VIOLET-BLUE LIGHT AND *STREPTOCOCCUS MUTANS* BIOFILM-INDUCED CARIOUS LESIONS

Grace Gomez Felix Gomez

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Richard L. Gregory, Ph.D., Chair

Andrea Zandona, DDS., MSD., Ph.D.

Doctoral Committee

Masatoshi Ando, D.D.S., Ph.D.

Chao-Hung Lee, Ph.D.

November 30, 2017

Gregory Anderson, Ph.D.

DEDICATION

This dissertation work is fulfilled by God's law of love from my family, who have been my pillars of strength. I dedicate this work to my wonderful daughter Christabel C. Fernando, who walked along with me unknowingly sacrificing her childhood pleasures of life. I also submit this work to my husband Joseph P Anbumani for always visioning me with esteem and success. My heartfelt thanks to my mother Philomenal Gomez, for being always there for me with answers to my questions in Science, Mathematics, Chemistry and English. She was there for me, when I needed her the most, irrespective of her age and health conditions. I also thank my father L. Felix Gomez and my sister Annie C. Gomez for their emotional and moral support.

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Grace Gomez Felix Gomez

VIOLET-BLUE LIGHT AND *STREPTOCOCCUS MUTANS* BIOFILM INDUCED CARIOUS LESIONS

Dental caries is a continuum of disease process. Early carious lesions are reversible and preventable. The primary etiological factor of dental caries is oral biofilm also known as dental plaque. It is an aggregate of oral bacteria, and one of the principal cariogenic bacteria is a facultative anaerobic microbe, *Streptococcus mutans*, which is indispensable for the initiation of caries. Management of prevention of carious lesions at the microbial level begins with reducing, eliminating and inhibiting the attachment of oral biofilm. Non-invasive phototherapy is widely studied to control oral biofilm as an alternative method to overcome the emergence of antibiotic resistant strains.

In vitro studies demonstrated that Violet-Blue light with a peak wavelength of 405 nm had an inhibitory effect on *S. mutans* biofilm cells irradiated for 5 min. Metabolic activity of *S. mutans* cells was significantly reduced immediately after treatment with some recovery at 2 and 6 hrs. An *in vitro* translational study was conducted to determine the inhibitory effect of Violet-Blue light with twice daily treatments for 5 min over a period of 5 days on *S. mutans* biofilm cells grown on human enamel and dentin specimens. Bacterial viability was significantly reduced in the Violet-Blue light treated group for both dentin and enamel. Lesion depth, obtained by imaging fluorescence loss through Quantitative Light Induced Fluorescence (QLF-D) Biluminator and through transverse microradiography

(TMR), was significantly reduced in *S. mutans* grown in tryptic soy broth with 1% sucrose (TSBS) for dentin. Mineral loss obtained through TMR in the absence of sucrose (TSB) was significant with enamel. However, all the parameters in the Violet-Blue treated groups were numerically reduced, albeit some being not significant. Accurate Mass Quadrupole Time of Flight Mass Spectrometry was used to identify Protoporphyrin IX (PP-IX) in *S. mutans* biofilm that may play a role in the photoinactivation and emission of fluorescence within specific wavelengths of the visible spectrum namely Violet-Blue light.

Richard L. Gregory, Ph.D., Chair

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List of Abbreviations

S. mutans	*Streptococcus mutans*
PDT	Photodynamic therapy
PT	Phototherapy
TSB	Tryptic Soy Broth
TSBS	Tryptic Soy Broth +1% sucrose
LLLT	Low-level laser light therapy
Hp	hematoporphyrin
HpD	hematoporphyrin derivative
SAD	Seasonal affective disorder
TMJ	Temporomandibular joints
PACT	Photodynamic antimicrobial chemotherapy
QLF	Quantitative light induced fluorescence
He-Ne	Helium neon
ER	Erythrosine
RB	Rose Bengal
TBO	Toluidine Blue O
MB	Methylene Blue
MG	Malachite green
LED	Light emitting diodes
EPS	Extracellular polysaccharides
ATP	Adenosine triphosphate

ADP	Adenosine diphosphate
XTT	2, 3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt
IRB	Institutional Review Board
CFU	Colony forming units
MSSB	Mitis-Salivaris Sucrose Bacitracin
P. gingivalis	*Porphyromonas gingivalis*
F. nucleatum	*Fusobacterium nucleatum*
P. aeruginosa	*Pseudomonas aeruginosa*
P. acnes	*Propionibacterium acnes*
P. intermedia	*Prevotella intermedia*

