularly destructive stains of *Aggregatibacter actinomycetemcomitans* are also capable of destroying immune cells, such as monocytes, polymorphonuclear leukocytes, endothelial cells, lymphocytes, and red blood cells, in addition to disrupting the host's cell replication process (Haubek & Johansson, 2014).

Susceptibility is often related to a genetic factor that predisposes the host to the rapid destruction and bone loss of the periodontium (Baehni & Takeuchi, 2003; Dorfer, 2003; Trombelli & Tatakis, 2003; Venezia & Shapira, 2003). Other host variables which affect disease outcome include smoking, as well as certain systemic diseases, medications, and conditions (Belstrom etal., 2014; Filoche et al., 2010; Venezia & Shapira, 2003), including hormonal changes evident during puberty, pregnancy, menopause, oral contraceptive use (Trombelli & Tatakis, 2003), and orthodontics (Avila et al., 2009). Diabetes, in particular, plays an interactive role with periodontal disease. Diabetes increases the risk, severity, and prevalence of periodontal disease (Filoche et al,

2010). As with dental caries, age, diet, ethnicity, oral hygiene capabilities, and socioeconomic status of the host also plays a part in susceptibility to disease (Filoche et al., 2010).

The disruption and removal of oral biofilm via tooth brushing and mouth rinsing is always the first step in oral disease control. Effective dental biofilm removal is essential in reducing the risk of dental caries and periodontal disease (Baehni & Takeuchi, 2003; Kelly et al., 2008; Kuramitsu et al., 2007; Liljemark & Bloomquist, 1996; Marsh & Bradshaw, 1997; Mombelli, 2003; Trombelli & Tatakis, 2003). Unfortunately, it has been concluded that most people do not practice adequate oral hygiene, often due to a lack of manual skill and motivation (Baehni & Takeuchi, 2003; Trombelli & Tatakis, 2003). The use of power tooth brushes has been shown to be much more effective in the removal of supragingival plaque when compared with the effectiveness of manual tooth brushes (Hope & Wilson, 2003a).

However, when certain periodontal pathogens, such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis,* are detected, the use of antibiotics such as amoxicillin and metronidazole could be warranted (Dorfer, 2003, Socransky & Haffajee, 2002; Trombelli & Tatakis, 2003). Also, some physiological locations are extremely difficult to reach, such as furcations, deep pockets, and host tissues that have been invaded, thereby justifying the use of antibiotics (Trombelli & Tatakis, 2003). Ideally, the antibiotic therapy should be one that narrowly targets these pathogens (Mombelli, 2003).

Some types of patients are better candidates for adjunctive antibiotic use than others. The following criteria for selection include those who are: terminally ill, elderly, mentally and/or physically challenged, immuno-compromised, those that have inadequate oral home care while undergoing periodontal treatment, those that are undergoing periodontal surgery, patients at risk for bacteremia, and those patients that have orthodontic appliances (Trombelli & Tatakis, 2003). This adjunct treatment carries the risk of increasing antibiotic resistance of the pathogens and/or increasing the possibility of an allergic reaction from the host (Quirynen et al. 2003; Socransky & Haffajee, 2002; Venezia & Shapira, 2003). However, due to the invasiveness of the above mentioned bacteria, antibiotic use could be the necessary step toward their eradication (Dorfer, 2003). Concern exists that use of antibiotics and antimicrobials, such as chlorhexidine, may not eliminate a sufficient level of pathogens, and can inadvertently contribute to the increasing virulence of the survivors, thereby making future treatment even more difficult (Filoche et al, 2010).

However, antimicrobial mouth rinses, like chlorhexidine and delmopinol, can play a valuable role in periodontal treatment by reducing pathogenic reservoirs within the oral cavity (Baehni & Takeuchi, 2003, Donlan & Costerton, 2002). The efficacy of chlorhexidine is diminished at low oral pH levels, by calcium ions, and by older, more established biofilm; therefore, its effectiveness can differ between individuals (Marsh, 2003). Unfortunately, mouth rinsing is not effective subgingivally, reaching only 0.2mm below the gingival margin (Quirynen et al., 2003; Venezia & Shapira, 2003). Irrigators, like Waterpik, can reach up to 3 mm subgingivally and when these solutions are delivered into periodontal pockets, via an ultrasonic scaler, depths up to 9 mm could be

reached, further reducing the bacterial load (Venezia & Shapira, 2003). Besides its ability to reach deeper into the diseased pocket, the use of a subgingival irrigator allows for an increased contact time between the medicament and subgingival environment, although multiple applications could be necessary (Quirynen et al., 2003). Interestingly, research has suggested that by reducing the periodontal bacterial load a shift in the microenvironment can occur, one that favors cariogenic bacteria. This could play a role in root caries etiology (Quirynen et al., 2003).

Pneumonia. The incidence of pneumonia has been correlated with inadequate oral hygiene, oral pathogens, and periodontal disease, especially in high risk groups such as the elderly and immune-compromised patients (Paju & Scannapieco, 2007). The pathogens can take the form of bacteria, viruses, or fungi that can be originally found residing in the mouths of patients. If a sufficient reservoir exists in the oral cavity there may be a migration of microbes from the biofilm, either by their shedding or sloughing, and subsequent inhalation (Avila et al., 2009). Additionally, inflammatory chemicals resulting from the periodontal inflammation, such as cytokines, can also be aspirated into the lungs. These chemicals can promote inflammation that contributes to the infective process (Paju & Scannapieco, 2007). If the health of the individual is significantly weakened, especially following influenza, there could be a progression to pneumonia. This is frequently seen in hospitals and nursing homes (Paju & Scannapieco, 2007).

It has been suggested that an inverse relationship exists between pneumonia risk and oral hygiene. As oral hygiene practices improve, the risk of pneumonia decreases because the pathogenic reservoir in oral biofilm has been reduced (Paju & Scannapieco, 2007). This information is of practical importance because influenza, followed by

pneumonia, is the leading cause of mortality amongst the institutionalized elderly (Paju & Scannapieco, 2007). Pneumonia can account for 13-48% of infections occurring in nursing home facilities and 10% of infections found in intensive care units (Paju & Scannapieco, 2007). However, reduction of oral biofilm from dental surfaces or dentures, whether by mechanical and/or chemical means, has been shown to reduce the risk of nosocomial pneumonia by 40% (Avilia et al., 2009; Paju & Scannapieco, 2007). The significance of this reduction is not surprising since one cubic millimeter of oral biofilm contains approximately 100 million bacterial cells (Allaker, 2010; Paju & Scannapieco, 2007).

Planktonic Cells

Free floating, unattached bacteria become covered with salivary substances, such as proteins and mucins. These salivary products, in turn, promote adhesion to surfaces within the mouth. The ability of different planktonic bacterial species to bind to one another in a purposeful manner is referred to as coaggregation (Donlan & Costerton, 2002; Jakubovics & Kolenbrander, 2010; Kolenbrander et al., 2004; Marsh, 2005; Rickard, Gilbert, High, Kolenbrander & Handley, 2003; Socransky & Haffajee, 2002; Whittaker, Klier, & Kolenbrander, 1996). It is not a random selection, rather bacterial species recognize their own species or other specific species that will provide a mutual advantage for survival (Jakubovics & Kolenbrander, 2010; Socransky & Haffajee, 2002). Sometimes these clusters of cells are formed from the same species or, as frequently happens, multiple species can coaggregate (Davey & O'Toole, 2000; Whittaker et al., 1996).

It has been suggested that clumps of multispecies coaggregated cells have a dramatic impact on the structure and species profile of the growing biofilm during adhesion. They also contribute to the variety of species represented in the biofilm, increasing both its diversity and complexity (Bowden & Hamilton, 1998; Davey & O'Toole, 2000; Foster & Kolenbrander, 2004; Marsh, 2003). Even obligate anaerobes were able to survive in a planktonic state if *Fusobacterium nucleatum* coaggregated with them (Bradshaw, Marsh, Watson, & Allison, 1998; Marsh & Bradshaw, 1997; Rickard et al., 2003). For example, *Streptococcus gordonii* and *A. naeslundii* have shown an ability to coaggregate with planktonic *P. gingivalis* (Demuth, Irvine, Costerton, Cook, & Lamont, 2001; Lamont et al., 2002). *S. gordonii* can also coaggregate with *Candida albicans*, to their mutual benefit (Ricker, Vickerman, & Dongari-Bagtzoglou, 2014).

A previous study has reported detecting more cariogenic and periodontal pathogens in saliva samples than in the oral biofilm (Suzuki, Yoshida, & Nakano, 2005). These coaggregated cell clusters can start in a planktonic state but can adhere to the acquired pellicle or to an existing biofilm (Liljemark & Bloomquist, 1996; Rickard et al., 2003; Socransky & Haffajee, 2002; Stoodley, Sauer, Davies, & Costerton, 2002). The ability of planktonic bacterial cells to adhere to a surface, such as an existing biofilm, is referred to as coadhesion (Jakubovics & Kolenbrander, 2010; Kolenbrander et al., 2002). The adhesins on the cell walls and membranes serve to bind to the receptors located on the surface of the acquired pellicle or still forming biofilm (Kolenbrander et al., 2002).

Planktonic cells respond to their environmental surroundings. When favorable conditions, such as nutrient availability, oxygen and pH levels, and availability of iron present themselves, planktonic bacteria will seek attachment (Davey & O'Toole, 2000;

Filoche et al., 2010; O'Toole et al., 2000). Based on these numerous variables, the species, strains, and bacterial community profile can be very different from one individual to the next (Filoche et al, 2010). In addition, what makes one individual ill might not cause a disease process in another individual (Filoche et al., 2010). Because of this variability, the most effective treatment plans would be those customized for each individual (Baehni & Takeuchi, 2003).

Stages of adherence

For bacteria to avoid being swallowed or expelled from the mouth they must attach themselves within the mouth and form mutualistic relationships with other bacterial cells. These relationships allow the bacteria to transform a hostile environment into one where they can thrive (Foster & Kolenbrander, 2004; Lamont et al., 2002; Li et al., 2004; Palmer et al., 2001; Quirynen et al., 2003; ten Cate, 2006). Surfaces within the oral cavity available for biofilm formation include the teeth, attached gingiva, oral mucosal surfaces, and tongue, as well as previously established biofilm (Quirynen et al. 2003). Each of these locations is environmentally unique from one another (Filoche et al., 2010; Quirynen et al. 2003).

Each location within the mouth can become a niche where different bacterial species can modify their surroundings to best serve their specific needs (Gilbert et al., 2014; Marsh, 2003). For example, a posterior tooth could have a different biofilm composition than an anterior tooth. Likewise, a lingual surface could have a different bacterial profile than a buccal surface (Gilbert et al., 2014). Also, a microbe that resides on the attached gingiva could become pathogenic if it relocated to a tooth surface or to

the gingival crevice (Liljemark & Bloomquist, 1996). Hence, a resident microbe may become an opportunistic pathogen (Bowden & Hamilton, 1998).

Additional layers build upon those layers already in existence (Whittaker et al., 1996). When bacteria become part of a biofilm, and are no longer in their planktonic state, they undergo a phenotypic transformation as they respond to their surroundings (Bowden & Hamilton, 1998; Davey & O'Toole, 2000; Marsh, 2003; Marsh, 2005; O'Toole et al., 2000; Yoshida, Ansai, Takehara, & Kuramitsu, 2005). This conversion of attached bacteria is attributed to a difference of gene expression compared to that of planktonic cells, thereby resulting in an altered phenotype (Dolan & Costerton, 2002; Kolenbrander et al., 2002; Loo, Corliss, & Ganeshkumar, 2000; Stoodley et al., 2002; Wecke et al., 2000; Wilson, 2001). According to Donlan and Costerton, "as many as 45 genes differ in expression between sessile cells and their planktonic counterparts" (2002, p. 168). Basic metabolic functions, such as growth rate, rate of respiration, oxygen usage, production of extracellular polymeric substances (EPS), and energy production, all change during this transformation (Donlan & Costerton, 2002; Wilson, 2001). Additionally, an increase in virulence, antibiotic resistance, and opportunistic behavior is exhibited (Loo et al., 2000).

The formation of oral biofilm follows specific phases as it develops (Filoche et al., 2010; O'Toole et al., 2000). As their conditions and surroundings evolve, so too does their response to it change. The bacterial species present exhibit different gene expression and behavior at each subsequent stage of biofilm formation (Stoodley et al., 2002). The first phase begins with the attachment of cells to a surface. Passive movement, via saliva, brings the bacteria in contact with the acquired pellicle (Marsh,

2003). The bacteria initially bond to the pellicle by weak van der Waals forces until they are able to produce polysaccharides, which provide stronger attachment (Liljemark & Bloomquist, 1996; Marsh, 2003).

Micro-colonies of bacteria may form as a result of twitching motility, enabling them to form a collaboration which strengthens their fledgling attachments to one another and to the surface while combining their efforts for extracellular polymeric substances (EPS) production (Rickard et al., 2003; Stoodley et al., 2002). EPS has been shown to absorb nutrients from the surrounding environment which further promotes the development of the bacterial colony (Davey & O'Toole, 2000; Marsh, 2005; Socransky & Haffajee, 2002). With the use of complementary enzymes, the presence of different bacterial species display an interdependent effort that allows them to work collaboratively and efficiently to breakdown and digest available food sources (Marsh, 2003). EPS also acts as a protective shield against dehydration, large swings in temperature and pH, and other environmental threats (Davey & O'Toole, 2000; Socransky & Haffajee, 2002).

The second phase quickly follows with the continued production of EPS, which consists of polysaccharides, nucleic acids, proteins, and salts (Allaker, 2010; Socransky & Haffajee, 2002). This sticky material allows cells to adhere to a surface, providing both the mechanism by which attachment is possible as well as the structural framework for the biofilm (Bowden & Hamilton, 1998; Marsh, 2003). Additionally, the greater the quantity of EPS, the more resistant the community may be to the effects of antibiotics (Davey & O'Toole, 2000; O'Toole et al., 2000). Conversely, the more the EPS is

disturbed, the greater the effect that antibiotics will have on the bacteria (O'Toole et al., 2000; Stoodley et al., 2002).

Thirdly, as the architecture of the biofilm progresses in diversity and complexity the bacterial residents multiply and new coaggregates adhere to the developing biofilm. At this stage, most of the growth within the biofilm is attributed to cell division and multiplication (Allaker, 2010; Bowden & Hamilton, 1998; Marsh, 2003). The next phase is a continuation of the biofilm development as it matures. Communication between the adhering species is a critical component of this entire process (Kolenbrander et al., 2002).

Juxtapositioned cells, those in close proximity to one another, interact via physiological signaling for each other's mutual benefit, thereby maximizing nutrients, gene transfer, and energy production while increasing pathogenicity and antibiotic resistance (Allaker, 2010; Davey & O'Toole, 2000; Marsh, 2003; Socransky & Haffajee, 2002; Stodley et al., 2002). Through their ability to communicate, the bacteria can also detect if their population is becoming overcrowded and if resources become compromised (O'Toole et al., 2000). Lastly, a mature biofilm will slough off or shed cells which potentially act as the seeds for colonization elsewhere (Filoche et al., 2010; Socransky & Haffajee, 2002; Stoodley et al., 2002).

Early Colonizers

The initial development of the acquired pellicle, comprised of an acellular layer of salivary proteins and enzymes produced following a complete removal of previous deposits, provides the foundation for bacterial adhesion (Al-Ahmad et al., 2007; Allaker, 2010; Bowden & Hamilton, 1998; Davey & O'Toole, 2000; Diaz et al., 2006; Donlan &

Costerton, 2002; Li et al., 2004; O'Toole et al., 2000; Socransky & Haffajee, 2002; Stoodley et al., 2002; ten Cate, 2006; Whittaker et al., 1996). The thickness of the acquired pellicle ranges from 0.1 to 1.0 microns (Liljemark & Bloomquist, 1996). This newly formed layer is considered to be nutritionally poor for most bacteria yet numerous proteins within the acquired pellicle serve as receptors for early pioneers during the first phase of biofilm formation (Allaker, 2010; Jakubovics & Kolenbrander, 2010; Kuramitsu, et al., 2007; Socransky & Haffajee, 2002).

Streptococci are able to utilize the acquired pellicle to adhere, reproduce in, and degrade the salivary products for nutrients (Diaz et al., 2006). Components of saliva include minerals, proteins, peptides, and glycoproteins (Kuramitsu et al., 2007). *Streptococci* have been found to bind well with amylase, which aids in the production of a newly forming biofilm (O'Toole et al., 2000). By their actions, an environment conducive for other species is created (Loo et al., 2000; Marsh, 2005; Rickard et al., 2003; ten Cate, 2006). Also, salivary nutrients are most efficiently broken down from the efforts of numerous species working together (Jakubovics, 2010).

The first bacterial species to start the biofilm are mostly Gram positive streptococci (Demuth et al., 2001; Diaz et al., 2006; Donlan & Costerton, 2002; Loo et al., 2000; Socransky & Haffajee, 2002; Yoshida et al., 2006). This process is enabled via adhesins on the cells which act as binding sites for the receptor sites located on the newly developing biofilm (Bowden & Hamilton, 1998; Li et al., 2004; Marsh, 2003; Marsh & Bradshaw, 1997; Rickard et al., 2003; Yoshida et al., 2006). Bacteria can have a variety of coaggregation adhesins, each appropriate for a different species. This variety permits

for flexible collaboration (Donlan & Costerton, 2002; Marsh, 2003; Marsh & Bradshaw, 1997; Rickard et al., 2003).