Chapter 1

General Introduction

1.1 History of Light Therapy or Phototherapy

“Let there be light”, and there was light, and it was considered good (Genesis 1:2). Light is considered divine in every spiritual teaching around the world. Light energy sustains all forms of life on earth and is known for its healing and destructive properties. Light consists of photons and is emitted by the movement of electrons between the shells of an atom. The properties and uses of light were discussed and developed as early as 400 BC. With an early atomic theory adopted by Democritus of Abdera from his mentor Leucippus. He stated that, “Universe is made of 2 elements namely, the atoms and the void in which they move”. He also had a philosophical approach to state, “Nothing occurs at random, but everything happens for a reason and by necessity” (Chalmers, 1998). Following atomic theory, fluorescence was discovered in 1577 by Nicholas Monardes, phosphorescence in 1603 by Vincenzo Cascariolo, postulation of the laws of refraction of light in 1637 by Rene Descartes, then the discovery of Ultraviolet radiation in 1801 by Johann Ritter (The Science of Phototherapy: An Introduction: Springer, 2005;). In 1903, Neil Finsen, who is considered the father of modern phototherapy, treated cutaneous tuberculosis with the combination of sunlight in addition to Ultraviolet light radiation from a carbon arc lamp (Daniell and

Hill, 1991; Roelandts, 2002). This was considered the birth of phototherapy. Later in 1900, Oscar Raab demonstrated photosensitization of *Paramecium caudatum* through a combination of sunlight and acridine orange. His first observation of photoinactivation was with a statement “I don't understand it Dr. Von Tappeiner, the paramecia were all wiggling just fine a minute ago, but now these over by the window seem to be dead”. There was a breakthrough with the invention of lasers by Theodre Maiman in 1960`s and this led to various avenues of light therapy studies, including phototherapy and photodynamic therapy (The Science of Phototherapy: An Introduction: Springer, 2005; N.S. Soukos et al 2011, D.P. Valenzeno, 1990).

Phototherapy is also called heliotherapy and has a long history in the field of dermatology. This has been in existence since 1500 BC, in various parts of the world. Black seeds from the plant *Psoralea corylifolia* (The Science of Phototherapy: An Introduction: Springer, 2005) in combination with sunlight exposure were used to treat skin conditions such as psoriasis and vitiligo. The compound psoralen, which is found in these plants, is a furocoumarin derivative, and acts as a photosensitizer in treating skin conditions. There are also thousands of natural photosensitizing compounds such as anthroquinones including hypericin, fagopyrin, chlorophyll, polyacetylenes, thiophene derivatives, quinones (cercosporin), angelicin, aflatoxin, alkaloids, terthienyl, and polyacetylenes (Konopka, 2007; Ebermann et al, 1996). The science of applying a photosensitizer in conjunction with any light of the electromagnetic radiation (Violet-Blue light, ultra violet light (UVA, UVB), red light) is known as photodynamic therapy (PDT). PDT

with synthetic compounds such as 5-aminolevulinic acid, its lipophilic derivative methyl amino levulinate, hematoporphyrin (Hp), derivatives of hematoporphyrin (HpD) are frequently used in the treatment of skin conditions such as actinic keratosis, bowens disease, basal cell carcinoma, cutaneous lymphoma, viral warts, leishmaniosis, psoriasis, scleroderma, necrobiosis lipoidica and Hailey-Hailey disease (Maria Claudia Almeida Issa et al, 2010; Leman & Morton, 2002).

1.2 Light Therapy Studies in Various Disciplines

In addition to applications in dermatology, PDT is also used as an adjunct treatment of various tumors. PDT is also used as a supplement in the treatment of brain tumors such as glioblastoma or astrocytoma, oligodendroglioma, and malignant glioma (Dougherty et al, 1998; Noske et al, 1991). It is used in the treatment of esophageal, lung, bladder, and papillary tumors as well. Preceding the application of light in the treatment of tumors, it was also widely applied in the late 1950s in the treatment of hyperbilirubinemia or jaundice in newborns. The visible light converts bilirubin into water soluble isomers and is excreted into the urine without much metabolic action through the liver (Stokowski, 2011).

In the 1980's, phototherapy was also used in the treatment of seasonal affective disorder (SAD), behavioral, bipolar, and other mood disorders (Kasper et al, 1989; Leibenluft et al, 1995). Low-level laser light therapy (LLLT) with a wavelength of 830 nm enhanced tissue repair and in the temporomandibular joints (TMJ) of induced arthritis in male Wistar rats. There was also a reduction in the

extracellular matrix degradation contributing to remodeling of joints (Lemos, et al, 2016). Signs of inflammation such as the number of leucocytes and vascular extravasation were reduced in rats which were experimentally induced with inflammation on treatment with an 810 nm infrared laser (Pallotta et al, 2012). Light of wavelengths 685 and 830 nm in an experimentally induced arthritis reduced edema formation, vascular permeability and hyperalgesia (De Morais et al, 2010). Helium Neon (He-Ne) laser was used as a treatment for rats with induced osteoarthritis in the knee which had an increased mucopolysaccharide synthesis after 2 months (Lin et al, 2006).

An in vitro study showed human cell line osteoblast proliferation and differentiation with 635 nm He-Ne laser irradiation (Stein et al, 2005). Osteoblast proliferation and viability have been studied with wavelengths of 635 to 809 nm, and 809 nm had an intermediate effect after 48 hrs which necessitated more laboratory studies on bone regeneration with standardized irradiation parameters (Bolukbasi et al, 2017).

Light therapy studies have also been explored in the prevention and control of myopia progression (Tori et al, 2017). Phototherapy has been widely applied in various fields of medicine and surgery, in detection, diagnosis and treatment. The principle of phototherapy and its mechanism are interrelated with the generation of fluorescence or phosphorescence and destruction of cytotoxic cells with the production of free radicals. Blue light of the visible region with a shorter wavelength ranging from 400 to 500 nm is used to irradiate. It causes the emission of light in the red region of the spectrum of longer wavelength, with lower energy, called

fluorescence. This principle of fluorescence is also applied for detection of abnormalities and in diagnosis followed by treatment.

There are 3 components which are indispensable for phototherapy or photodynamic therapy and for the generation of fluorescence. They are,

A. Light of specific wavelength

B. Photosensitizer

(1)Exogenous (Rose Bengal, Erythrosine, Toluidine Blue and more)

(2) Endogenous (flavins, porphyrins, cytochrome hemoproteins and much more)

C. Molecular oxygen

Light with the appropriate wavelength ranging from 400 to 500 nm is irradiated over a photoactive compound or a photosensitizer. The photosensitizer which is in its ground singlet state, absorbs the energy from photons of light emission. The photosensitizer undergoes a transition of reactions and gets excited from a ground singlet state to an excited singlet state. The excited molecules are further converted to an excited triplet state. The excited triplet has an affinity towards molecular oxygen. Under normal conditions, molecular oxygen is in its ground triplet state. This reaction generates excited singlet oxygen thereby releasing free radicals such as hydroxyl ions, superoxide ions, and hydrogen peroxide. These oxidation products can react with biological materials such as protein, lipids, and nucleic acids and cause cytotoxicity. Since the excited singlet state photosensitizers are short lived, they lose energy and go back to its original ground state by emitting light of longer wavelength in the red region and lower

energy. This is called fluorescence. The principle of fluorescence is used for detection and diagnosis of pathological conditions, and the production of free radicals is used in phototherapy. The interrelationship between the principle of fluorescence and the production of free oxygen radicals is illustrated **(Figure 1.1)**.

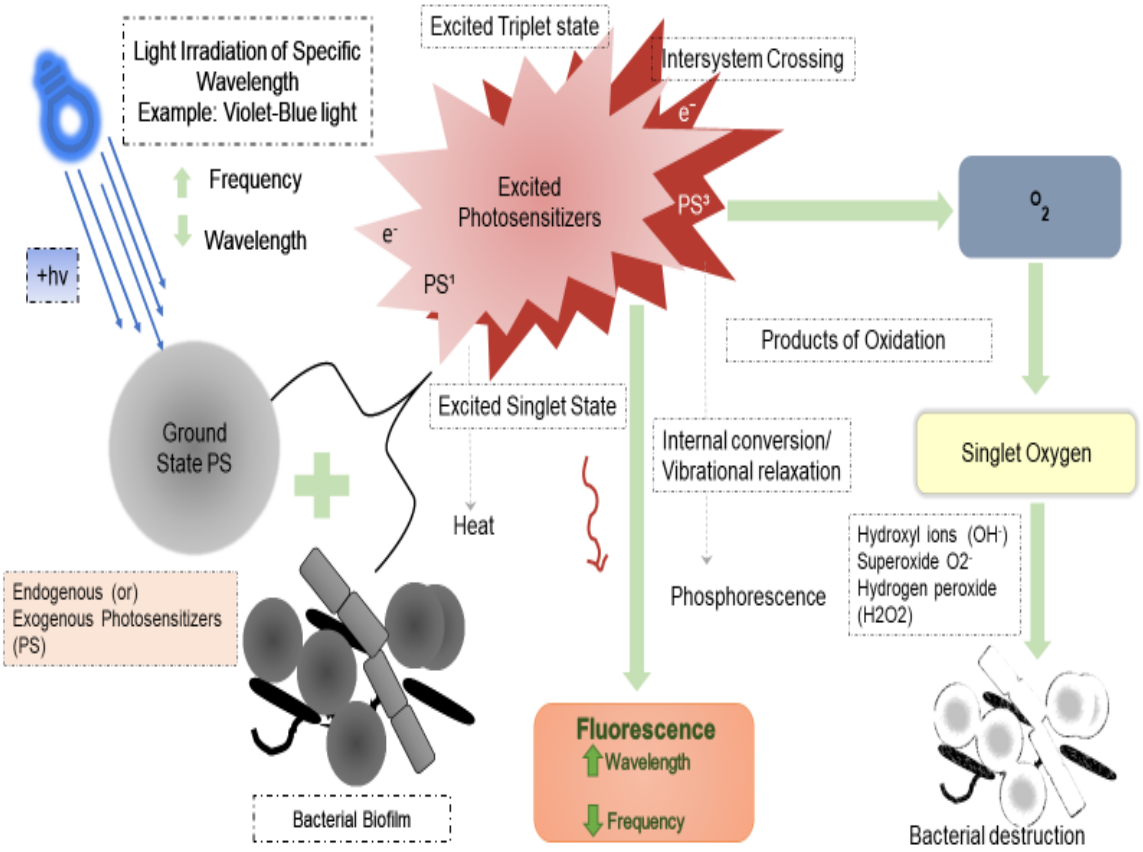

Figure 1.1 Interrelationship between fluorescence and photoinactivation.