The first layer of bacteria, referred to as early colonizers, is typically comprised of facultative anaerobes, such as *Streptococci* and *Actinomyces* (Diaz et al., 2006; Donlan & Costerton, 2002; Foster & Kolenbrander, 2004; Loo et al., 2000; O'Toole et al., 2000; Suzuki et al., 2005; Whittaker et al., 1996; Wilson, 2001, Yoshida et al., 2005; Zijinge et al., 2010), representing the most abundant group of bacteria present in oral biofilm (Filoche et al., 2010; Jakubovics, 2010; Wilson, 2001). *Streptococcus* species are unique in their broad ability to coadhere and coaggregate with many different species as well as host products (Kolenbrander et al., 2002).

Early colonizers include *Streptococcus oralis, Streptococcus sanguinis, Streptococcus gordonii,* and *Streptococcus mitis* (Allaker, 2010; Bowden & Hamilton, 1998; Kreth, Zhang, Herzberg, 2008; Kuramitsu et al., 2007; Liljemark & Bloomquist, 1996) which represent roughly 80% of the bacteria in this early stage of biofilm formation; 5-10% of the bacterial population is usually *Actinomyces naeslundii,* which is also Gram positive (Avila et al., 2009; Jakubovics & Kolenbrander, 2010; Kolenbrander et al., 2002; Palmer et al., 2001; Rickard et al., 2003; Socransky & Haffajee, 2002; ten Cate, 2006; Yoshida et al., 2006). Actinomyces *naeslundii* and *Streptococcus oralis* have a symbiotic relationship in that they work together to efficiently utilize salivary products as a nutrition source (Jakubovics, 2010; Kuramitsu et al., 2007). Primary sites for colonization include the dental pits and fissures, protected crevices, rough surfaces and dental anatomical defects (Liljemark & Bloomquist, 1996; Marsh, 2003; Socransky & Haffajee, 2002).

S. sanguinis and *S. gordonii* have an antagonistic relationship with *S. mutans* (Gilbert et al., 2014). The lactic acid produced by *S. mutans* is detrimental to *S. sanguinis* and *S. gordonii;* while *S. gordonii* and *S. sanguinis* produce hydrogen peroxide as a metabolic product, which is detrimental to *S. mutans* and various other periodontal pathogens (Gilbert et al., 2014; Jakubovics & Kolenbrander, 2010; Kuramitsu et al., 2007). *S. mitis* and *S. oralis* also produce hydrogen peroxide; therefore, they too are deleterious to some periodontal pathogens (Li et al., 2004).

Studies suggest that if *S. sanguinis* is able to become established in the biofilm in sufficient numbers then disease-causing pathogens, such as *S. mutans* and *P. gingivalis,* will not be present in sufficient numbers to cause disease (Jakubovics & Kolenbrander, 2010; Kuramitsu et al., 2007). After the initial adherence of a bacterial layer, more bacteria continue to adhere, multiply, and accumulate (Wilson, 2001). Cell multiplication in the newly formed biofilm is believed to occur when the cell density reaches a certain threshold. It has been suggested that this coordinated reproduction is initiated through chemical communication (Liljemark & Bloomquist, 1996).

Coaggregation and co-adherence creates metabolic collaborations between specific plaque species, often between facultative anaerobes and obligate anaerobes (Bradshaw et al. 1998; Marsh, 2003; Marsh & Bradshaw, 1997). This is accomplished by cells recognizing and binding to one another via one cell's adhesins coupling with a different cell's receptors (Foster, Palmer, & Kolenbrander, 2003; Li et al., 2004). Adhesins can take the form of pili, fimbriae, fibrils, and other cell projections (Liljemark & Bloomquist, 1996; Socransky & Haffajee, 2002). Conversely, receptors are comprised of mucins, glycoproteins, agglutinins, and enzymes (Kolenbrander et al., 2002; Liljemark

& Bloomquist, 1996). Gram positive species commonly coaggregate with Gram negative species (Attaker, 2010). The biofilm will continue to thicken and become an increasingly complex structure with each additional layer and species (Al-Ahmad et al., 2007; Diaz et al., 2006; Dorfer, 2003; Marsh, 2005; Palmer et al., 2001; Yoshida et al., 2006).

The early colonizers create a micro-environment that is more suitable for species that join the biofilm at a later stage (Brogden, Guthmiller, & Taylor, 2005; Burne et al., 2009; Jakubovics, 2010; Kuramitsu et al., 2007; Li et al., 2004; Marsh & Bradshaw, 1997; Marsh, 2005; ten Cate, 2006). For example, *Streptococcus mutans* is not an initial colonizer and will not join the biofilm unless the conditions favor a lower pH (Jakubovics & Kolenbrander, 2010; Burne et al., 2009; Yoshida et al., 2006). Studies have suggested that when *S. mutans* is frequently subjected to low pH its phenotype will become more cariogenic, thereby contributing to an environment that favors acidophilic species (Bowden & Hamilton, 1998; Burne et al., 2009; Gilbert et al., 2014; Kuramitsu et al., 2007), such as *Bifidobacteria*, *Lactobacillus* and *Streptococcus sorbinus* (Allaker, 2010; Jakubovics & Kolenbrander, 2010), as well as *Prevotella*, *Scardovia,* and *Actinomyces* species (Gilbert et al., 2014). This is an example of microbial interference, when an environment has been modified by a microbe to the extent that the micro-environment favors certain organisms while antagonizing the presence of other species (Brogden et al., 2005; Kreth et al., 2008).

The later colonizing bacteria have more specific biological requirements and cannot adhere until the conditions are conducive to their survival (Whittaker et al., 1996; Wilson, 2001). Research conducted by Bowden and Hamilton has suggested that the

greater the microbial diversity of biofilm, the more successful it will be at withstanding fluctuations within the oral environment (1998).

Late Colonizers

Unlike other species, fusobacteria are able to aggregate with all other types of bacteria. After the foundation has been laid by the early colonizers, a critical bacterial species, *Fusobacterium nucleatum,* acts as an intermediary link for the late colonizers to adhere to the biofilm (Al-Ahmad et al., 2007; Bradshaw et al., 1998; Davey & O'Toole, 2000; Jakubovics, 2010; Lamont et al., 2002; O'Toole et al., 2000; Rickard et al., 2003; Socransky & Haffajee, 2002; Whittaker et al., 1996; Zijinge et al., 2010). *Fusobacterium nucleatum* is the most common Gram negative species found in healthy mouths and is found in even greater amounts in periodontally unhealthy mouths (Jakubovics & Kolenbrander, 2010; Kolenbrander et al., 2002). *Fusobacterium nucleatum* has shown the ability of binding with host cells, including immunoglobulin A (Avila et al., 2009).

As biofilm ages, the percentage of bacterial residents becomes increasingly Gram negative anaerobes and more resistant to antimicrobials (Bradshaw et al., 1996; Demuth et al., 2001; Diaz et al., 2006; Donlan & Costerton, 2002; Filoche et al, 2010; Loo et al., 2000; Marsh, 2005; Marsh & Bradshaw, 1997; Syed & Loesche, 1978). Their ability to not only live but thrive within an aerated environment such as the mouth is testament to their ability to develop mutualistic relationships with oxygen tolerant species (Bradshaw et al., 1998; Diaz et al., 2006; Donlan & Costerton, 2002; Filoche et al., 2004; Marsh, 2005).

Aerobes and facultative anaerobes consume sufficient oxygen within the bacterial community to allow the oxygen sensitive species to adhere (Bradshaw et al., 1996; Bradshaw et al., 1998; Kuramitsu et al., 2007; Li et al., 2004). For example, *P. gingivalis* is oxygen intolerant while *Fusobacterium nucleatum* is able to endure an environment of 20% oxygen (Kuramitsu et al., 2007). *Fusobacterium nucleatum* is able to consume the oxygen that would be toxic to *P. gingivalis* while also producing carbon dioxide as a byproduct, which in turn is utilized by *P. gingivalis* (Jakubovics, 2010; Kuramitsu et al., 2007).

By this stage, anaerobes, such as *Prevotella intermedia*, *Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola,* and *Aggregatibacter actinomycetemcomitans* are able to join the biofilm via their attachment to *Fusobacterium nucleatum* (Allaker, 2010; Foster & Kolenbrander, 2004; Kuramitsu et al., 2007; ten Cate, 2006). The relationship of *P. gingivalis* and *T. denticola,* which are regularly found together, is just one example of how different species can form symbiotic associations (Jakubovics, 2010; Marttila et al., 2014). These periodontal pathogens are well known for their destructive capabilities (Filoche et al. 2010; Kuramitsu et al., 2007; Socransky & Haffajee, 2002). For example, *Treponema denticola* produces enzymes and cytotoxins that are capable of destroying host tissues, while inactivating host defenses (Marttila et al., 2014).

With advancements in laboratory techniques, more species have been able to be cultivated and can play a greater role in the disease process than previously suspected, such as *Filifactor alocis* (Filoche et al., 2010). Other studies have suggested that viruses, such as Epstein-Barr, herpes simplex, cytomegalovirus, and papillomavirus, play a

contributing role in periodontal disease (Avila et al., 2009; Socransky & Haffajee, 2002). It is suspected that these viruses hinder the host immune response, thereby making the host more susceptible to the periodontal pathogens (Socransky & Haffajee, 2002).

It has been suggested that these pathogens work in association with each other to initiate a disease state, such as periodontitis (Kuramitsu et al., 2007; Marsh, 2005; Perez-Chaparro et al., 2014; Suzuki et al., 2005). If it were not for *Fusobacterium nucleatum* these anaerobic pathogens would be unable to successfully become residents within the biofilm (Suzuki et al., 2005). When *Fusobacterium nucleatum* was absent, other facultative anaerobes increased as a percent of the bacterial population (Bradshaw et al., 1998). Also, pH plays a critical role in determining what species will thrive. *Fusobacterium nucleatum* and *Prevotella intermedia* are able to tolerate a pH range from 5.0 to 7.0, while *P. gingivalis* cannot tolerate a pH below 6.5 (Kuramitsu et al., 2007). *Fusobacterium nucleatum* and *Prevotella intermedia* are able to produce ammonia which buffers the pH to a level that *P. gingivalis* can withstand (Kuramitsu et al., 2007).

P. gingivalis can also form partnerships with *S. gordonii* and *Actinomyces naeslundii* via adhesion (Lamont et al., 2002), as well as *Treponema denticola* (Marttila et al., 2014). *S. gordonii* is able to sustain a neutral to slightly basic pH through its metabolism of arginine, thereby protecting the acid sensitive *P. gingivalis* (Demuth et al., 2001). Both *P. gingivalis* and *S. gordonii* have been detected within dentinal tubules; a partnership that contributes to periodontitis and possibly endodontic infections (Demuth et al., 2001; Kolenbrander et al., 2002). *P. gingivalis* and *S. mutans* could never adhere to one another due to their opposing environmental needs (Demuth et al., 2001; Lamont et al., 2002).

Studies have implicated *P. gingivalis*, *Treponema denticola*, and *Aggregatibacter actinomycetemcomitans* of being capable of invading epithelial cells of the mucosal lining, gingiva and sulcus (Lamont, et al., 2002; Marttila et al., 2014; Whittaker et al., 1996). These pathogens gain a tremendous survival advantage by their ability to enter epithelial cells in the oral cavity. They are protected within the cells from the host's immune defense plus they are provided a nutrient rich environment. Therefore, they are nurtured and protected while replicating within the host's infected cells and are able to carry out their cellular destruction unabated (Whittaker et al., 1996).

Supragingival Environment

Regardless of how many different species may be present in the biofilm, they still only comprise approximately 10-50% of the total volume. The remaining portion consists of water and polysaccharides (Donlan & Costerton, 2002; Hope & Wilson, 2003b; Socransky & Haffajee, 2002; Wilson, 2001). How the biofilm develops and functions is dependent on its exposure to environmental forces, availability of nutrients, oxygen level, and bacterial profile (Burne et al., 2009; Loo et al., 2000; Davey & O'Toole, 2000; Wilson, 2001). Supragingival bacteria are nourished primarily by carbohydrates derived from the saliva and host ingested foods (Bowden & Hamilton, 1998; Jakubovics & Kolenbrander, 2010). Competition for carbohydrate resources persists between the microbial species (Moye, Zeng, & Burne, 2014).

Salivary flow has been shown to play a significant role in determining what species are favored throughout the mouth; rate and force of flow, as well as nutrient delivery, vary in different locations within the oral cavity (Jakubovics, 2010). Channels

that allow for the movement of water, waste removal, delivery of nutrients, and communication chemicals, traverse through the biofilm (Davey & O'Toole, 2000; Donlan & Costerton, 2002; Hope & Wilson, 2003b; Kolenbrander et al., 2002; Marsh, 2003; Marsh, 2005; Socransky & Haffajee, 2002; ten Cate, 2006). This level of architecture would not be possible unless there is communication between the multispecies residents of the biofilm (Jakubovics & Kolenbrander, 2010; Socransky & Haffajee, 2002; Stoodley et al., 2002). These features can vary throughout the makeup of the biofilm and are responsible for the gradient of life sustaining components listed above.

Cells living deeper within the biofilm have reduced metabolic activity when compared to cells living near the periphery. This has been attributed to a difference of available nutrients (Socransky & Haffajee, 2002; Stoodley et al., 2002). Deeper regions within the biofilm are more compact, having less oxygen and nutrients available for bacteria. With increasing biofilm age, a decrease in channels occurs, further diminishing nutrients and oxygen within the community (Socransky & Haffajee, 2002). These gradients of oxygen and nutrients affect which bacteria can survive within the biofilm, as well as their gene expression (Bradshaw et al., 1998; Marsh, 2003; Marsh, 2005; Marsh & Bradshaw, 1997; Wilson, 2001). This micro-environment favors the anaerobes (ten Cate, 2006).

A previous *in vivo* study has shown that after seven days *Fusobacterium nucleatum* is detected with increasing numbers in biofilm while the *Streptococci* species are seen as a decreasing percentage of the total amount of bacteria present (Zijinge et al., 2010). The anaerobic population will continue to increase at the expense of aerobic species (Bowden & Hamilton, 1998). If supragingival biofilm is left undisturbed for

three weeks, it will become similar in form and structure to subgingival biofilm (Zijinge et al., 2010).

Subgingival Environment

Bacterial species have distinctive shapes that are easily seen under a microscope. Approximately 450 species can be found in subgingival biofilm, many of which also reside in supragingival biofilm (Kolenbrander et al., 2002). *Prevotella* are shaped like rods, *Tannerella* species are filamentous, *Spirochetes* are shaped like cork screws, while *Synergistetes* have a large cigar shape. This last bacterial species has been detected in contact with the host's eukaryotic cells and has an appearance similar to that of polymorphonuclear leukocytes (Zijinge et al, 2010). *Synergistetes* is suspected of interacting with host cells and may play a part in the immune response. According to Zijing et al. (2010), this species has represented 3-11% of the bacterial population.

The microbes dwelling subgingivally, within a diseased periodontal pocket, are largely comprised of anaerobes, spirochetes, such as *Treponema denticola*, and motile Gram negative species (Baehni & Takeuchi, 2003; Kolenbrander et al., 2002; Marsh, 2005; Marsh & Bradshaw, 1997; Marttila et al., 2014; Suzuki et al., 2005; ten Cate, 2006; Wecke et al., 2000). These pathogens are not evident in a periodontally healthy sulcus (Donlan & Costerton, 2002). *Fusobacterium nucleatum*, a critical ally of pathogenic anaerobes, has been detected in large numbers in subgingival plaque (Foster & Kolenbrander, 2004). An inverse relationship exists between the depth of the periodontal pocket and the amount of oxygen that is available; having a profound effect on what bacterial species can thrive in this environment (Marsh & Bradshaw, 1997). Subgingival

plaque, which has been permitted to accumulate and remain largely undisturbed, is capable of causing the host to launch an inflammatory response, even without tissue invasion (Mombelli, 2003; Yilmaz, 2008).

An increase in gingival crevicular fluid production follows this inflammatory response. Gram negative periodontal pathogens, such as *P. gingivalis, Treponema* species, and *Tannerella forsythia,* can use this protein-rich fluid as a nutrient source (Bowden & Hamilton, 1998; Jakubovics & Kolenbrander, 2010; Marsh, 2003; Marsh, 2005; Marsh & Bradshaw, 1997; ten Cate, 2006; Wilson, 2001). Nutrients found in crevicular fluid include glycoproteins, blood products, and albumin (Donlan & Costerton, 2002; Kuramitsu et al., 2007). The host's inflammatory response causes more than just an increase in crevicular fluid. Chemical mediators, such as prostaglandins and cytokines, are produced by the immune system to aid in the fight against these bacterial pathogens. However, they are also capable of inadvertently causing further destruction of the periodontium (Wilson, 2001).

Successful periodontal treatment must address the removal of subgingival biofilm and the massive reduction of periodontal pathogens (Donlan & Costerton, 2002; Mombelli, 2003). Even with the dramatic reduction of periodontal pathogens following oral debridement, the microbial population will once again reach destructive levels after approximately 12 weeks (Quirynen et al. 2003). This research provides the justification of three month periodontal maintenance recall appointments. Bacterial multiplication and recolonization resulting from invaded host tissues and dentinal tubules, and/or migration of microbes from other locations within the mouth, can all contribute to a resurgence of pathogenic microbes (Quirynen et al., 2003).

Invasiveness

Certain bacterial species have shown an ability to invade host cells within the oral cavity. One explanation is that the bacteria can alter the host cell's endocytosis mechanisms (Rudney, Chen, & Sedgewick, 2005). Cells taken from the buccal mucosa of research subjects have revealed the presence of *P. gingivalis, Aggregatibacter actinomycetemcomitans*, and *Tannerella forsythia* (Donlan & Costerton, 2002; Lamont et al., 2002; Rudney, Chen, & Sedgewick, 2005; Rudney, Chen, & Zhang, 2005; Yilmaz, 2008). For those subjects that did not have active periodontal disease, the presence of these pathogens was greater in the buccal cells than that found in their supragingival plaque (Avila et al., 2009; Rudney, Chen & Sedgewick, 2005; Yilmaz, 2008).

Other invasive bacterial species detected within buccal cells include *Prevotella intermedia, Eikenella corrodens, Fusobacterium nucleatum, Treponema denticola, Campylobacter* and streptococci, among others, that fall within 11 distinct bacterial classifications (Marttila et al., 2014; Rudney, Chen, & Zhang, 2005). It has been suggested that these latter species may have gained access to the buccal cells due to their coaggregation with the former bacteria rather than by independent invasion (Rudney, Chen, & Sedgewick, 2005).

Research has suggested that upon entering the buccal epithelial cell, the bacteria are able to change the flow of calcium in the epithelial cell while also producing enzymes that breakdown proteins involved in cytokine functions, thereby mediating the host's responses. Host cell behavior is further modified in favor of the pathogens by the suppression of the host cell's programmed cell death (apoptosis). It is in the interest of

the bacteria to keep the invaded host cell alive as long as possible (Yilmaz, 2008). Additionally, the invading bacteria are able to move to neighboring host cells via filaments, all the while avoiding the attention of the host's immune system (Yilmaz, 2008). For example, *P. gingivalis* has been shown to have fimbriae that contribute to its invasiveness, as well as effecting the host's production of cytokines (Socransky & Haffajee, 2002). The result is a successful interference and repression of the host's defenses (Davey & O'Toole, 2000).

This invasiveness is not to imply that all the cells from the buccal epithelial samples were invaded. However, all samples tested contained invaded cells. These findings suggest that the invaded buccal cells may act as a reservoir of oral pathogens that can re-infect the periodontal pocket after periodontal treatment (Rudney, Chen, Sedgewick, 2005; Rudney, Chen, & Zhang, 2005). Other potential reservoirs include the remaining surfaces within the mouth, such as the gingiva, tongue, and tonsils (Liljemark & Bloomquist, 1996; Venezia & Shapira, 2003).

Climax community

Once a biofilm reaches a certain stage of growth in a stable oral environment it can develop a level of homeostasis where the consortium of residents has established balanced communication, competition, coaggregation, and nutrient usage that fit the needs of the bacterial community (Bowden & Hamilton, 1998; Davey & O'Toole, 2000; Diaz et al., 2006; Marsh, 2003; Marsh & Bradshaw, 1997; Rickard et al., 2003; ten Cate, 2006; Wilson, 2001). Usually more than 10 days is required for biofilm to reach this

level of maturity (Stoodley et al., 2002). If left undisturbed for two to three weeks, biofilms can reach thicknesses of 50-100 μm (Donlan & Costerton, 2002).

The nature of the oral environment will determine what bacterial species will have a competitive edge over other types of bacteria. Food sources, pH levels, and oxygen levels can all influence which species are promoted or antagonized (Marsh et al., 2014; Wilson, 2001). When the oral micro-environment becomes more favorable to pathogens, a shift from health to disease will occur (Filoche et al., 2010; Kuramitsu et al., 2007; Liljemark & Bloomquist, 1996; Marsh, 2003; Marsh & Bradshaw, 1997), a situation referred to as the ecological plaque hypothesis (Moye, Zeng, & Burne, 2014).

Creating an environment that is favorable to pathogens will promote disease by permitting their proliferation (Perez-Chaparro et al., 2014). However, by maintaining an oral environment that is compatible with health one can reduce the risk of pathogens gaining a foothold, effectively preventing a disease state consistent with the ecological plaque hypothesis (Filoche et al., 2010; Marsh, 2005; Wilson, 2001), where the balance of health and disease can be determined by the local environment (Perez-Chaparro et al., 2014). A healthy oral environment can be protected by establishing an oral environment that is unfavorable to pathogens while encouraging microbes compatible with health (Marsh, 2005; Marsh & Bradshaw, 1997; Ventura et al., 2009).

If periodontitis and dental caries are already established, they can be managed and treated by inhibiting the pathogens while the environment is modified to favor bacterial species compatible with health (Filoche et al., 2010; Kuramitsu et al., 2007; Marsh & Bradshaw, 1997; Ventura et al., 2009). It is possible to reinstate health by changing the

environment from one that favors disease to one that discourages it by raising the pH, eliminating sources of inflammation, or supplementing with probiotics (Kuramitsu et al., 2007). Also, a critical component of health promotion is good oral hygiene. It is by far the most effective and safest means of reducing the risk of oral disease (Avila et al., 2009; Baehni & Takeuchi, 2003; Jakubovics & Kolenbrander, 2010).

This shift of the oral environment from a diseased state to one of health requires effective biofilm removal, fluoride supplementation, dietary changes that minimize consumption of fermentable carbohydrates, antimicrobial agents, and stimulation of salivary flow (Baehni & Takeuchi, 2003; Filoche et al., 2010; Jakubovics & Kolenbrander, 2010; Marsh & Bradshaw, 1997; Socransky & Haffajee, 2002). It has been suggested that removal or prevention of the supragingival biofilm will affect the bacterial profile of the subgingival community, thereby reducing its pathogenicity (Socransky & Haffajee, 2002). This situation will encourage benign bacterial species to multiply to the level that pathogenic species can no longer compete for limited resources (Liljemark & Bloomquist, 1996).

These bacterial communities can be subjected to strong forces that contribute to determining the shape, strength, and density of the biofilm, as well as possibly disrupting their attachment (Stoodley et al., 2002; ten Cate, 2006). Mechanical and fluid forces within the oral cavity can cause a portion of the biofilm to break away from the colony (Donlan & Costerton, 2002). However, cell detachment may occur deliberately. Some studies have suggested that when biofilms experience a shortage of nutrients dissolution of EPS can occur in order to release cells from the community, thereby allowing them to seek more nutrient rich environments (Bowden & Hamilton, 1998; Marsh, 2003; O'Toole

et al., 2000; Stoodley et al., 2002). It is possible for these clusters to reattach elsewhere in the mouth, thereby recolonizing a different location (Filoche et al., 2004; Socransky & Haffajee, 2002; Stoodley et al., 2002; Wilson, 2001).

Quorum sensing

Research suggests that the lipoproteins used in binding cells to one another could play a part in their communication (Whittaker et al., 1996). The ability of bacterial cells to communicate with one another is referred to as quorum sensing, with cell density being a determining factor for gene activation and production of chemical signaling (Jakubovics & Kolenbrander, 2010; Jakubovics, 2010; Socransky & Haffajee, 2002). This communication can happen through several avenues, including exchange of genetic material (horizontal gene transfer via sex pili, transformation, transduction, transposons, and integrons), chemical interaction, and physical contact (co-adhesion and coaggregation) (Foster & Kolenbrander, 2004; Kolenbrander et al., 2002; Kuramitsu et al., 2007; Liljemark & Bloomquist, 1996, Marsh, 2005; Quirynen et al. 2003; Socransky & Haffajee, 2002). One means is by the production of an auto-inducer which diffuses to neighboring cells and can stimulate specific gene expression (Bowden & Hamilton, 1998; Loo et al., 2000; Socransky & Haffajee, 2002; Wilson, 2001). Oral biofilm has been likened to a genetic reservoir for the possible exchange and reconfiguration of bacterial genes in resident species (Marsh, 2005).

Once the establishment of the biofilm is well underway, these auto induces can influence the colony's pathogenicity, structure, utilization of life sustaining materials, and defense mechanisms against the host and antibiotics; giving them a distinct survival

advantage (Bowden & Hamilton, 1998; Kuramitsu et al., 2007; Marsh, 2005; ten Cate, 2006; Wilson, 2001; Yoshida et al., 2005).). However, the competition for survival between different microbial species continues. For example, *S. gordonii* is able to antagonize *S. mutans* by interfering with the latter's ability to engage in quorum sensing (Gilbert et al., 2014; Kuramitsu et al., 2007). Because of this level of communication, the biofilm's residents are able to determine if nutrients are becoming limited or if their waste products are becoming too abundant. This information allows them to determine if an adjustment of their metabolic rate is warranted (Marsh, 2005; ten Cate, 2006).

The exchange of genetic information between cells can alter their pathogenicity and antibiotic resistance (Bowden & Hamilton, 1998; Wilson, 2001). The host's complement activation may be blocked and phagocytosis may be rendered inept by the biofilm's defenses. Because of these abilities, host defense mechanisms are often thwarted by biofilms (Marsh, 2005; Wilson, 2001). Unfortunately, the host's attempts at eradicating the infection can serve to destroy its own tissues, as is evidenced by the progression of periodontal disease. Bacteria which are antibiotic resistant and/or inaccessible within the biofilm can persist and are responsible for chronic infections (Marsh, 2005; Yoshida et al., 2005).

Antibiotic resistance

Bacteria within biofilms have been shown to be up to 1,000 times more resistant to the effects of antibiotics than planktonic bacteria (Allaker, 2010; Avila et al., 2009; Bowden & Hamilton, 1998; Davey & O'Toole, 2000; Jakubovics & Kolenbrander, 2010; Marsh, 2003; Marsh, 2005; Quirynen, et al., 2003; Socransky & Haffajee, 2002; ten Cate,

2006). Antibiotic resistance can occur by several means. The gene expression of the bacteria may make them less susceptible to the antibiotic. Bacteria within a biofilm also have a reduced growth rate that makes them less susceptible than the faster growing planktonic varieties (Davey & O'Toole, 2000; Donlan & Costerton, 2002; Jakubovics & Kolenbrander, 2010; Liljemark & Bloomquist, 1996; Marsh, 2003; Marsh, 2005; Marsh et al., 2014; O'Toole et al., 2000; Socransky & Haffajee, 2002; ten Cate, 2006).

An antibiotic can be ineffective in penetrating the outer surface of the biofilm, can be rendered ineffective by bacterial enzymes, and/or can be expelled from the bacteria via pumps in the cell wall (Baehni & Takeuchi, 2003; Bowden & Hamilton, 1998; Davey & O'Toole, 2000; Marsh, 2003; Marsh, 2005; Mombelli, 2003; O'Toole et al., 2000; Socransky & Haffajee, 2002; ten Cate, 2006; Wilson, 2001). These abilities may be shared between different bacterial species by horizontal plasmid gene transfer during conjugation (Bowden & Hamilton, 1998; Davey & O'Toole, 2000; Donlan & Costerton, 2002; Marsh, 2003; Marsh, 2005; Quirynen et al., 2003). Horizontal gene transfer can occur easily within the mouth due to the structure and architecture of oral biofilm (Filoche et al., 2010, Marsh, 2005). Another means of acquiring new genetic information can occur as a result of viral bacteriophage's transduction (Davey & O'Toole, 2000).

Usage of sub-lethal antibiotic doses increases the ability of oral bacteria to mutate and become resistant (Marsh et al., 2014; Quirynen et al. 2003). The speed with which new species are becoming resistant is a great concern to the medical community and has far-reaching effects for global health (Quirynen et al. 2003). Another adverse risk associated with any type of antibiotic use is the possible disruption of benign and

beneficial bacteria, allowing opportunistic microorganisms to become established (Marsh et al., 2014).

Locally administered antibiotics can be at a concentration that may be toxic if they were given systemically. Unfortunately, the increased production of crevicular fluid can flush the medicine out of the pocket prematurely (Mombelli, 2003; Quirynen et al. 2003). By comparison, systemic antibiotics can be justified if the pathogens are dispersed throughout the oral cavity and have invaded tissues that cannot be reached by a local application (Socransky & Haffajee, 2002; Trombelli & Tatakis, 2003). However, due to the host's metabolic processes, they could be at an insufficient concentration by the time they reach their target (Mombelli, 2003). Allergic reactions and adverse side effects must always be considered when prescribing any type of antimicrobial. Unfortunately, *C. albicans* biofilms have also demonstrated behavior similar to that of bacterial biofilms in that they can exhibit resistance to antifungal medications (Allaker, 2010; Olsen, 2014; O'Toole et al., 2000).

Antimicrobial Effects of Silver

Silver's Mode of Action

While the actual mechanism of silver's effectiveness has not been understood until relatively recently, its ability to fight infection, reduce inflammation, and promote wound healing, was recognized early and exploited as a result (Demling & DeSanti, 2001). Scientists now know that it is the silver ions, or free radicals, that provide the antimicrobial action. These silver ions hinder a bacterial cell's ability to produce energy due to the interference of its respiratory enzyme system (Allaker, 2010; Demling &

DeSanti, 2001). Silver has an affinity to thiol groups (-SH) which are critical components of bacterial enzymes (Feng et al., 2000, Matsumura, Yoshikata, Kunisaki, & Tsuchido, 2003; Silvestry-Rodriguez, Sicairos-Ruelas, Gerba, & Bright, 2007). Silver also interferes with the bacterial cell's DNA, causing problems with replication (Allaker, 2010; Feng et al., 2000; Matsumura et al., 2003) by binding with the phosphate groups within the DNA (Clement & Jarrett, 1994). By understanding the basic mechanisms of how silver ions work, the door has been opened for more extensive research on its effectiveness on specific pathogens and how best to utilize this medicament.

The antimicrobial benefits that silver has on reducing contamination of potable water precedes the time of Alexander the Great, while the medicinal use of silver was first recorded in 750 A. D. (Maillard & Denyar, 2006). In 1700, silver nitrate was used in the treatment of venereal diseases, abscesses, and wounds until modern antibiotics largely replaced this medicament in the 1940's (Rai, Yadav, & Gade, 2009). In order to gain a better understanding of how silver nitrate worked, 19th century scientists conducted lab experiments on specific microbial species (Russell & Hugo, 1994). Silver ions (Ag+) were shown to have a huge potential for medical use because they have demonstrated a deleterious effect on a wide range of pathogenic microbes, such as viruses, including herpes simplex and HIV-1, fungi, protozoan, and of course, bacteria (Russell & Hugo, 1994). In the case of HIV-1, the nanoparticles measuring 1-10 nm are able to bind to the receptor sites on the virus, known as gp 120 glycoprotein knobs, which then inhibits the virus' ability to bind with the host's CD4 cell receptor sites (Elechiguerra et al., 2005). Also, silver damages the viral protein and the viral nucleic acids, while the antifungal

effects work in a similar manner as to what damage bacterial species undergo when exposed to silver ions (Maillard & Denyar, 2006).

Early experiments revealed silver's ability to kill *Spirogyra* (a filamentous green algae species found in fresh water), thwart the germination of *Aspergillus niger* spores (a common fungus responsible for food spoilage) (Russell & Hugo, 1994), and prevent eye infections in newborn babies (Maillard & Denyar, 2006). Scientific research has determined that silver has a formidable bactericidal effect on at least 16 different bacterial species (Sondi & Salopek-Sondi, 2004). Counted in this group is *E. coli,* a pathogen well known to the general public.

The medicinal use of silver is perhaps best known for its use in dressings applied to large wounds, especially those resulting from burns (Maillard & Denyar, 2006). *Pseudomonas aeruginosa,* a ubiquitous pathogen that can cause opportunistic infections, sepsis, and death in burn victims, can be destroyed by silver-containing medicaments (Maillard & Denyar, 2006). *Ps. aeruginosa* has been shown to be very susceptible to the damaging effects of silver when treated with silver nitrate (Liau, Read, Pugh, Furr, & Russell, 1997). Wound dressings that contained 0.5% silver nitrate were shown to reduce septicemia and mortality resulting from *Ps. aeruginosa* infections in severe burns (Clement & Jarrett, 1994). The silver ions penetrate into the *Ps. aeruginosa* cells, interact with the thiol groups, disrupt the functions of the cell membrane, and inhibit function of the cell's enzymes and cell division (Allaker, 2010; Rai, Yadav et al., 2009; Russell & Hugo, 1994).

Proteins within the bacteria can also become denatured as a result of treatment with silver particles (Sondi & Salopek-Sondi, 2004). Cell division is disrupted when the cell's DNA bases bind with the silver ions from silver nitrate. The hydrogen bonds between the nitrogenous pairs are displaced by the silver ions thereby inhibiting DNA replication (Allaker, 2010; Rai, Yadav et al., 2009; Richards, 1981; Russell & Hugo, 1994; Silvestry-Rodriguez et al., 2007). If enough base pairs interact with the silver ions, then the cell is damaged beyond repair (Russell & Hugo, 1994). These mechanisms are shown to occur in many different bacterial species when exposed to sufficient levels of silver, the higher the concentration of silver particles, the greater the destruction to the bacterial cells (Sondi & Salopek-Sondi, 2004).