Light with a specific wavelength irradiated on a ground state photosensitizer (photoactive compound) absorbs the energy from photon of light undergoes a transition from a low-energy to an excited singlet state. The transfer of energy from the activated photosensitizer to available oxygen results in the formation of singlet

oxygen resulting in production of free radicals and toxic oxygen species. These very reactive chemical species can react with the biological materials damage proteins, lipids, nucleic acids, and other cellular components leading to cytotoxicity. The life span of the excited singlet state of the photosensitizer will be very short. The singlet state will lose its energy and will go back to its ground state by emitting light longer wavelength and lower energy known as fluorescence.

1.3 Light Therapy on Bacteria and Microorganisms

In 1899, Oscaar Raab a medical student from Germany observed that acridine orange as a chemical agent killed *Paramecium caudatum* (Raab, 1900; Soukos et al, 2011). The lady with the lamp, Florence Nightingale, believed in the importance of sunlight to the sick and stated that light should shine in through windows at least on one side of the patient's bed. In the 1800's, ultraviolet violet (UV) light was discovered and one of the scientists who worked with UV light, Finsen, mentioned previously, received a Nobel prize in 1903 for his work. It was done by filtering sunlight to heal lupus vulgaris caused by tuberculosis (Roelandts, R. 2002). Later in the 1970s, phototherapy or light therapy without the use of photosensitizer was employed. It was used in treatment of wounds and in the inactivation of bacteria (Percival et al, 2014).

Light inhibition of microorganisms such as *Acanthamoeba polyphaga*, *Candida albicans*, *Mycobacterium massiliense*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* with wavelengths of 450, 520, and 630 nm was studied

and had a phototherapeutic effect. There was a 94% growth inhibition suggesting a bactericidal effect (Decarli et al, 2016). Microbial reduction through phototherapy in conjunction with photosensitizers is called Photodynamic Antimicrobial Chemotherapy (PACT). This mode of treatment is highly effective in pathogenic infections such as osteomyelitis caused by *S. aureus* (Lozanao et al 2015; Reis et al 2015).

Antibiotic resistant microorganisms such as methicillin resistant *S. aureus* and *P. aeruginosa* were killed effectively with methylene blue and a non-thermal activating light with a wavelength of 670 nm (Biel et al, 2011). Malachite green with light was effective on *Bacillus* and *Listeria* which causes food spoilage and contamination (Brovko et al, 2009). *Candida dubliensis*, found in immunocompromised individuals, and *Sporothrix schenckii* complex, which causes sporotrichosis, were found to be susceptible to light in the presence of photosensitizers (Gasparett et al, 2010 and Gilabarte et al, 2014).

1.4 Light Therapy on Oral Bacteria

In the 1990's, PDT was used on oral microorganisms (Okamoto et al, 1992), although there was one published study in 1970 with light alone on dental plaque (Orstavik, 1979). Various studies have been conducted with a wide range of oral bacterial species namely, Candida species such as *C. albicans* and *C. glabrata* periodontal pathogens such as *Porphyromonas gingivalis*, and subgingival cariogenic organisms such as *Streptococcus sanguis* and *Streptococcus mutans*

in the presence of photosensitizers such as methylene blue, rose bengal and curcumin. Every organism has its own characteristics to get inactivated by a specific wavelength, intensity, and time duration (Soria-Lozano et al, 2015, de Miranda et al, 2015; de Figueiredo Freitas et al, 2017). Aggressive periodontitis caused by *Aggregatibacter actinomycetemcomitans* can be inactivated by indium-gallium-aluminum-phosphate laser with a wavelength of 662 +/- 0.1 nm, energy density of 6 J/cm^2, and Radaclorin as a photosensitizer (Moslemi Neda et al., 2015). Light was also used to disinfect prostheses such as surgical guides or prosthetic dentures. *E. coli* and other microorganisms growing on acrylic resin dentures were killed by light in combination with photosensitizers (de Freitas pontes et al, 2014).