Two common bacterial species used in research experiments are *Escherichia coli*, a Gram negative species, and the Gram positive species *Staphylococcus aureus.* The latter species has become a large concern because some strains are now resistant to various antibiotics. Generally referred to as methicillin-resistant *Staphylococcus aureus* (MRSA), these strains are actually resistant to the penicillin family and cephalosporin antibiotics. *S. aureus* infections are more commonly found in individuals that have had invasive procedures, tattoos, or have open wounds. In general, antibiotic resistance of bacteria has become a growing concern in the medical community. According to Rai, Deshmukh, et al. (2012), "it was proved that the silver nanoparticles are the powerful weapons against the multiple drug resistant (MDR) bacteria such as *Pseudomonas aeruginosa*, ampicillin-resistant *Escherichia coli*, erythromycin-resistant *Streptococcus pyogenes*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Staphylococcus aureus* (VRSA)" (p. 841-842). Silver ions effectively destroyed

the function of the cell's surface membrane and led to an efflux of metabolites from the cell (Cho, J.-E. Park, Osaka, & S.-G. Park, 2005; Clement & Jarrett, 1994). Lok et al. (2006) also reported a huge loss of potassium and phosphorus from *E. coli* cells during this process. The loss of phosphorus and inactivation of proteins and enzymes contributed to a depletion of ATP which further reduces the sustainability of the cell (Allaker, 2010; Rai, Yadav et al., 2009).

Most antibiotics damage specific sites on bacteria. This specificity allows the bacterial cell to adapt the susceptible area to become antibiotic-resistant. However, becoming resistant to the effects of silver is more complicated because this metal interferes with a wide range of cell functions at the same time (Rai, Yadav et al., 2009), making it more difficult for bacterial species to evolve at multiple sites at the same time. The cell is destroyed before it has time to adapt (Allaker, 2010; Pal, Tak, & Song, 2007).

The oxidation of glucose by *E. coli*, a Gram negative species, is suppressed by silver ions from an application of silver nitrate (Bragg & Rainnie, 1974). Silver also causes the development of pits in the bacterial membranes, resulting in excessive permeability. This increased permeability makes it impossible for the cell to maintain control of transport regulation through its membrane, ultimately leading to death of the cell (Lok et al., 2006; Rai, Yadav et al., 2009; Sondi & Salopek-Sondi, 2004). The production of a type of free radical, reactive oxygen species, also results from silver's destruction of an enzyme used in the cell respiration process, thereby causing further damage to the cell (Allaker, 2010; Kim et al., 2007; Matsumura et al., 2003; Pal et al., 2007). Both *E. coli* and *S. aureus* have demonstrated that their DNA becomes

condensed during treatment with silver ions, in an effort by the cells to protect this vital material (Feng et al., 2000; Rai, Yadav et al., 2009). Moreover, DNA replication cannot occur when the DNA is in condensed form (Feng et al., 2000; Rai, Yadav, et al., 2009). Studies have also suggested that *E. coli* was more susceptible to the effects of silver ions because it is Gram negative, while *S. aureus,* which is Gram positive, is less susceptible due to its more formidable cell wall construction (Allaker, 2010; Kim et al., 2007). Silver does not penetrate the cell walls of Gram positive species as easily as it does the cell walls of Gram negative species due to the difference in their cell wall and membrane construction (Feng et al., 2000; Kim et al., 2007; Rai, Yadav et al., 2009).

Nanoparticles

The search for effective antimicrobials continues. This search has included the exploration of nanoparticles. Their minute size creates a huge surface area that is able to interact with the cell walls and membranes of bacteria and other microbial pathogens. The smaller the particle size (1 to 10 nanometers) the greater the antimicrobial effect (Allaker, 2010). Nano-sized antimicrobial particles incorporated into implant coatings can serve to reduce infection after implantation of the device. Additional applications could include their incorporation into orthodontic cements and dentures (Allaker, 2010). The water channels within biofilms could also be exploited by nanoparticles in order to gain access to deeper layers of the bacterial community (Allaker, 2010).

Allaker (2010) has shown that metal ions have a positive charge while bacterial cell membranes have a negative charge. This situation makes for a natural attraction between them, allowing them to interact to the detriment of the bacteria (Allaker, 2010).

The extremely small size of nanoparticles allows them to enter cells, tissues, and organs, something that larger particle sized substances are unable to do (Allaker, 2010). The smaller the particle, the greater the surface area that can interact with pathogens, the greater the interaction, the greater the antimicrobial effect is expected to be (Allaker, 2010; Rai, Deshmukh et al., 2012; Rai, Yadav et al., 2009). This size difference explains why silver nanoparticles are more effective than silver nitrate (Lok et al., 2006).

A nanometer represents one billionth of a meter (10^{-9}) (Rai, Yadav et al., 2009); a nanoparticle of silver can range in size from 1 to 100 nm, having an estimated 10,000 to 15,000 silver atoms (Rai, Deshmukh et al., 2012). Research has shown these minute particle sizes to be very effective antimicrobials, even with antibiotic resistant strains, regardless of whether they are Gram positive or negative species (Rai, Deshmukh, et al., 2012). When the diameter of the nanoparticles employed for treatment is decreased to 5 to 20 nm, HIV-1 viral replication is suppressed (Rai, Deshmukh et al., 2012). Placement of silver nanoparticles on burn wounds has also been purported to minimize scarring and have an improved cosmetic outcome (Rai, Yadav et al., 2009).

Silver ions also have been credited with the ability to affect the host response by decreasing inflammation, aid in wound healing, and suppressing biofilm development (Rai, Deshmukh et al., 2012). The silver ions hinder the bacteria's ability to produce EPS by means of inhibiting enzymes responsible for the synthesis of glucans. Glucans are a primary component of biofilm, which provides the bacteria with the ability to adhere to surfaces (Mei, Li et al., 2014).

Research revealed that the smaller the nanoparticle the more effective it was because of greater surface area, especially if the nanoparticle size is less than 10 nm (Allaker, 2010; Morones et al., 2005). However, scientists wanted to know if the shape of the nanoparticle had an impact on effectiveness as well. Particles that were rod shaped, spherical, and triangular were compared. It was determined that effectiveness was highest in the triangular shaped nanoparticles, then the spherical, and then the rod shaped particles, in descending order (Allaker, 2010; Rai, Deshmukh, et al., 2012). The triangular shaped particles had rounded corners so they are referred to as being truncated. The truncated triangular shaped silver particles were shown to be much more effective in inhibiting *E. coli* and at substantially lower doses than what was exhibited by the spherical and rod shaped particles (Pal et al., 2007). This difference in effectiveness was attributed to the amount of surface area of the particles that could interact with the bacteria, the truncated triangles had the greatest amount of surface area of the three shapes (Pal et al., 2007).

Combination Therapy and Bacterial Resistance

When silver nanoparticles are used in conjunction with antibiotics, the results were more profound than if either had been used alone; the effectiveness of the antibiotics was enhanced with the addition of the silver (Rai, Deshmukh et al., 2012; Silvestry-Rodriguez, Sicairos-Ruelas, Gerba, & Bright, 2007), especially against Gram negative species (Rai, Yadav et al., 2009). This effect can be exploited against those pathogens that are exhibiting an increasing resistance to pharmaceuticals, whether they are antibiotics or antifungals. This situation is critically important because the synthesis

of new antibiotics is a lengthy and costly process while the ability of bacteria to become resistant can occur in a short time, comparatively. Resistance can be acquired by vertical transmission and/or horizontal transmission via transduction, transformation, or conjugation (Rai, Deshmukh et al., 2012). New generations of microbes are created in such a short time that the resistance can be spread quickly.

Bacterial resistance can manifest itself in several ways. Methods of resistance include adaptation of the cell wall/membrane to reduce penetration of the antibiotics, an enhanced ability to pump the antibiotics out of the cell before damage can occur, rendering the medicament ineffective, and/or receptor site alterations (Rai, Deshmukh et al., 2012). Resistance to silver can occur if the bacterial cell wall becomes difficult for the ions to bind with, therefore, resulting in less permeability (Russell & Hugo, 1994). Another means are efflux pumps that can actively transport the silver out of the cell (Silver, 2003). Also, a reduction of silver ions, the active therapeutic agent, to the inactive metallic form, renders the silver ineffective (Maillard, & Denyer, 2006).

While the search for an effective antimicrobial continues, one that does not illicit a resistant response from pathogens, there is concern that some pathogens could actually be building a defense against silver. Burn units first became aware of silver resistant microbes, such as *P. aeruginosa,* in 1966 (Maillard & Denyer, 2006; Silvestry-Rodriguez et al., 2007). Since 1975, there have been less than 20 reported cases of silver resistance (Chopra, 2007). The ability may have been spread from one bacterial cell to another via plasmid mediated conjugation (Chopra, 2007). Although silver resistance appears to be

relatively isolated, unlike antibiotic resistance, the situation needs to be addressed before resistance increases.

Dosing Effects and Healing

When dosing, silver concentrations need to be of a sufficient level to rapidly kill the pathogens; sub-lethal levels risk the formation of resistance (Chopra, 2007; Rai, Yadav et al., 2009). Gallant-Behm et al. (2005) recommend evaluating the efficacy of antibacterial agents via their ability to kill rapidly (log reduction tests) rather than making decisions based on zones of inhibition. By ensuring a fast-kill of the pathogens, the risk of developing silver resistance is reduced (Gallant-Behm et al., 2005). Mulligan, Wilson, and Knowles (2003) conducted a study using soluble, silver containing phosphate glasses ($Ca_{20}Na_{34}P_{45}Ag_{1,}$ $Ca_{20}Na_{30}P_{45}Ag_{5}$, $Ca_{20}Na_{27}P_{45}Ag_{10}$, and $Ca_{20}Na_{22}P_{45}Ag_{15}$) to determine efficacy against *S. sanguis.* These glasses had a predictable, linear rate of dissolution thus allowing for the controlled release of silver ions (Mulligan et al., 2003). While this was an *in vitro* study, the authors suggested that use of silver-containing phosphate glasses would be beneficial in fighting periodontal disease by their placement in diseased pockets. The authors concluded that the phosphate glass with the largest amount of silver, $Ca_{20}Na_{22}P_{45}Ag_{15,}$ was most effective in reducing levels of *S. sanguis.* Mulligan et al. determined that the higher molarity (15 mol) glass had a better ability to penetrate through the biofilm and layers of dead bacteria, thus proving to have the most effective concentration level (Mulligan et al., 2003). However, the amount of silver administered must not be so great that toxicity occurs in the patient.

Conversely, Thomas et al. (2009), conducted research that addressed the issue of wound healing after various antiseptics demonstrated the ability to repress fibroblast activity, which is critical to wound healing. They suggested that the larger the dose of an antiseptic, such as iodine, hydrogen peroxide, silver sulfadiazine, silver nitrate, and chlorhexidine, the less fibroblast activity occurred during wound healing. Thomas et al. attributed this result to the cytotoxic effects of the antiseptic. However, silver sulfadiazine, silver nitrate, and chlorhexidine had a less severe impact on fibroblasts than did iodine or hydrogen peroxide. The goal of the study was to determine what level of each antiseptic would be the least toxic to fibroblasts while still providing an antibacterial effect. Results indicated that 1 μM silver nitrate and 1 μM silver sulfadiazine actually encouraged fibroblast activity while greater concentrations of these silver agents (5 μM, 10 μM, and 20 μM) had an increasingly toxic effect on the fibroblasts. Therefore, the authors recommend using the lowest doses of the silver medicament in order to speed healing (Thomas et al., 2009).

Toxicity of Silver

In extremely small concentrations silver is not toxic to humans (Rai, Yadav et al., 2009) and has been found to be less toxic than other metals (Allaker, 2010). A toxic effect to fibroblasts can occur at 30 μg/mL (Allaker, 2010), whereas a silver overdose can occur with a daily dose of 50 to 200 ppm (The Danger of Silver Nitrate, 2004). Based on research studies conducted on mice, the World Health Organization (WHO) defines an acute exposure, oral lethal dose$_{50}$ (oral LD_{50}), of silver to occur in the range of 50 to 100 mg/kg (1996). Therefore, it has been approximated that an acute lethal dose of silver

nitrate for humans is 10 grams (WHO, 1996). Toxicity can occur as a result of industrial exposure, misuse of silver-containing medicaments, or in those patients with large burns that are treated with silver wound dressings (Hollinger, 1996).

A silver toxicity, which occurs infrequently, is called argyria (Fung & Bowen, 1996). The best known sign of argyria is skin discoloration. The absorbed silver is reduced to silver sulfide, which when coupled with stimulation of melanocytes, reacts to sunlight in a fashion similar to processed photographic film (Panyala, Pena-Mendez, & Havel, 2008). Discoloration of the skin may be permanent (Fung & Bowen, 1996). Deposits of silver may be found in the skin, eyes, liver, spleen, kidneys, adrenal glands, nervous system, and gingiva (Fung & Bowen, 1996; Hollinger, 1996; Panyala et al., 2008). Signs include a grayish blue discoloration of the skin, toxicity of lymphocytes, reduction in bone marrow cells, transient leukopenia, possible delayed wound healing, and a reduction of collagen production in synovial cells (Hollinger, 1996). The gingiva is the first site that argyria becomes visible, while the route of elimination is by feces (Fung & Bowen, 1996). Silver exits the liver via bile, which is then incorporated into the feces (WHO, 1996). Silver elimination happens very slowly due to a biological half-life that can be as long as 50 days (WHO, 1996).

The Environmental Protection Agency (EPA) recommends that the maximum daily exposure of silver should be less than 25 µg per day for infants that are five kg (11 pounds) to less than 350 µg per day for 70 kg adults (154 pounds) (Fung & Bowen, 1996). The EPA's Reference Dose for "oral silver exposure is 5 µg/kg/day, with a critical dose estimated at 14 µg/kg/day for the average person" (Fung & Bowen, 1996, p.

120). The amount of silver in drinking water that has been tested in the United States, which has not been treated with silver, has ranged from undetectable to five µg/liter (WHO, 1996). However, water that had been disinfected with silver had measurable silver levels of 50 µg/liter or greater (WHO, 1996). The majority of food, on the other hand, has trace amounts of silver, in the range of 10-100 µg/kg (WHO, 1996).

According to Fung and Bowen (1996), "the average amount of exposure required to develop argyria is reported to be 3.8 grams of elemental silver. An average fatal dose is about 10 grams although some subjects have apparently survived even after exposure to 30 grams" (p. 124). Other studies have suggested that absorption of silver from a single application has low absorption due to its superficial binding properties (White & Cutting, 2008). All medicaments containing silver should be used judiciously after weighing the risks and benefits.

Silver, Dental Caries and Periodontal Disease

While many pathogens are opportunistic and may cause infections on a systemic level, there are those pathogens that are ubiquitous and cause wounds on a much smaller scale, albeit no less problematic. *Streptococcus mutans* is the bacteria that have been implicated in causing dental caries, resulting in dental pain, loss of tooth structure, loss of oral function (eating, speaking, smiling), and reduction of self-esteem. On a global scale, 95% of humans have experienced this infectious disease (Espinosa-Cristobal et al., 2009), making dental caries the most common infectious disease on the planet.

As with many diseases, some populations experience a disproportionate incidence of this disease. Those of low income, low socioeconomic status, racial minorities, the

infirm, and rural populations carry the heaviest burden. It has been consistently found that 20% of the population has 80% of the dental caries (Duffin, 2012). Traditional, invasive restorative dentistry has failed to curtail this disease process. The secondary intervention nature of restorative dentistry attempts to repair the damage after it is well under way. Preventive measures have helped but have not come close to meeting its potential in thwarting the disease process (Espinosa-Cristobal et al., 2009). For treatment to be successful, the most common cause of dental caries, as well as periodontal disease, must address the presence of oral biofilm (Addy, 1994).

Oral biofilm, as discussed earlier, is a complex bacterial community adhering to oral structures. The older and heavier the accumulation, the more complex and pathogenic the biofilm becomes. Numerous pathogens reside in plaque; one notable resident is *Streptococcus mutans* (Mei, Chu, Lo, & Samaranayake, 2013). Oral hygiene requires the daily disturbance and removal of this biofilm, via tooth brushing and flossing, in order to reduce the risk of oral diseases, such as dental caries and periodontal disease. However, these practices could be insufficient to totally eliminate risk of caries and periodontal disease.

With the knowledge of silver's antimicrobial properties, it was a natural progression that this medicament would be applied to the field of dentistry. Silver nitrate has a long history of use in dentistry, primarily as an antibacterial agent against *S. mutans.* Early pioneers in modern dentistry, G. V. Black, W. D. Miller, and Percy Howe, used silver nitrate to arrest carious lesions (Duffin, 2012). Silver nitrate became known as Howe's solution in the early 20th century because Dr. Howe was so well known for

using this solution on a large scale for arresting uncomplicated (no pulpal involvement) dental caries (Duffin, 2012). The silver ions effectively killed the *S. mutans,* eradicating the infection and halting the destruction of the tooth (Mei, Chu et al., 2013; Silvestry-Rodriguez et al., 2007). This process is painless, non-invasive, and inexpensive.