Besides cariogenic microorganisms and periodontal bacteria, phototherapy is also employed to disinfect root canals and endodontic applications. They are effective on *Enterococcus faecalis* and *Fusobacterium nucleatum* with methylene blue, Toluidine blue, eosin or curcumin (Manoil et al, 2016; Peng et al, 2007; Soukos et al, 2006; Pileggi et al, 2013). The primary focus of this dissertation project was to investigate specifically the effect of violet to blue light on *S. mutans*, a primarily oral cariogenic bacterium without the application of an external photosensitizer.

1.5 Dental Caries and *S. mutans*

The oral microbiome is composed of 13 phyla, and one of the phyla is the firmicutes, which are predominantly found and include 227 taxonomic species (Dewhirst et al, 2010). Firmicutes are further divided into the classes of bacilli, clostridia, erysipelotrichia, negativicutes, and thermolithobacteria. The class bacillus includes the genus streptococcus containing mostly gram positive, opportunistic and saprophytic bacteria with cell walls. The classification of streptococci is based on their ability to hemolyze blood cells.

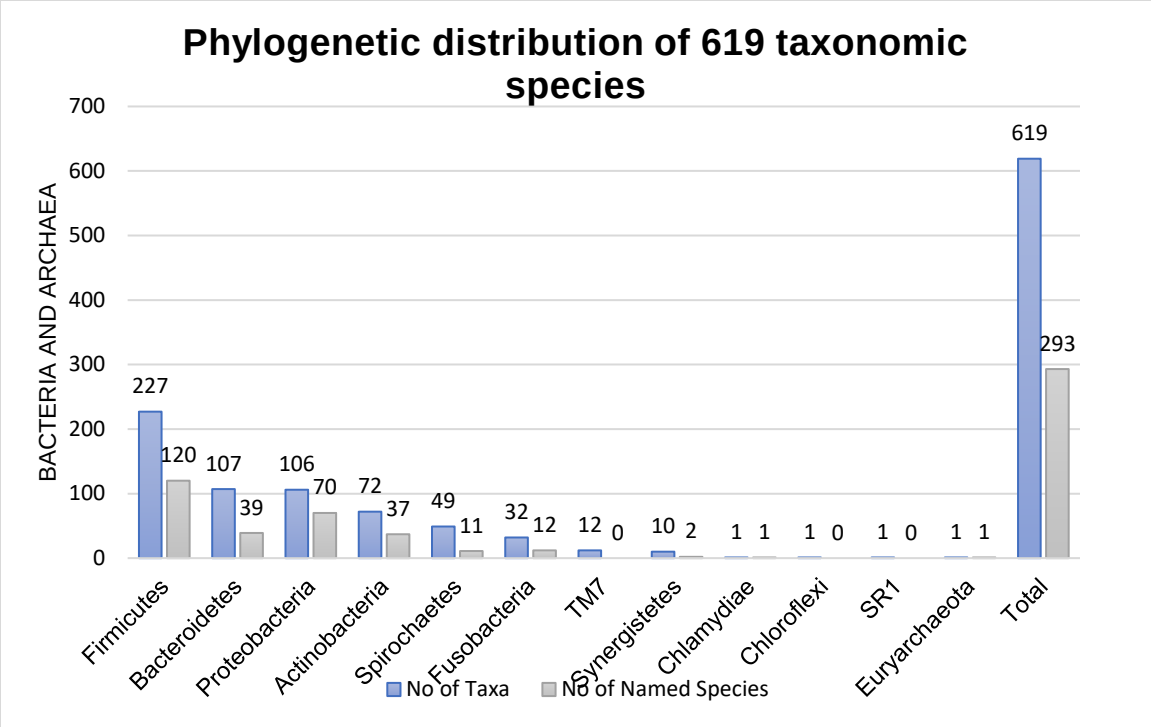

Figure 1.2 Phylogenetic distribution of 619 taxonomic species.

Taxa including cultivated, named, unnamed and uncultivated species are distributed within the 13 phyla based on Human Oral Microbiome database (HOMD). Adapted and Modified from Dewhirst et al, 2010.

The ecosystem of the oral cavity has around 619 prokaryotic (taxonomic) species which have been identified through 16s rRNA gene cloning studies. This Oral microbiome is classified into 13 phyla and among them Firmicutes are predominantly found which is further classified into class Bacilli and has the genus Streptococcus **(Figure 1.2)**.

The streptococcus genus represents about 53% of all genera in the oral cavity (Peterson et al, 2014). Within the genus streptococcus is the species *S.*

mutans. Epidemiological studies have observed the causal association of *S. mutans* and dental decay. *S. mutans* is present in the oral cavity from the time of the first tooth erupt in the mouth. With its unequivocal ability to metabolize sugars, it produces organic acids. Therefore, its presence is indicative of its cariogenicity. Even before *S. mutans* was found, Miller in 1890's stated that there is no sharp line of distinction between a pathogenic and a non-pathogenic bacterium. Any bacterium brought into the host in sufficiently large masses for a longer period can elicit an inflammatory reaction. In 1891, W.D Miller in his book "The human mouth as a focus of infection" reported that "The unanimous verdict of investigations is decay of teeth that is the most widespread disease, in which the human body is subject, and the prognosis is exceedingly good." He also added that "It often becomes a disease of grave nature when it affects the young or weak" (Miller, W.D, 1890).

In 1924 *S. mutans was* isolated from a carious lesion from humans by J. Killian Clarke. *S. mutans* was consistently observed in all early lesions. Out of 50 teeth with early lesions, *S. mutans* was present in 40 pit and fissure caries and in 10 approximal enamel lesions (Clarke, J.K, 1924).

Dental caries is a pan epidemic disease seen then and now affecting everyone irrespective of age, gender and ethnicity. The incidence of caries in adults has declined in developed countries; however, childhood caries remains a public health problem (Selvitz, 2007). A national call to action to promote oral health was to find new approaches to prevent dental caries (National Call to Action, 2003). Dental plaque or oral biofilm is an aggregate of microbes, and in conjunction

with its interaction with host factors, it is one of the etiological factors of dental caries. Biofilms are stated as “masses of microorganisms that bind to and multiply on a solid surface, typically with a fluid bathing the microbes” (Huang et al, 2011). The biofilm creates a sessile life on surfaces. The first step involves an acquired pellicle formation, which is a thin proteinaceous film formed by surface salivary proline rich proteins, mucins, salivary agglutinins, alpha amylase, lysozyme and other proteins on a clean tooth surface. Oral streptococci are initial or early colonizers. Upon attachment, oral streptococci secrete extracellular polysaccharide substances (EPS) which help them during the binding process (Huang et al, 2011).

Late colonizers then come into action and recognize protein receptors or polysaccharide receptors on the surface of the early colonizers, aggregate, coaggregate and thereby form a micro community. The final phase is dispersion which takes place when the nutrients are limited or due to host defense mechanisms. Also, it may occur due to competition from other oral bacteria. The biofilm cells which are dislodged will find a suitable niche and start a new life. *S. mutans* is one of the primary cariogenic bacteria involved in oral biofilm formation. Early dental caries is reversible and preventable but later, more mature carious lesions are irreversible.

1.6 Light Therapy on *S. mutans* Biofilm

Various preventive treatments are currently used, which have been shown to reduce, inhibit, and even eliminate oral biofilm. Non-invasive phototherapy/photodynamic therapy is a method currently studied against microbial infections to overcome the emergence of antibiotic-resistant bacterial strains. “A clean tooth never decays”, so the non-operative mode of treatment at the microscopic level is necessary to control caries and prevent the progression of caries (Fejerskov and Kidd, 2009). Visible light within the spectral range from 380 to 700 nm is commonly used to inhibit or kill bacteria. The quantitative light induced fluorescence (QLF) used in this thesis study is an early caries detection light device with an excitation peak wavelength of 405 nm and a spectral range of 380 to 440 nm, and it is based on fluorescence technology. I was given an opportunity to analyze QLF images in 2012 for a longitudinal study which looked at the progression of caries (Zandona et al, 2013) for a four-year period. The images were acquired with a light source employing a Xenon arc lamp with a wavelength from 380-520 nm. Since the light used is towards the ultraviolet spectrum, Violet-Blue light were reviewed for its inhibitory effects on *S. mutans*. This led us to study the effect of light on *S. mutans*. During the analysis of QLF images, an orange to red fluorescence was observed in several images. This observation was also described and studied by other researchers (al-Khateeb et al, 1997; Buchalla, 2005; Heinrich-Weltzien et al, 2003; Konig et al, 1998). The orange to red fluorescence emanated from dental plaques and was related to carious lesions

observed in QLF images. The fluorescence was postulated to be due to bacterial metabolic byproducts called porphyrins. Researchers have shown previously that dental plaque on the surface of the tooth and in carious lesions fluoresce in the orange to red region of the electromagnetic radiation. Dental plaque is a mixture of oral microorganisms that contributes to the origin of fluorescence, which is an etiological factor of dental caries. *S. mutans*, the primary cariogenic bacterium, was also hypothesized to produce and contribute to the overall fluorescence. Auto fluorescence of *S. mutans* was studied at an excitation wavelength of 385 or 405 nm with an emission wavelength at 770 or 800 nm, which confirmed the presence of a possible fluorophore responsible for absorption of Violet-Blue light.