The greatest patient complaint was an unattractive blackening of the site (Mei, Li et al, 2013). If this staining became an issue for the patient, restorative dentistry could remove the blackened material and a tooth colored restorative material can be placed. Also, the arrested caries site leaves a distinct margin of previously diseased tissue that is clearly defined. Thus, during the restorative preparation, no excess tooth structure is inadvertently removed, local anesthesia is often unnecessary, and the patient generally tolerates the procedure better (Duffin, 2012).

In recent years, thousands of Oregon Medicaid patients, primarily children, have had their dental caries arrested with 25% silver nitrate, amounting to a total of approximately 20µl per patient, per application (Duffin, 2012). These simple procedures have saved thousands of dollars in restorative care, as well as greatly reducing the pain, fear, and dread that patients experience from dental work (Duffin, 2012). A retrospective sampling of those patients that have received silver nitrate treatment for carious lesions revealed that 98% of the lesions remained arrested up to four years later. Because of these results, this medicament is well suited for use in public dental health (Duffin, 2012).

Silver's Effect on Supragingival and Subgingival Pathogens

Oral biofilm comes in two distinct forms: supragingival and subgingival. Removal of supragingival biofilm or medicinal inactivation of pathogenic bacteria found in this type of plaque is regarded as a disease prevention method. On the other hand, medicinal treatment and/or removal of plaque below the gingival margin (subgingival) are viewed as therapeutic treatment (Addy, 1994). Prevention and treatment must address the pathogens in both types of biofilm, supragingival and subgingival (Slots & Jorgensen, 2002). The suppression of biofilm by silver ions (Rai, Deshmukh et al., 2012), as discussed earlier, is a critical part of the formula for reducing oral diseases, such as periodontal disease and dental caries.

The bacterial composition of a healthy mouth is very different than that of a diseased mouth. The bacteria found in a healthy mouth are generally Gram positive, non-motile cocci that dwell supragingivally. As biofilm accumulates and ages it increases subgingivally as well. The bacterial species become increasingly motile and Gram negative. Cocci are replaced as the dominant bacterial form with flagellated, filamentous species, vibrios, and spirochetes (Zijnge et al., 2010). As this infective process increases, inflammation of the gingiva becomes evident, showing the classic signs of bleeding, reddened, and swollen tissue. If this process continues unabated, the infection can spread to the periodontium, the support apparatus of the teeth. Loss of alveolar bone is non-reversible; if it continues then the long term prognosis of the teeth is jeopardized. However, if localized regenerative surgery is performed and if it is to have any possibility

of success, the periodontal pathogens too must first be effectively reduced in number, thereby making an incurable disease manageable and controllable (Killoy, 1998).

Calculus is mineralized biofilm that cannot be brushed or flossed away. Calculus is microscopically rough and always covered with bacteria. Periodontal pathogenic species such as *Tannerella, Actinomyces, Fusobacteria, Synergistetes, B. forsythus, P. intermedia,* and *P. gingivalis, Treponema, Eubacterium,* etcetera, are among those living in established, subgingival oral biofilm (Slots & Jorgensen, 2002; Zijnge et al., 2010). As it accumulates subgingivally, periodontal ligaments and alveolar bone continue to be destroyed. The immune host response to this infection, coupled with the infection itself, can have far reaching health consequences.

The ability of a subgingival medicament to effectively reduce periodontal pathogens is dependent on three specific traits. First, the antimicrobial must be able to be placed at the bottom of the periodontal pocket. Second, the medicine must be sufficiently concentrated to have a bactericidal effect. Third, the medicament must remain in the pocket at these concentrations for a sufficient length of time to be of clinical benefit (Finkelman & Williams, 1998). When an antimicrobial is able to meet these three requirements by maintaining a therapeutic concentration for an adequate time period it is said to have zero order kinetics (Greenstein & Polson, 1998). Establishing zero order kinetics can be a challenge because the effectiveness of any delivery system is negatively affected by the increase of crevicular fluid during the inflammatory process, which can create a turnover rate of forty times per hour (Medlicott, Rathbone, Tucker, & Holborow, 1994). This last scenario can lead to first order kinetics, a rapid reduction in the

medicament's therapeutic level (Greenstein & Polson, 1998). The increase in crevicular fluid is believed to contribute to the subgingival antimicrobials being flushed out of the periodontal pockets prematurely (Medlicott et al., 1994).

Silver nitrate impregnated periodontal wafers were created for use in a 21-day, silver nitrate, *in vivo* clinical trial. The trial was designed to gauge the antimicrobial effectiveness, silver staining, subgingival retention, plaque analysis, and presence of silver in crevicular fluid, resulting from the wafers (Bromberg et al., 2000). The study's participants consisted of nine patients who had a minimum of four periodontal pockets of 5 mm or greater. Each patient had four wafers placed in periodontal pockets, one per site. Bromberg et al. (2000) endeavored to determine the silver nitrate's efficacy against the periodontal pathogens residing in the patients' pockets. They were also concerned with the pathogens developing antibiotic resistance, as has happened with some antibiotics.

The study by Bromberg et al. revealed a significant reduction of both aerobic and anaerobic bacteria. Although most wafers were not retained after three days, the silver present in the gingival crevicular fluid of the periodontal pocket continued to be bactericidal at least until the 21st day (Bromberg et al., 2000). No adverse reactions were reported other than minimal staining. Four of the nine patients experienced staining of tooth structure at the wafer deposition site; three out of the four had it occur at only one site. All sites that showed evidence of staining either resolved without intervention or were eliminated with dental polishing (Bromberg et al., 2000).

Adjunctive use of antibiotics in periodontal treatment has not always proven effective. Therefore, the search for other antimicrobial agents, such as metal ions, naturally resulted in their being tested. Spacciapoli et al. (2001) were also concerned by the increasing frequency of antibiotic resistance among pathogens and the persistent nature of periodontal microbes. This concern resulted in their constructing an *in vitro* experiment using silver nitrate, zinc chloride, and copper chloride to determine the antibacterial effect on Gram-positive streptococci and Gram-negative periodontal pathogens (Spacciapoli et al., 2001). The *in vitro* study sought to mimic the environment of a periodontal pocket by using a growth medium similar to the exudate found in diseased pockets (Spacciapoli et al., 2001). Confirmation of silver nitrate's bactericidal abilities was demonstrated against both Gram-negative aerobic and anaerobic periodontal pathogens (Spacciapoli et al., 2001). The results revealed that the silver nitrate was effective against the following periodontal pathogens: *P. gingivalis, P. intermedia, P. denticola, B. forsythus, Fusobacterium nucleatum vincentii, Campylobacter gracilis, Campylobacter rectus, Eikenella corrodens, and A. actinomycetemcomitans* (Spacciapoli et al., 2001). The zinc chloride and copper chloride antimicrobial effects, however, were considered unsuccessful on periodontal pathogens (Spacciapoli et al., 2001).

Conversely, the silver nitrate was deemed less effective against the Gram-positive Streptococci bacteria. This reduced effectiveness is not considered to be problematic, considering that a healthy mouth is populated predominantly by Gram-positive, supragingival, streptococcal species (Spacciapoli et al., 2001). Due to the effectiveness against the periodontal pathogens, the authors concluded that silver nitrate has the potential of being an effective medicament in the fight against periodontitis (Spacciapoli

et al., 2001). This conclusion was bolstered by the determination that the small size of the silver ions allowed them to penetrate biofilm (Spacciapoli et al., 2001).

Summary

The use of silver against periodontal pathogens has been the focus of several studies. While these destructive pathogens form a specific and unique group, they too, have been shown to be vulnerable to the effects of silver ions. Deep, actively diseased periodontal pockets are frequently inhabited by anaerobic bacteria in addition to aerobic pathogens residing closer to the gingival margin. Most periodontal pathogens are Gram negative, although some species are Gram positive. Placement of silver nitrate impregnated periodontal wafers subgingivally into diseased pockets of at least 5mm was effective in reducing the bacterial load when compared to baseline measurements (Bromberg et al., 2000; Straub et al., 2001). Both the aerobic and anaerobic bacteria, and particularly the Gram negative species, were susceptible to the effects of the silver ions (Bromberg et al., 2000; Straub et al., 2001). The quantity of the silver ions was non-toxic and, unlike antibiotics, would not cause a risk of an allergic reaction from the host and would not promote resistance by the bacteria (Bromberg et al., 2000). These findings are encouraging and lend themselves to further exploration.

Use of silver nitrate, already used to arrest caries in Oregon, has the potential to thwart the accumulation of biofilm responsible for the inception of oral diseases such as dental caries and periodontal disease. This suppression of biofilm production would reduce the risk for caries as well as periodontal disease in our populace. Conducting an *in vivo* study with healthy volunteers would allow researchers to analyze the effectiveness

of silver nitrate when applied supragingivally. The potential is to gauge whether this silver medicament could forestall the disease process before 5mm pockets have an opportunity to develop. It would be worth the effort to see if the effectiveness of silver nitrate could be used as a primary intervention in the fight against periodontal disease and dental caries.

CHAPTER 3

Research Design

The purpose of this study was to determine if the silver ions in silver nitrate, when applied *in vivo,* decreased oral biofilm accumulation and gingival inflammation in healthy volunteers during a two week period in which no oral hygiene homecare was performed on the posterior teeth. Everyone produces oral biofilm and is potentially at risk for developing periodontal disease and caries, given the right circumstances. Therefore, it was felt that the results of the study might be generalizable to the public. The high risk groups such as children, the elderly, and disabled were not included in the sampling. The intent of this study was to analyze any effect that silver ions could have on biofilm formation, not to further increase the risk of dental caries and periodontal disease for people that were already high risk and vulnerable. In reality, if silver ions are effective in reducing oral biofilm then the vulnerable, high risk populations would have the most to gain.

This experimental study utilized a control and a treatment group. Each study participant belonged to one group exclusively. Because the treatment consisted of silver nitrate, it was anticipated that silver ions could migrate to other areas within the oral cavity, thereby compromising a split-mouth experimental design.

The treatment group received a one-time, single drop of silver nitrate at baseline. According to the literature, one drop is considered of sufficient quantity for all four posterior sextants. A thin layer of this medicament was brushed onto the teeth of the first, third, fourth, and sixth sextants of the volunteer. When used clinically, one drop of silver nitrate is enough to treat approximately eight carious lesions (Advantage Dental, 2011; Duffin, 2012).

Conversely, the control group had a one-time, single drop of saline solution brushed onto the teeth of their first, third, fourth, and sixth sextants. One drop was sufficient to thinly coat all posterior teeth. All other components of the research were identical. The entire length of the study was two weeks in duration, with three measurements: baseline, week one, and week two. Research steps for both the experimental and control groups were as follows:

- Consent forms filled out and collected, questions and concerns addressed
- Participants randomly assigned into two groups, treatment and control, with a minimum of 15 people in each group

  Baseline, week one and two:
- Salivary samples collected from each participant, identified with a number rather than a name, and frozen.
- Gingival Index (GI) recorded, identified with a number rather than a name.

- Disclosing agent applied.
- Modified Quigley Hein Plaque Index (MQHPI) recorded, identified with a number rather than a name.
- Intra-oral photos taken of all posterior regions of the oral cavity, identified with a number rather than a name.
- After the baseline plaque index was recorded, each individual was instructed to thoroughly brush and floss prior to the placement of the silver nitrate or saline solution; this allowed all study participants to begin the study without previously acquired oral biofilm. The baseline GI and MQHPI of each participant established their unique baseline from which future comparisons were made.
- Treatment group had one drop of silver nitrate (20 μl) applied with a micro-brush to all posterior dental surfaces, and then sodium fluoride varnish was immediately applied over those surfaces. Participants were instructed not to eat or drink for one hour plus not brush or floss the posterior teeth for two weeks. Nor was the use of mouthwash permitted during the two week period. However, participants could brush and floss the anterior teeth from the mesial of #6 (upper right canine) to the mesial of #11 (upper left canine), and from the mesial of #22 (lower left canine) to the mesial of #27 (lower right canine), using only a toothbrush moistened with water and no toothpaste (see picture below). This was necessary in order to avoid interference from the antimicrobial ingredients in toothpaste, such as triclosan, and to avoid possible interaction of the toothpaste with silver ions. Tongue brushing, however, was permitted with a tooth brush or tongue scraper moistened with water (see Figure 1).

- Control group had saline solution brushed on all intact posterior dental surfaces at baseline, and then sodium fluoride varnish was immediately applied over those surfaces.
- All other study protocols were exactly the same for the control group as those set for the treatment group.

Research Setting

Participants were required to go to the office of Oral Health Outreach (OHO), in Wilsonville, Oregon for the collection of data. OHO, which is owned by Steven Duffin, DDS, provides the dental health assessment, dental hygiene care, cavity preparation and treatment, denture care, and patient education to its patients. OHO has mobile dental units and equipment that allows the dental professionals to go to the patients' place of residence, whether it is a private home or group home setting, such as an assisted living residence. Dr. Duffin also has a microbiology laboratory in the OHO office. This laboratory setting has allowed Dr. Duffin to continue his research on oral microbes, pathogens, and dental caries. Necessary equipment and armamentarium simulated a typical dental operatory.

Research Context

Patrick Braatz, the Oregon Board of Dentistry's executor director, recently confirmed that silver nitrate application is permitted by dentists in the state of Oregon, but not by auxiliary staff, such as dental hygienists or dental assistants (Bannow, 2013). Therefore, a local dentist applied the silver nitrate medicament to the treatment group participants, and saline solution to the control group. Both the silver nitrate and saline

solution were premeasured and placed in pre-numbered ampules by a microbiologist. The ampules were numbered 100 to 130. No one, except the microbiologist, knew which numbered ampule contained which fluid. This arrangement created the double blind nature of the study. Neither the dentist, principal investigator, nor the participants, knew which group they were in. It was not until the completion of the data gathering that participants were identified as to which group they had been assigned: the control or the experimental.

Research Participants

Sample Description. Recruitment was based on a convenience sample. The primary investigator approached potential participants from friends, family, acquaintances, and co-workers. Study requirements were fairly simple. Selection criteria of participants included:

- Health history form completed. Volunteers were healthy, with no health complications.
- Caries risk assessment form completed. Participants had to have low caries risk in order to participate.
- The majority of posterior teeth were present in all four quadrants, i. e. three to four teeth.
- Agree to not brush or floss the posterior teeth, nor use mouthwash for two weeks. When a piece of food became trapped interproximally the volunteer could use floss at that site only.

Exclusion criteria:

- No antibiotic use in previous three months.
- No pregnant or lactating women, as a precaution.
- Potential participants with extensive posterior composite restorations were excluded from the study.
- No orthodontia treatment at time of study.

Study participants were only healthy adults. The rationale for this decision was to protect any individuals that could have an increased risk associated with two weeks of accumulated oral biofilm. A healthy immune system is able to withstand the temporary bacterial insult while someone with a compromised immune system perhaps could not. An individual that is bedridden with impaired immunity could be at risk of bacterial aspiration, which could contribute to the risk of pneumonia. Likewise, oral pathogens could gain access to the vascular system via a diseased gingival sulcus. By only allowing for study participants that had good general and oral health, the risks of a systemic or even localized infection were minimal.

Human Subjects Protection. In addition to the participants having filled out individual consent forms (Appendix C), approval from the Human Subjects Committee (HSC), Idaho State University's Internal Review Board (IRB), was required prior to conducting the silver nitrate study. During the data collection, all information that could be used to identify an individual's identity was kept confidential. Participants were identified by a randomly assigned number. Study participants did not know if they were in the treatment or control group due to blinding. Data were entered into a computer spread sheet according to the respective identifying numbers. All participants were

informed, at the beginning and throughout the study period, that they had the option of discontinuing and withdrawing from the study without penalties.

Data Collection

The Gingival Index (GI) and Modified Quigley Hein Plaque Index (MQHPI) are both considered to be highly reliable indices that have been widely accepted in research studies. The GI has been used in many clinical trials and has been shown to have good reproducibility and sensitivity when used by trained examiners (Rebelo & de Queiroz, 2011). "The GI has gained wide acceptance as a simple, accurate, and reproducible method for evaluating gingival health or disease in epidemiological and clinical research" (Wei & Lang, 1981, p. 355). According to Marks et al. (1993), the gingival index is the most frequently used index in assessing gingival health.

The MQHPI is "widely used and recommended. It is recognized as a reliable index for measuring plaque" (Kelly et al., 2008, p.443), while Marks et al. (1993) stated that, "it is the most sensitive indicator for dental deposits" (p. 58). The GI and MQHPI was determined for each study participant and entered into the computer file. A number was assigned to each participant to ensure confidentiality. The GI measured the condition of the gingival health based on color, edema, and bleeding.

A score from zero to three was assigned to four locations around each selected tooth (buccal, lingual, mesial, and distal). These scores are (Rebelo & de Queiroz, 2011, p. 42):

- Score 0: gingiva of normal texture and color, no bleeding
- Score 1: mild inflammation: slight change in color and slight edema but no bleeding on probing

- Score 2: moderate inflammation: redness, edema and glazing, bleeding on probing
- Score 3: severe inflammation: marked redness and edema, ulceration with tendency to spontaneous bleeding

The four scores for each tooth were added and divided by four. An individual's total score was calculated by simply adding the scores for each tooth and dividing that number by the total number of teeth (Rebelo & de Queiroz, 2011). The final score reflected the range of gingival health, from being healthy to having severe inflammation. These scores are (Rebelo & de Queiroz, 2011, p. 42):

- Mild inflammation: 0.1-1.0
- Moderate inflammation: 1.1-2.0
- Severe inflammation: 2.1-3.0

The MQHPI has a scoring system from zero to five. It was used to assess plaque accumulation on the buccal and lingual surfaces, except third molars (Malmö University, n. d.). The buccal and lingual surfaces are divided into three sections, the mesial, middle, and distal. This creates a total of six areas per tooth. Scoring for the surfaces was as follows, (Malmö University, n. d., Figure 2):

- Score 0: no plaque
- Score 1: separate flecks of plaque at the gingival margin
- Score 2: a continuous band of plaque, measuring up to one mm, at the cervical, gingival margin
- Score 3: a continuous band of plaque wider than one mm but less than one-third of the tooth's crown

- Score 4: plaque covers at least one-third but less than two-thirds of the tooth's crown
- Score 5: plaque covers two-thirds or more of the tooth's crown

The MQHPI of the six surfaces per tooth (buccal mesial, middle, and distal plus lingual mesial, middle, and distal) were combined and divided by six to determine the score per tooth. The total index score was calculated by adding the scores of each tooth and dividing that number by the total number of teeth assessed (Cugini, Thompson, & Warren, 2006).

Measurements were obtained at baseline, week one, and week two. Saliva samples were analyzed via mass spectrometry to determine quantities of silver ions present in the oral cavity. Additional salivary samples were collected at each data session for the purpose of analyzing the bacterial species profile for each volunteer. This information also provided data as to what bacterial changes occurred during that two week period.

The principal investigator (PI) served as the only examiner/rater during data collection. A copy of the GI and MQHPI parameters was kept next to the PI during all data gathering sessions in order to maximize consistency. Due to the subjectivity of scoring plaque and gingival indices, photos were taken to record a visual standard by which scoring during the study could be contrasted, i.e., mild, moderate, or severe inflammation, as well as teeth with plaque scores ranging from zero to five. The intra-oral photos served to both reduce subjectivity, as well as provide documentation of clinical data. Personal identifiers were eliminated and replaced with the candidate number. Kelly et al. (2008) suggested that when MQHPI scores were determined using intra-oral photos, the scores were similar to those that were determined clinically. The

conclusion was that using intra-oral photographs "proves to be a simple and effective technique to assess the reliability of the Modified Quigley Hein Plaque Index as modified by Turesky et al., thus improving the reproducibility of epidemiological studies" (Kelly et al., 2008, p. 447).

Statistical Analysis

This double-blinded, two tailed, randomized, prospective study has a 95% confidence interval. The statistical test used was a repeated measure ANOVA. Anonymous numerical identifiers were provided to each participant so that neither the participants nor the principal investigator knew to which group they had been randomly assigned. As mentioned earlier, the silver nitrate and saline solutions were premeasured into glass ampules and randomly assigned numbers, 100 to 130. Not even the dentist who applied the medicament knew to which group each participant belonged. Using a number for each ampule thus served to mask their contents. Comparisons were made for each group as well as between the two groups; the latter providing a group membership measurement variable. An equal number of participants were randomly assigned to each group.

The independent variable was an application of one drop of silver nitrate. Participants either received it (treatment group) or they did not receive it (control group). There were three dependent variables to consider: plaque score, gingival inflammation, and salivary silver content. Each study participant had their MQHPI and GI measured three times to determine the repeated measure analysis of variance, while presence of salivary ions was determined via mass spectrometry. Presence of silver ions was reported

in descriptive terms since it was expected, but not assumed, to be negligible in the control group.

Assumptions, Limitations, Delimitations

Assumptions. All of the participants in this study use English as their primary language. Therefore, communication, both written and verbal, was not expected to be a barrier to understanding the medical history form, nor the consent form, as well as implications of the study. All participants were encouraged to ask questions throughout the entire process so it was assumed that any concerns, confusion, or questions were voiced and promptly addressed. Diet was not a controlled variable; therefore different dietary choices of the participants could have had an impact on the individuals' dental biofilm formation. However, this issue was not expected to weaken the study because the comparison in biofilm formation and gingival inflammation was also comparative for each individual in that two week period. There will always be a certain amount of variation in dental plaque formation and retention in the general population due to differences in diet, dental crowding and malocclusion, the amount of movement of each person's tongue, lips, and cheeks, and body chemistry.

Limitations. It was beyond the principal investigator's control as to what foods and liquids were consumed by participants. Some may have consumed more carbohydrates than others while some likely ate more fibrous and raw foods than the others. This variable may have impacted the amount of oral biofilm that formed and how much was removed during the mastication process. A limitation of the study was the

relatively small sample size. For this reason, these findings cannot be generalized to the broader community based on this study alone.

Other limitations of this study included the length of time participants were required to refrain from mechanical plaque removal and the use of toothpaste and mouth rinses (two weeks). Recruitment was a challenge due to this two week requirement.

Delimitations. The inclusion criteria set for this study controlled for most of the limitations previously listed. Participants were generally healthy adults that had most of their posterior teeth, limited composite restorations, were not high risk for dental caries or periodontal disease, and had not been recently prescribed antibiotics.

Summary

This chapter provided a description of the study design, sample population, data collection instruments and study time frame. The following section of this thesis document includes a manuscript entitled, "The antibacterial effects of silver nitrate on oral biofilm". The manuscript reports results, discussion and conclusions in lieu of chapters 4 and 5. This manuscript will be submitted for publication in the *Journal of Dental Hygiene*. Complete author guidelines for the *Journal of Dental Hygiene* are listed in Appendix E.

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The Antibacterial Effects of Silver Nitrate on Oral Biofilm

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Abstract

Purpose: The purpose of this study was to determine if the silver ions in silver nitrate, when applied *in vivo,* decreased oral biofilm accumulation and gingival inflammation in healthy volunteers during a two week period in which no oral hygiene homecare was performed.

Methods: This double-blinded, randomized, prospective study used thirty volunteers assigned to either a control (saline) or experimental (silver nitrate) group. A onetime, single drop of 25% silver nitrate or saline was applied to all posterior teeth and participants refrained from oral hygiene procedures during the two week period. Gingival inflammation and plaque accumulation were assessed for each participant using the Gingival Index (GI) and Modified Quigley Hein Plaque Index (MQHPI) at baseline, week one, and week two. Data were analyzed using repeated measure ANOVA. Additionally, salivary samples were collected at each data collection session and analyzed employing mass spectrometry for the purpose of quantifying the presence of silver ions.

Results: Both the control and treatment groups developed a significant amount of plaque (control group $p=<0.0001$, treatment group $p=<0.0007$). The control group also had a significant worsening of gingival inflammation ($p=0.03$), while the treatment group did not ($p=0.25$). Silver ions were not detected in any of the salivary samples.

Conclusion: Silver nitrate, when applied to intact tooth surfaces, may have the ability to prevent a worsening of gingival inflammation during a two-week cessation of oral hygiene. Additional studies are needed to determine the effect of silver nitrate on periodontal disease parameters, and on specific bacterial species.

Keywords: oral biofilm, oral plaque, oral microbes, oral pathogens, silver nitrate, silver ions, metallic ions, and anti-microbial agents.

This study supports the NDHRA priority area, **Health Promotion/Disease Prevention:** investigating emerging research and science that could potentially reduce the risk of oral disease in susceptible populations.

Introduction

Oral biofilm, commonly referred to as dental plaque, is quite complex in structure and harbors many microbes of varying pathogenicity. As the biofilm ages and moves subgingivally, the existing species become increasingly anaerobic and more pathogenic. Although these pathogens cause oral diseases, such as dental caries and periodontal disease, studies have shown that their impact can go beyond that of the oral cavity and affect the overall systemic health of patients if the disease process is not halted.[1] In the case of dental caries and periodontal disease the most direct approach has been to remove the reservoir of pathogens, namely the biofilm. The removal of biofilm requires its physical disruption via a mechanical approach such as regular tooth brushing and professional debridement. Adjunctive use of antibiotics is frequently utilized to further reduce the subgingival populations of pathogenic bacteria. Unfortunately, there is evidence of a substantial increase of antibiotic resistant oral pathogens, making antibiotics less effective as adjunctive therapy.[2] Therefore, more studies which specifically address the susceptibility of pathogens residing in multispecies biofilms to topically-applied antimicrobial agents are needed.

Silver and Silver Ions

Silver ions, within the silver nitrate solution ($AgNO_3$), have been credited with decreasing bacterial counts in oral lesions. Silver ions exert several mechanisms of antimicrobial action. They have an affinity to thiol groups (-SH), critical components of bacterial enzymes, disabling the production of energy due to interference with the cell's respiratory enzyme system.[3-7] Silver also interferes with the bacterial cell's DNA by

binding with the phosphate groups within the DNA, causing problems with replication.[3,5,6,8] Proteins within the bacteria can also become denatured as a result of treatment with silver particles.[9] Cell division is disrupted when the cell's DNA bases bind with the silver ions from silver nitrate. The hydrogen bonds between the nitrogenous pairs are displaced by the silver ions thereby inhibiting DNA replication.[3,7,10-12] These mechanisms are shown to occur in many different bacterial species when exposed to sufficient levels of silver, the higher the concentration of silver particles, the greater the destruction to the bacterial cells.[9]

While most antibiotics damage specific sites on bacteria, this specificity allows the bacterial cell to adapt the susceptible site and become antibiotic-resistant. However, bacterial resistance to the effects of silver is more complicated because the metal interferes with a wide range of cell functions at the same time, making it more difficult for bacterial species to evolve at multiple sites simultaneously.[10] Bacterial cells are destroyed before they have time to adapt.[3,13] *In vitro* and *in vivo* studies have demonstrated the efficacy of silver in arresting active dental caries as well as in preventing new carious lesions.[14-16] Silver nitrate has historically been used to arrest caries and is currently used with patients in Oregon.

Silver, Dental Caries and Periodontal Disease

While many pathogens are opportunistic and may cause infections on a systemic level, there are those pathogens that are ubiquitous and cause wounds on a much smaller scale, albeit no less problematic. *Streptococcus mutans* is the bacteria that have been implicated in causing dental caries, resulting in dental pain, loss of tooth structure, loss of

oral function (eating, speaking, smiling), and reduction of self-esteem. On a global scale, 95% of humans have experienced this infectious disease[17], making dental caries the most common infectious disease on the planet.

As with many diseases, some populations experience a disproportionate incidence of this disease. Those of low income, low socioeconomic status, racial minorities, the infirmed, and rural populations carry the heaviest burden. It has been consistently found that 20% of the population has 80% of the dental caries.[18] Traditional, invasive restorative dentistry has failed to curtail this disease process. The secondary intervention nature of restorative dentistry attempts to repair the damage after it is well under way. Preventive measures have helped but have not come close to meeting its potential in thwarting the disease process.[17] For treatment to be successful, the most common cause of dental caries, as well as periodontal disease, must address the presence of oral biofilm.[19]

The bacterial species responsible for periodontal disease are different than those that cause dental caries. Some well know periodontal pathogens include *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*[20-22], and *Tannerella* forsythia.[23-30] *Treponema denticola,* a virulent periodontal spirochete, is also frequently present in individuals with severe and/or refractory periodontitis.[31]

The effectiveness of silver ions against periodontal pathogens is unknown. Therefore, it is with this question in mind that the effectiveness of a limited application of silver nitrate was applied. This study sought to determine whether or not the silver ions in silver nitrate could decrease dental plaque accumulation, prevent gingival

inflammation, and demonstrate substantivity during a two week period where no posterior dental homecare was performed.

Methods and Materials

The Institutional Review Board (IRB) at Idaho State University approved this study, # 4035. A non-probability, convenience sample of volunteers was recruited. Volunteers were first appointed for a screening visit to determine eligibility for the study. Thirty eligible adults were enrolled for a series of three appointments over a two-week period. Inclusion and exclusion criteria were as follows:

Inclusion criteria:

- Minimum 18 years old of age, healthy, with no health complications
- Low caries risk
- Three or more posterior teeth present in all four quadrants
- Agree to not brush or floss the posterior teeth, or use mouthwash for two weeks

Exclusion criteria:

- No antibiotic use in previous three months
- No pregnant or lactating women
- Extensive posterior composite restorations (no more than two per quadrant)
- No orthodontia treatment at time of study

Data was collected three times, at one week intervals. Baseline, week one and week two data collection included:

- Salivary samples collected
- Gingival Index (GI)
- Modified Quigley Hein Plaque Index (MQHPI)
- Intra-oral photos of all posterior regions of the oral cavity

The GI measured the degree of gingival inflammation surrounding each of the posterior teeth of the study participants. Numbers, which are on a continuous scale, were assigned according to the criteria shown in Table I.[32] The MQHPI, also on a continuous scale,

measures the amount of plaque that covers the tooth, as graphically represented in Figure 2 and described in Table II.[33]

The data collection proceeded in the following order. First, a salivary sample was collected from each participant by the principal investigator (PI). All labeled and dated samples were immediately placed in a -20° C freezer, whose sole purpose was to store the samples. Then the GI was determined and recorded for each participant. The MQHPI was then determined by applying a plaque disclosing agent (GC Tri Plaque ID Gel®; GC Corporation, Tokyo, Japan) to the posterior teeth. After the recording of the MQHPI was complete intra-oral photos were then taken for future reference. The GI and MQHPI were calculated by the principal investigator. All data were entered into an Excel Spreadsheet.

Following baseline data collection, the PI instructed each individual to thoroughly brush and floss prior to application of either the treatment or control solution. Each individual was visually checked by the PI to ensure that all of the disclosing agent, and thus the plaque, had been removed prior to application of the treatment (Silver Nitrate Solution 25%, Gordon Laboratories, Philadelphia, Pennsylvania, USA) or control (saline) solutions. Following the application of either solution, 5% sodium fluoride varnish (Kolorz Clear Shield®; DMG America LLC, Englewood, New Jersey, USA) was immediately applied over the posterior teeth. These solutions and fluoride varnish were applied by an Oregon licensed general dentist, who was also blinded as to which solution the individual participants received. After application of the drop of silver nitrate (treatment) or saline (control) solution and fluoride varnish, participants were instructed

not to eat or drink for one hour. They were then instructed to not brush or floss the posterior teeth and not rinse with mouthwash for two weeks.

However, participants could brush and floss the anterior teeth from the mesial of #6 (upper right canine) to the mesial of #11 (upper left canine), and from the mesial of #22 (lower left canine) to the mesial of #27 (lower right canine), using only a toothbrush moistened with water and no toothpaste. If a piece of food became trapped interproximally, participants were instructed to use floss at that site only. Tongue brushing was permitted with a toothbrush or tongue scraper moistened with water.

The salivary samples were used to determine the presence of salivary silver ions for all participants. There was the potential of silver ions originating from numerous sources, including industrial exposure, amalgam restorations, medicines/supplements, etcetera, as well as the silver nitrate for those in the treatment group. A baseline was necessary in order to determine if any silver ions were present prior to the silver nitrate application (treatment group only), and if so, whether or not the application of silver nitrate made an appreciable difference. Additionally, substantivity could then be determined. Therefore, three samples were required per participant: baseline, week one, and week two. Frozen samples were delivered to, and analyzed by, the Biochemistry and Molecular Biology Lab at the Oregon Health and Science University (OHSU) in Portland, Oregon. Any presence of silver ions could be detected in parts per billion via mass spectrometry.

Results

Gingival Inflammation

Table III highlights the results of the changes in gingival inflammation for both the control and treatment groups. The gingival inflammation of the control group progressed from the mild to moderate range over the course of the study. However, the GI of the treatment group did not exhibit a significant change. This group's gingival inflammation remained in the low moderate inflammation range throughout the two week study period. The GI scores were analyzed using repeated measure ANOVA. The increase in the GI of the control group (saline solution) was statistically significant ($p=0.03$) during the two week duration of the study, while the GI index of the treatment group was not statistically significant ($p=0.25$).

Plaque Accumulation

Both groups experienced a significant increase in the amount of plaque accumulation, as represented in Table IV. From their respective baselines, the control group acquired slightly more plaque than the treatment group. For both groups, the greatest increase in plaque accretion occurred between baseline and week one (control $p = 0.0004$; treatment $p = 0.02$), but the overall plaque accumulation for the entire two week period remained very significant (control $p = <0.0001$; treatment $p = <0.0007$). However, no significant difference in plaque accumulation existed between the control and treatment groups.

Salivary Silver Ions

The authors expected some level of silver to be detected in the samples due to the sensitivity of the mass spectrometer, which can identify ions in the parts per billions. The

samples were processed twice to be certain. Debris from the oral cavity that had been filtered out of the liquid portion of the saliva was also checked for silver. However, none of the salivary samples analyzed via mass spectrometry revealed any detectable silver ions (data not shown).