1.7 Research Objectives

Blue light of the visible region of the electromagnetic radiation has antimicrobial properties. Based on the pioneering work of Dr. Andrea Ferreira Zandona (Zandona et al, 2013) on QLF, which uses a Violet-Blue Light to acquire images based on fluorescence technology, and the observation of orange to red fluorescence on the acquired QLF images, an extensive literature review was conducted, and we embarked on a project to look at the effect of Violet-Blue light on bacteria, specifically *S. mutans*. Phototherapy studies had been previously conducted with *Porphyromonas gingivalis* and an acne-causing bacterium, *Propionibacterium acnes*. A few studies were conducted with *S. mutans* in conjunction with hydrogen peroxide and a plasma arc dental curing light with a

wavelength of 400-500 nm. PDT studies with the use of photosensitizers such as Toluidine Blue, Erythrosine, and Rose Bengal on *S. mutans* have been common. However, studies without the use of photosensitizers were much fewer. We undertook this project with QLF technology, primarily an early caries detection device, which uses Violet-Blue light. We also borrowed the delayed antibacterial effect concept from others (Steinberg et al, 2008; Feuerstein et al, 2006; and Chebauth Taub et al, 2012). None of the light therapy studies have used QLF as a light source for phototherapy studies. The proposed thesis project was further accomplished by an in vitro study determining the effect of Violet-Blue light on *S. mutans* biofilm. The photoinactivation of *S. mutans* biofilm was studied through quantitation of colony forming units and determination of the kinetics of the growth of *S. mutans* at 2 and 6 h after reincubation. Further, the metabolic activity of *S. mutans* with the treatment of Violet-Blue light was measured through a colorimetric tetrazolium assay after 0, 2 and 6 h of reincubation. These experiments were followed by a translational study that was designed to test the effects of Violet-Blue light in *S. mutans* biofilm-induced carious lesions on human dentin and enamel specimens. Based on the observation of orange-red fluorescence on the QLF images, a retrospective study was conducted to determine the association between the orange/red fluorescence on surfaces that progressed to cavitation compared to the surfaces that did not progress to cavitation as determined by the International Caries Detection and Assessment System (ICDAS). If carious lesions could fluoresce, we proposed that cariogenic bacteria, specifically *S. mutans,* contribute to the overall fluorescence. We hypothesized that a fluorophore or

chromophore within the bacterium which absorbs the Violet-Blue light. A fluorometric assay was developed to evaluate the autofluorescing properties of *S. mutans* biofilm and clinical isolates of caries active and caries resistant strains of *S. mutans.* Finally, the fluorophore in *S. mutans,* which might be responsible for the absorption of Violet-Blue light leading to photo-mediated destruction of the bacteria and emission of the fluorescence observed in the biofilms, was detected with Quadrupole Time of Flight Liquid Chromatography/Mass Spectrometry (QTOF-LC/MS).

Chapter 1 of this dissertation discusses the published studies on *S. mutans* with blue light and also with an UV spectrum close to 380 nm. Few studies with light alone have been performed. Though PDT studies have been found to be more effective, we wanted to elucidate the effect of Violet-Blue light from a QLF device on *S. mutans*.

In Chapter 2 - a literature review was conducted to determine the effect of blue light within the 380 to 500 nm wavelength range with and without photosensitizers on *S. mutans*.

Chapter 3 discusses the effectiveness of Violet-Blue light at 380-440 nm to inhibit or kill early *S. mutans* biofilm without the presence of photosensitizers. Based on the results on the inhibition of biofilm formation, reduction in colony forming units and growth rate in the treated groups, we hypothesized that there must be absorption of light by a biological molecule, a fluorophore or a chromophore within the bacterium. We also examined metabolic activity and glucan binding protein expression as an extension of the previous study.

Chapter 4 explores the metabolic activity and glucan binding protein expression of *S. mutans* in response to violet to blue light. We performed a colorimetric tetrazolium assay after 0, 2 and 6 hrs of reincubation of *S. mutans* biofilm following a 5-minute treatment with Violet-Blue light.

Chapter 5 describes a translational study to determine the inhibitory effects of Violet-Blue light on carious lesions induced by *S. mutans* on dentin specimens. Lesion depth for treated and the untreated groups of dentin specimens are given as supplemental information.

Chapter 6 further explores the effect of Violet-Blue light on carious lesions induced by *S. mutans* on human enamel specimens.

Chapter 7 is focused on identifying the role of the fluorescence observed in carious lesions. If Violet-Blue light has photo destructive properties on *S. mutans*, the bacterium should have a biological molecule, a chromophore or fluorophore that has the ability to absorb the blue light from electromagnetic radiation. A four-year longitudinal observational study (Zandona et al, 2013) was conducted to determine the surrogate of carious lesions that progress rapidly in a high-risk population using QLF. An analysis of QLF images revealed an orange to red fluorescence on these images as observed by other researchers (Lennon et al, 2006, Coulthwhite et al, 2006, Bittar et al, 2014). So, we carried out a retrospective analysis to determine whether there is an association between the intrinsic orange to red fluorescence and the progression of carious lesions.

Chapter 8 examines the autofluorescing properties of *S. mutans.* Following the previous study, we proposed that if carious lesions fluoresce with blue light and

if the cariogenic bacterium *S. mutans* gets inactivated by the light, there must be a biological molecule or a fluorophore which might cause this phenomenon. This fluorophore may contribute to the overall orange to red fluorescence observed in dental plaque and in carious lesions. Based on this, we studied the fluorescent capacity of *S. mutans* from subjects with high and low *S. mutans* counts. Some work related to the influence of various media and hemin on *S. mutans* fluorescence was also conducted.