Discussion

To the best of the authors' knowledge, this study had not been previously conducted. As such, there were no preconceived ideas as to what to expect from the treatment group. Under normal circumstances, with the cessation of oral homecare, one would expect gingival inflammation to increase while dental plaque also thickened and covered a greater surface area of the teeth, as was seen in the control group. While this situation was expected from the control group, to what degree, if any, would the silver nitrate affect the treatment group? The treatment group developed plaque in a similar fashion to the control, although to a slightly less, non-significant degree. However, the extent and quantity of plaque does not reflect the bacterial composition within it. With that said, the bacterial species profile of one sample of dental plaque does not necessarily equate to another sample.

As the data revealed, the control group experienced a significant increase in gingival inflammation over the course of the two week study period. However, the treatment group did not. Their gingival inflammation did not significantly change, in spite of an increase of plaque. This result leads one to consider the possibility that the virulence of the plaque differed between the two groups. Perhaps the silver, from the silver nitrate, affected the difference.

Since no silver ions were detected in the salivary samples of week one (one week after the treatment group received an application of the medicament), it is unknown how long the silver ions remained in the oral cavity. It could have been minutes, hours, or days, but certainly not a week; otherwise the ions would have been detected by the mass spectrometry. A clue as to the possible substantivity of the silver nitrate could be inferred from the remarks of two study participants, who, in retrospect, belonged to the treatment group. These two people independently stated at the second data collection session that for three or four days their teeth felt "squeaky" clean, in spite of a cessation of oral homecare on the posterior teeth. These statements suggest a possible substantivity of several days.

These subjective remarks also suggest that the silver nitrate might have affected the early stages of dental plaque adhesion, which in turn could have influenced the bacterial inhabitants of that community. The initial development of the acquired pellicle, comprised of an acellular layer of salivary proteins and enzymes produced following a complete removal of previous deposits, provides the foundation for bacterial adhesion.[3,20,24,28,34-41]This newly formed layer is considered to be nutritionally poor for most bacteria yet numerous proteins within the acquired pellicle serve as receptors for early pioneers during the first phase of biofilm formation.[3,28,42,43]

The early colonizers create a micro-environment that is more suitable for species that join the biofilm at a later stage.[24,25,40,43-47]The later colonizing bacteria have more specific biological requirements and cannot adhere until the conditions are conducive to their survival.[41,48] Unlike other species, fusobacteria are able to aggregate with all other types of bacteria. After the foundation has been laid by the early colonizers, a critical

bacterial species, *Fusobacterium nucleatum,* acts as an intermediary link for the late colonizers to adhere to the biofilm.[28,34,36,38,41,46,49-52] *Fusobacterium nucleatum* is the most common Gram negative species found in healthy mouths and is found in even greater amounts in periodontally unhealthy mouths.[42,53]

As biofilm ages, the percentage of bacterial residents becomes increasingly Gram negative anaerobes and more resistant to antimicrobials.[20,25,37,47,54-58] By this stage, anaerobes, such as *Prevotella intermedia, Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola,* and *Aggregatibacter actinomycetemcomitans* are able to join the biofilm via their attachment to *Fusobacterium nucleatum*.[3,40,43,59] These periodontal pathogens are well known for their destructive capabilities.[28,43,56]

The question remains, did the silver nitrate interfere with the adhesion process of the early colonizers, which in turn deterred or possibly delayed the arrival of the later, more pathogenic bacterial species into the oral plaque? A bacterial analysis of the plaque's bacterial species profile would be necessary to determine which species are present, the benign ones associated with health, or the later, more virulent arrivals that are associated with disease.

There are several limitations to this study. The total number of participants, of which there were thirty, is small. In order to have a greater statistical power a larger cohort is necessary. Also, ideally, the study's duration should have been longer. Additionally, because the participants were not sequestered, the PI had to trust each of them to hold to the agreement that they would adhere to the requirements of the study. Every participant was asked at the data collection sessions of week two and week three if they had deliberately or accidently brushed and/or flossed their posterior teeth, used

mouthwash or used toothpaste. All individuals stated that they had not, and that they had followed the verbal and written instruments they had been given. The objective data collected appeared to confirm these affirmations; however, the PI had no means to verify their compliance.

Diet was not addressed in this study. Participants were permitted to eat and drink whatever they chose. Therefore, there is the potential that some participants ate more fibrous foods than others; foods that could disrupt adhering dental plaque. In fact, several of the participants stated that their teeth felt so "disgusting" that they ate far more raw vegetables than they usually consumed, all in the attempt to naturally cleanse their posterior dental surfaces. Another factor that was not addressed was the varying degrees of malocclusion among the participants. While there were no severe cases of posterior malocclusion, any amount can contribute to the reduced self-cleansing of the dentition.

Conclusion

Silver ions have been shown to have a deleterious effect on bacteria. The question has been raised as to what effect, if any, silver ions may have on oral bacteria, and specifically, what effect they may have on the sequelae of gingivitis, periodontal disease, and dental plaque formation. This *in vivo* study attempted to investigate these questions, namely by assessing what effect silver nitrate may have on the development of gingival inflammation and oral plaque accumulation when compared to a control group. Thirty, healthy volunteers were divided into two groups, a control group that received a one-time application of saline solution, and a treatment group that received a one-time application of 25% silver nitrate, onto their posterior teeth.

Subsequent changes to their gingival health and plaque scores were assessed over a two week period. Based on the results of repeated measure ANOVA, a 20 µL 25% silver nitrate application may have the ability to prevent a worsening of gingival inflammation during a two week cessation of oral hygiene, in spite of a significant worsening of plaque scores. Conversely, the control group had a significant increase in gingival inflammation, in addition to a significant increase in their plaque scores. Based on these findings, more studies are needed to determine the effect of silver nitrate on periodontal disease parameters, such as gingival inflammation, and on specific bacterial species. New studies should be of longer duration, with more participants, with more frequent salivary samples taken and analyzed, and with various concentrations of silver nitrate.

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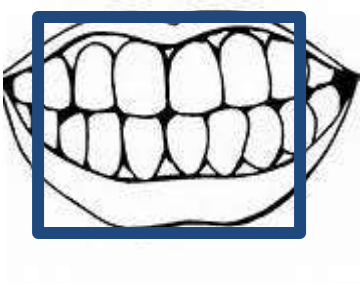
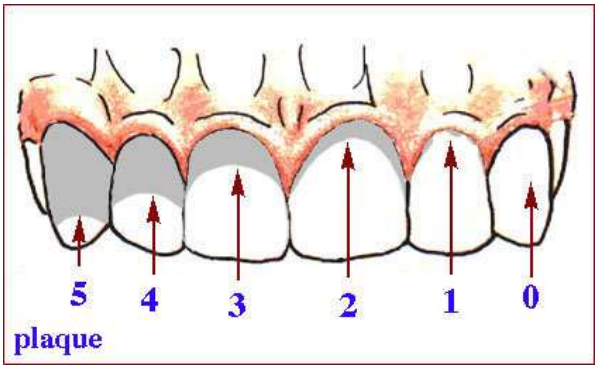
FIGURES

Figure 1: Anterior Teeth, #6-11, #22-27



FIGURES (continued)

Figure 2: Modified Quigley Hein Plaque Index Numeric Representation



TABLES

Table I: Gingival Inflammation Index Numeric Values and Range

GI #	Numeric Value
0	Absence of inflammation: normal gingival
1	Mild inflammation: slight change in color and slight edema
2	Moderate inflammation: redness, edema, and glazing, bleeding on probing
3	Severe inflammation: marked redness and edema, ulceration, tendency towards spontaneous bleeding
GI range	**Condition**
<0.1	No inflammation (excellent)
0.1 to 1.0	Mild inflammation (good)
1.1 to 2.0	Moderate inflammation (fair)
2.1 to 3.0	Severe inflammation (poor)

TABLES (continued)

Table II: MQHPI Categories

#	Numeric Descriptor
0	No plaque present.
1	Separate flecks of plaque at the cervical margin.
2	A thin, continuous band of plaque (up to 1 mm) at the cervical margin.
3	A band of plaque wider than 1 mm but covering less than one-third of the surface.
4	Plaque covering at least one-third but less than two-thirds of the surface.
5	Plaque covering more than two-thirds of the surface.

TABLES (continued)

Table III: Summary of Changes of Gingival Index

	N	Mean	Standard Deviation	p value
Control Baseline	15	0.98200000	0.48903988	n/a
Treatment Baseline	15	1.17000000	0.40194172	n/a
Control Week One	15	1.11600000	0.21326710	0.03*
Treatment Week One	15	1.25000000	0.22258225	0.31**
Control Week Two	15	1.26466667	0.17500476	0.03*
Treatment Week Two	15	1.27333333	0.24679855	0.25**

*significant increase in gingival inflammation (control group)

**no significant increase in gingival inflammation (treatment group)

TABLES (continued)

Table IV: Summary of Changes of MQHP Index

Data Session	N	Mean	Standard Deviation	p value
Control Baseline	15	2.01933333	0.53747381	n/a
Treatment Baseline	15	2.18133333	0.58456170	n/a
Control Week One	15	2.66333333	0.33506218	0.0004*
Treatment Week One	15	2.60133333	0.36556935	0.02*
Control Week Two	15	2.87533333	0.40172604	<0.0001*
Treatment Week Two	15	2.90200000	0.27477783	<0.0007*

*significant increases in plaque scores for both control and treatment groups

APPENDIX A

PATIENT NAME _____ Birth Date _____

Although dental personnel primarily treat the area in and around your mouth, your mouth is a part of your entire body. Health problems that you may have, or medication that you may be taking, could have an important interrelationship with the dentistry you will receive. Thank you for answering the following questions.

Are you under a physician's care now?	Yes	No	If yes, please explain: _____
Have you ever been hospitalized or had a major operation?	Yes	No	If yes, please explain: _____
Have you ever had a serious head or neck injury?	Yes	No	If yes, please explain: _____
Are you taking any medications, pills, or drugs?	Yes	No	If yes, please explain: _____
Do you take, or have you taken, Phen-Fen or Redux?	Yes	No	
Are you on a special diet?	Yes	No	
Do you use tobacco?	Yes	No	
Do you use controlled substances?	Yes	No	
Do you need to pre-medicate?	Yes	No	If yes, please explain: _____

Women: Are you Pregnant/Trying to get pregnant? Yes No Taking oral contraceptives? Yes No Nursing? Yes No

Are you allergic to any of the following?

Aspirin Penicillin Codeine Acrylic Metal Latex Local Anesthetics

Other If yes, please explain:

Do you have, or have you had, any of the following?

AIDS/HIV Positive	Yes	No	Cortisone Medicine	Yes	No	Hemophilia	Yes	No	Renal Dialysis	Yes	No
Alzheimer's Disease	Yes	No	Diabetes	Yes	No	Hepatitis A	Yes	No	Rheumatic Fever	Yes	No
Anaphylaxis	Yes	No	Drug Addiction	Yes	No	Hepatitis B or C	Yes	No	Rheumatism	Yes	No
Anemia	Yes	No	Easily Winded	Yes	No	Herpes	Yes	No	Scarlet Fever	Yes	No
Angina	Yes	No	Emphysema	Yes	No	High Blood Pressure	Yes	No	Shingles	Yes	No
Arthritis/Gout	Yes	No	Epilepsy or Seizures	Yes	No	Hives or Rash	Yes	No	Sickle Cell Disease	Yes	No
Artificial Heart Valve	Yes	No	Excessive Bleeding	Yes	No	Hypoglycemia	Yes	No	Sinus Trouble	Yes	No
Artificial Joint	Yes	No	Excessive Thirst	Yes	No	Irregular Heartbeat	Yes	No	Spina Bifida	Yes	No
Asthma	Yes	No	Fainting Spells/Dizziness	Yes	No	Kidney Problems	Yes	No	Stomach/Intestinal Disease	Yes	No
Blood Disease	Yes	No	Frequent Cough	Yes	No	Leukemia	Yes	No	Stroke	Yes	No
Blood Transfusion	Yes	No	Frequent Diarrhea	Yes	No	Liver Disease	Yes	No	Swelling of Limbs	Yes	No
Breathing Problem	Yes	No	Frequent Headaches	Yes	No	Low Blood Pressure	Yes	No	Thyroid Disease	Yes	No
Bruise Easily	Yes	No	Genital Herpes	Yes	No	Lung Disease	Yes	No	Tonsilitis	Yes	No
Cancer	Yes	No	Glaucoma	Yes	No	Mitral Valve Prolapse	Yes	No	Tuberculosis	Yes	No
Chemotherapy	Yes	No	Hay Fever	Yes	No	Pain in Jaw Joints	Yes	No	Tumors or Growths	Yes	No
Chest Pains	Yes	No	Heart Attack/Failure	Yes	No	Parathyroid Disease	Yes	No	Ulcers	Yes	No
Cold Sores/Fever Blisters	Yes	No	Heart Murmur	Yes	No	Psychiatric Care	Yes	No	Venereal Disease	Yes	No
Congenital Heart Disorder	Yes	No	Heart Pace Maker	Yes	No	Radiation Treatments	Yes	No	Yellow Jaundice	Yes	No
Convulsions	Yes	No	Heart Trouble/Disease	Yes	No	Recent Weight Loss	Yes	No			

Have you ever had any serious illness not listed above? Yes No If yes, please explain: _____

_____

_____

Comments: _____

_____

_____

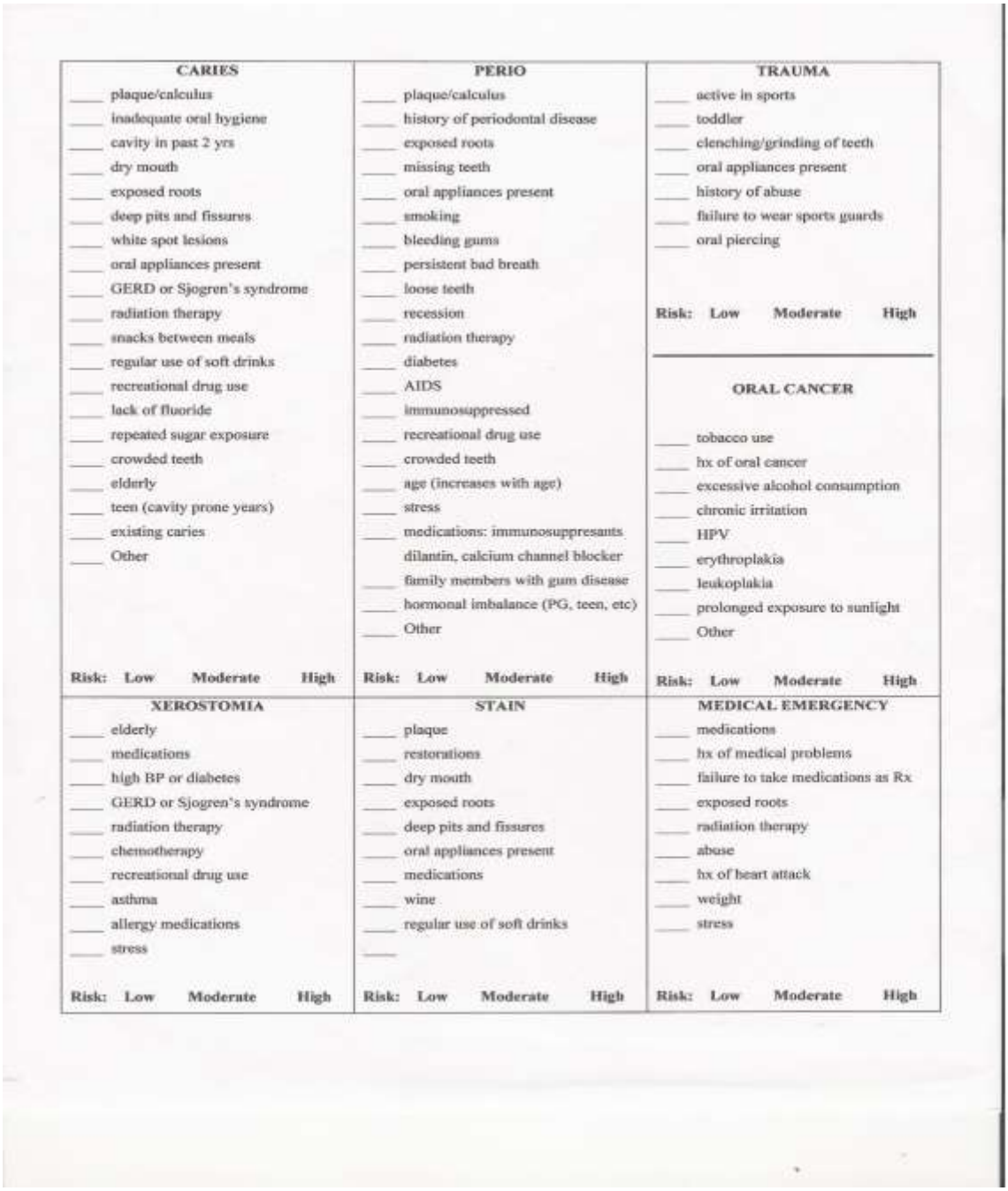
_____

To the best of my knowledge, the questions on this form have been accurately answered. I understand that providing incorrect information can be dangerous to my (or patient's) health. It is my responsibility to inform the dental office of any changes in medical status.

SIGNATURE OF PATIENT, PARENT, or GUARDIAN _____ DATE _____

APPENDIX B

HEALTH & ORAL HEALTH SCREENING FORM



CARIES

____ plaque/calculus
____ inadequate oral hygiene
____ cavity in past 2 yrs
____ dry mouth
____ exposed roots
____ deep pits and fissures
____ white spot lesions
____ oral appliances present
____ GERD or Sjogren's syndrome
____ radiation therapy
____ snacks between meals
____ regular use of soft drinks
____ recreational drug use
____ lack of fluoride
____ repeated sugar exposure
____ crowded teeth
____ elderly
____ teen (cavity prone years)
____ existing caries
____ Other

Risk: Low Moderate High

PERIO

____ plaque/calculus
____ history of periodontal disease
____ exposed roots
____ missing teeth
____ oral appliances present
____ smoking
____ bleeding gums
____ persistent bad breath
____ loose teeth
____ recession
____ radiation therapy
____ diabetes
____ AIDS
____ immunosuppressed
____ recreational drug use
____ crowded teeth
____ age (increases with age)
____ stress
____ medications: immunosuppresants
dilantin, calcium channel blocker
____ family members with gum disease
____ hormonal imbalance (PG, teen, etc)
____ Other

Risk: Low Moderate High

TRAUMA

____ active in sports
____ toddler
____ clenching/grinding of teeth
____ oral appliances present
____ history of abuse
____ failure to wear sports guards
____ oral piercing

Risk: Low Moderate High

ORAL CANCER

____ tobacco use
____ hx of oral cancer
____ excessive alcohol consumption
____ chronic irritation
____ HPV
____ erythroplakia
____ leukoplakia
____ prolonged exposure to sunlight
____ Other

Risk: Low Moderate High

XEROSTOMIA

____ elderly
____ medications
____ high BP or diabetes
____ GERD or Sjogren's syndrome
____ radiation therapy
____ chemotherapy
____ recreational drug use
____ asthma
____ allergy medications
____ stress

Risk: Low Moderate High

STAIN

____ plaque
____ restorations
____ dry mouth
____ exposed roots
____ deep pits and fissures
____ oral appliances present
____ medications
____ wine
____ regular use of soft drinks
____

Risk: Low Moderate High

MEDICAL EMERGENCY

____ medications
____ hx of medical problems
____ failure to take medications as Rx
____ exposed roots
____ radiation therapy
____ abuse
____ hx of heart attack
____ weight
____ stress

Risk: Low Moderate High

APPENDIX C

Silver Nitrate Study Informed Consent Form

The purpose of this study is to determine if the silver ions in silver nitrate, when applied *in vivo,* have the ability to decrease oral biofilm accumulation and gingival inflammation in healthy volunteers during a two week period in which no oral hygiene homecare will be performed. The purpose of this investigation is to determine if the application of silver nitrate has the potential to thwart the development of gingivitis, which is the reversible, first step in the development of periodontal disease. Results of this investigation could be beneficial in determining dental care, especially for special needs populations. During this two week period participants have the potential of developing the reversible condition of gingivitis and/or discoloration of demineralized dental surfaces or staining of composite margin restorations. I:

- Completed the health history form
- Completed the caries risk assessment form.
- Have the majority of my posterior teeth
- Am willing to not brush or floss my posterior teeth, nor use mouthwash for two weeks.
- Will not drink or eat for one hour after receiving my one time application of the medicament

I:

- Have no known allergy to silver nitrate or fluoride
- Had no antibiotic use in the previous three months
- Am not pregnant or lactating
- Have no extensive composite restorations

1) I, _____, consent to the silver nitrate study to be completed by Monika Alcorn, RDH.
2) The procedure(s)/treatment(s) have been explained to me.
3) I have been informed of the purpose of the study.
4) I understand that the following risk(s) may result from the procedure:
   a. Plaque accumulation and gingivitis from not brushing or flossing for two weeks.
   b. Possible staining of posterior tooth surfaces for those participants in the treatment group.
5) I understand that I am free to withdraw from the study at any time and that my information will remain confidential.
6) All my questions have been satisfactorily answered.

Patient Signature: _____ Date: _____

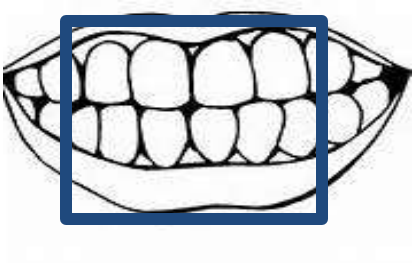
Witness Signature: _____ Date: _____

APPENDIX D

Participant Oral Homecare Instructions

As a participant in the Antibacterial Effects of Silver Nitrate study it very important that you adhere to the following instructions during the two week trial period:

1. Do not brush or floss the posterior teeth (premolars and molars).
2. Brushing and flossing of the anterior upper and lower teeth is permitted as long as the toothbrush and floss does not come in contact with the any part of the premolars or molars. Note the tooth structures that can be brushed and flossed illustrated below, within the blue box:



3. No use of antibiotics. If it becomes necessary within the two week trial period then withdrawal from the study must occur.
4. No use of toothpaste.
5. No use of mouthwash.
6. Tongue brushing is permitted.

Please call Monika Alcorn, 503-270-6816, with any questions or concerns you might have during the course of this study. Once again, thank you for your participation.

APPENDIX E

Journal of Dental Hygiene Guidelines

Author Guidelines

Statement of Purpose

The Journal of Dental Hygiene (JDH) is the refereed, scientific publication of the American Dental Hygienists' Association (ADHA). It promotes the publication of original creative work related to dental hygiene research, education and evidence-based practice. The JDH supports the development and dissemination of a unique dental hygiene body of knowledge through scientific inquiry in basic, behavioral, clinical and translational research.

Author Guidelines

Starting with the Summer 2004 issue, the JDH has been published online. The online format provides searching capabilities to JDH readers by establishing a link to dental hygiene research indexed through the National Library of Medicine and PubMed.

Manuscript Requirements

Manuscripts are evaluated for quality, depth and significance of research, comprehensive evaluation of the available literature, and the expertise of the author(s) in the given subject. Content must provide new information and be of general importance to dental hygiene. The JDH discourages submitting more than one article on related aspects of the same research. If multiple papers are submitted from the same project, significant differences in the papers must be evident.

Originality

Manuscripts must be original, unpublished, owned by the author and not submitted elsewhere. Authors are responsible for obtaining permission to use any materials (tables, charts, photographs, etc.) that are owned by others. Written permission to reprint material must be secured from the copyright owner and sent to ADHA when the manuscript is accepted for publication. The letter requesting permission must specifically state the original source, using wording stipulated by the grantor.

Manuscript Categories

The JDH publishes original scientific investigations, literature reviews, theoretical articles, brief reports and special feature articles related to dental hygiene. Specific categories of articles are as follows: Original Research Reports, Literature Reviews, Short Reports, Critical Issues in Dental Hygiene, and Innovations in Education and Technology. All submissions are reviewed by the editor and by members of the Editorial Review Board.

Original Research Reports – Limited to 4,000 words (excluding cover page, abstract, references and tables/figures).

Include reports of basic, behavioral, clinical and translational studies that provide new information, applications or theoretical developments. Original Research Reports include an Abstract, Introduction (including the review of the literature and ending with a statement of the study purpose), Methods and Materials, Results, Discussion, and Conclusion.

Abstract: Approximately 250 words. Use the headings "Purpose" (purpose), "Methods" (design, subjects, procedures, measurements), "Results" (principal findings) and "Conclusion (i.e. Major conclusions)." The abstract must be able to stand alone. References should therefore be avoided.

Text: The body of the manuscript should be divided into sections preceded by the appropriate subheading. Major subheadings should be in capital letters at the left-hand margin. Secondary subheads should appear at the left-hand margin and be typed in upper and lower case and in bold face.

Introduction (including the literature review): Cite a variety of relevant studies that relate to the need for the current study and its significance. References should be as current as possible, unless a hallmark study is included. Compare findings of previous studies, clearly indicating all sources of concepts and data. When a source is directly quoted, use quotation marks. However, use of quotation marks should be limited. End this section with a clear statement of the purpose of the study, hypothesis or research objectives.

Methods and Materials: Describe the research design (e.g. randomized controlled trial) and procedures (e.g. IRB approval, target population, inclusion/exclusion criteria, recruitment, informed consent, variables to be tested, instruments, equipment, procedures and method of data analysis). Specify the measurements and statistical tests used as well as related levels of significance. Furthermore, assure an adherence to all pertinent federal and state regulations concerning the protection of the rights and welfare of all human and animal subjects.

Results: Summarize all relevant data and study findings. Do not repeat in the text the data reported in tables and figures verbatim, but do refer to the data and emphasize important findings (e.g. Table 1 shows that most of the subjects were African American and between the ages of 12 and 16).

Discussion: Evaluate and interpret the findings. Compare them with those of other related studies. Discuss how they relate to dental hygiene practice, profession, education or research. Include overall health promotion and disease prevention, clinical and primary care for individuals and groups and basic and applied science. Discuss study limitations; implications for dental hygiene practice, education, and research; and recommendations or plans for further study.

Conclusion: State the conclusions, theories, or implications that may be drawn from the study. This section should be one to two paragraphs or can be listed as bulleted points.

Literature Reviews – Limited to 3,000 words (excluding cover page, abstract, references and tables/figures).

A presentation of relevant and primary published material on a specific topic constitutes a comprehensive literature review. Such a review includes a summary and critique of the current status of the topic, and the aspects requiring further study.

Abstract: Literature reviews begin with a non-structured abstract — a brief statement of purpose, content summary, conclusions and recommendations.

Short Reports – Limited to no more than 2,000 words (excluding cover page, abstract, references and tables/figures). Illustrations should be limited to a total of no more than two (e.g. two figures, two tables, or one figure and one table).

The JDH publishes short reports related to dental hygiene. Short reports are limited in scope and should begin with a brief, non-structured abstract that describes the topic.

Text: A concise introduction (which includes a literature review), detailed description of the topic or activity, and discussion, conclusion and recommendations must also be included. References are necessary to support the rationale and methods presented.

A short report may describe a clinical case study, an educational innovation, a research method, a concept or theory, or other current topics.

Clinical Case Study: A report that describes a unique aspect of patient care not previously documented in the literature. Such reports usually focus on a single patient or groups of patients with similar conditions. Suitable topics include, but are not limited to, innovative preventive methods or programs, educational methods or approaches, health promotion interventions, unique clinical conditions, or pathologies and ethical issues.

Theoretical Manuscript: A report that provides a well-supported explanation for natural phenomena that clarify a set of interrelated concepts, definitions, or propositions about dental hygiene care or processes. Such reports provide new knowledge, insight, or interpretation; and discussion, conclusions, and recommendations. These reports begin with a non-structured abstract. At least four keywords are listed at the end of the abstract.

Critical Issues in Dental Hygiene – Limited to 4,000 words (excluding cover page, abstract, references and tables/figures).

The purpose of this category is to highlight challenges and opportunities pertinent to the future directions of the profession of dental hygiene.

Text: Articles in this category should follow the basic structure for text outlined for Original Research Reports.

Innovations in Education and Technology – Limited to 4,000 words (excluding cover page, abstract, references and tables/figures).

The purpose of this category is to feature short reports of innovative teaching applications and techniques as well as new technologies available for increased communication and learning in dental hygiene education.

Text: Articles in this category should follow the

basic structure for text outlined for Original Research Reports.

Manuscript Submission

Authors submitting a manuscript to the JDH should utilize the BenchPress system, located at http://submit-jdh.adha.org/. Specific instructions for submission will be outlined on the BenchPress website. There is no charge for submission. Receipt of submission will be acknowledged by email.

All papers are reviewed by the editor and assigned to three reviewers. The editor reserves the right to return, without review, any manuscript that does not meet JDH criteria for formal review.

The review process takes approximately ten to twelve weeks, depending on the need for authors to make revisions. All reviewer comments, as well as notification of acceptance or rejection, are submitted to the corresponding author. For any questions about the manuscript submission process, contact Staff Editor Josh Snyder at joshs@adha.net.

Manuscript Preparation and Style

Standard usage of the English language is expected. Manuscripts should contain one-inch margins, double spacing and Verdana 10 pt. font. All pages should be numbered, beginning with title page and ending with references.

Title Page: A title page must include: 1) title of article, which should be concise yet informative, 2) first name, middle initial and last name of each author, with academic credentials, 3) each author or coauthor's job title, department and institution or place of employment (if other than academic), 4) disclaimers/disclosures, if any, 5) name, address, all contact information of author responsible for correspondence about the manuscript, and 6) funding sources for the project, equipment, drugs, etc.

Blinding Manuscripts: All information that can identify the author(s) (such as author name, institutional affiliation, IRB approval, acknowledgements, etc.) should be included in the title page. Manuscripts must be blinded and any of the above identifying information should be removed from the text for the review process. If a manuscript is accepted for publication, this information can be added back into the manuscript for publication.

Keywords: When submitting a manuscript, please choose four to six keywords from the list provided by BenchPress. These key words will be used for indexing purposes during the review process. If a manuscript is accepted for publication, a more detailed list of key words can be provided.

National Dental Hygiene Research Agenda: Identify how the study supports a specific topic area and related objective from the National Dental Hygiene Research Agenda (NDHRA). For example: This study supports the objective: Assess strategies for effective communication between the dental hygienist and the client, under Health Promotion/Disease Prevention. NDHRA statements can be found at: http://www.adha.org/downloads/Research_agenda%20-ADHA_Final_Report.pdf

Author Biography: Please include a brief biographical sketch of each author at the end of the cover letter. List names, credentials, titles, affiliations and locations. Example: "Mary B. Jones, RDH, MA, is assistant professor and clinic director, Department of Dental Hygiene; Bill R. Smith, DDS, MEd, is associate professor, Department of Pediatric Dentistry. Both are at the University of Minnesota in Minneapolis."

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Acknowledgments: Be brief and straightforward. Example: "The authors thank Jane Smith, RDH, for her assistance in developing the survey instrument." Anyone making a substantial contribution to the conduct of the research or the resulting report should be appropriately credited as an author.

Acronyms: Spell out abbreviations and acronyms on first mention followed by the abbreviation in parentheses. Limit the overall use of abbreviations in the text.

Medication, Product or Device Names: Throughout the text, use generic, nonproprietary names for medications, products and devices. At the first mention, state the generic name followed in parentheses by the trade name with the register® or trademark™ symbol and the manufacturer's name and city/state.

Example: Chlorhexidine (Peridex®; 3M ESPE, Minneapolis, MN) coded or abbreviated as CHX

Visual Aids

Do not embed tables and figures in the body of the text. These should be provided as separate files, per BenchPress instruction. All tables and figures must be blinded for the review process.

Tables: All tables must have a title that is brief but self-explanatory. Readers should not have to refer to the text to understand a table. The main body of text should not overly depend on the tables. Indicate explanatory notes to items in the table with reference marks (*, #). Cite each table in the text in the order in which it is to appear. Identify tables with Roman Numerals (example: Table I).

Figures: Includes charts, graphs, photographs and artwork. All should include a brief caption and use Arabic numerals (example: Figure 1). Cite each figure in the text in the order in which it will appear.

Photographs: High-resolution digital photos are preferred, with a resolution of at least 300 pixels per inch.

References

The JDH follows National Library of Medicine (NLM) citation style. Please refer to http://www.wsulibs.wsu.edu/quickguides/nlm for specifics.

Each reference should be numbered in the order it first appears in the text. If a source is cited more than once, the first reference number it is given is used throughout. Each reference in the text should be in superscript format. Continuous references should be connected with a dash (example: 7,8-10). ADHA editorial staff does not assume responsibility for verifying references. For more information and detailed examples, please visit the International Committee of Medical Journal Editors at www.icmje.org. Please ensure that every reference cited in the text is also present in the reference list and vice versa. Citation of a reference as "in press" implies that the item has been accepted for publication.

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Examples of reference citations:

Example Article in a Journal: Michalowicz BS, Hodges JS, DiAngelis AJ, et al. Treatment of periodontal disease and the risk of preterm birth. N Engl J Med. 2006;355(18):1885-1894.

Smith MA, Jones BB. Curette sharpness: a literature review. J Dent Hyg. 1996;77:382-390.

Book citations: Spolarich AE, Gurenlian JR. Drug-induced adverse oral events. In: Daniel SJ, Harfst SA, Wilder RS, ed. Mosby's Dental Hygiene: Concepts, Cases and Competencies. 2nd ed. St. Louis, MO. Mosby/Elsevier Publishing. 2008. p. 259-276.

Internet citations: NLM requires the standard elements of a citation for an Internet resource, with a few modifications. The main elements required:

Polgreen PM, Diekema DJ, Vandeberg J, et al. Risk factors for groin wound infection after femoral artery catheterization: a case-control study. Infect Control Hosp Epidemiol [Internet]. 2006 Jan [cited 2007 Jan 5];27(1):34-7. Available from: http://www.journals.uchicago.edu/ICHE/journal/issues/v27n1/2004069/2004069.web.pdf

Poole KE, Compston JE. Osteoporosis and its management. BMJ [Internet]. 2006 Dec 16 [cited 2007 Jan 4];333(7581):1251-6. Available from: http://www.bmj.com/cgi/reprint/333/7581/1251?maxtoshow=&HITS=10&hits=10&RESULTFORMAT=&andorexactfulltext=and&searchid=1&FIRSTINDEX=0&sortspec=relevance&volume=333&firstpage=1251&resourcetype=HWCIT

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