Chapter 9 details the detection of the fluorophore, protoporphyrin IX, in *S. mutans* biofilm using sophisticated Liquid chromatography/ Mass Spectrometry (LC/MS).

Chapter 10 concludes with a discussion of conclusive findings of the projects and future investigations.

Chapter 2

Light Therapy in the Control of *Streptococcus mutans* Biofilm

A Literature Review

Dental caries is a biofilm mediated and diet modulated disease. A complex mixed bacterial flora comprises the biofilm. Currently *Streptococcus mutans* is considered to play a major role in the caries process. Oral biofilm is typically removed by mechanical and chemotherapeutic approaches with each having limitations. New novel antibacterial treatments are being researched. Light therapy including phototherapy [PT] and photodynamic therapy [PDT] are among these novel treatments. The aim of this literature review is to present an overview of light therapy in the control of *S. mutans* biofilm. There are limited studies that focus on the effectiveness of light therapy on *S. mutans*. An electronic literature search was done with Endnote in the database PubMed by using the combined terms: “Blue light”; “*Streptococcus mutans*”; “phototherapy”; “photodynamic therapy”; “light therapy”; and “biofilm”. Twenty-three articles in English published between 1979 and 2015, that met the inclusion criteria, were found and reviewed. The studies were predominantly *in vitro*, and some were *in situ* and *in vivo* clinical studies. Based on this review, it was determined that various factors mediate photoinactivation and bacterial killing. Studies differed in the following: photosensitizers [PS] with varied absorption spectrum, multiple light sources with different wavelengths, incubation time of the bacteria, duration of exposure, power

density or intensity of the light, distance between the light source and the *S. mutans* biofilm, the amount of bacterial biofilm during irradiation, various volumes of solution above the biofilm, sucrose content and other factors. The conclusive finding of this review was that light in combination with a PS has a more potent antibacterial effect than the use of light alone. However, more studies are needed to reach a common agreement on mediating killing or inhibition of *S. mutans* by light. Based on the known side effects of PS, commonly used PS in dental practice should be considered. However, in general, more studies on the effectiveness of light without the addition of exogenous PS are required.

2.1 Introduction

Dental caries is a preventable, chronic, and biofilm mediated disease modulated by diet with a pandemic distribution (Edelstein, 2006, Loesche, 1986, Selwitz et al, 2007). It has a multifactorial etiology caused by the interaction of host factors, diet, and microorganisms. The oral microbiota has a commensal relationship with the human host and may become pathogenic due to environmental changes, especially diet, affecting the equilibrium of the microorganisms, thereby initiating the disease process. These oral microbes tend to aggregate to form biofilms commonly called dental plaque causing oral conditions such as dental caries, gingivitis, periodontitis, and other diseases (Avila et al, 2009).

Bacteria in biofilms are typically more resistant to antimicrobial treatments than their planktonic counterparts [reviewed Huang et al, 2011). Although the understanding of dental caries has progressed significantly since the postulation of the “non-specific plaque hypothesis” (Loesche, 1979), the long-standing treatment philosophy in clinical dentistry is still based on this theory. An over-accumulation of dental plaque can be removed by mechanical methods like tooth brushing, flossing, and by interdental brushing. In addition, chemical antimicrobial mouth rinses can be used to remove plaque. Complete removal of dental plaque is not achieved by the above methods; however, the removal of biofilm of friendly bacteria is not desired as the non-pathogenic bacteria typically prevent colonization of pathogenic bacterial species. The examination of Koch’s postulates on a specific bacterium causing a single known disease led to the development of the “specific plaque hypothesis” which suggests a causal relationship between *S. mutans* and dental caries (Clarke, 1924, Gibbons, 1964, Simon, 2007). The current understanding of dental caries as an ecological shift in the tooth-surface biofilm modulated by dietary patterns leading to a mineral imbalance between plaque fluid and tooth does not refute the important role of *S. mutans* (Marsh, 1994, Kidd and Fejerskov, 2004).

A biofilm-forming microorganism such as *S. mutans* was initially isolated by Clarke (Clarke, 1924). It is a facultative anaerobic, gram-positive streptococcus, and is considered to be the primary cariogenic bacterium among other oral microbes. Research has been conducted to control and balance biofilm through novel approaches, thereby combating antibiotic resistance (Ten Cate and Zaura,

2012). Current investigations include the use of probiotics, which include live microorganisms [for example: *Lactobacillus casei* and *Bifidobacterium dentium* used in cheese and yogurt, respectively] in sufficient doses to reduce the number of *S. mutans*. Prebiotics such as arginine, xylitol, and urea are used to raise the pH of the oral cavity and reduce biofilm formation. Nano technological approaches [i.e., specifically targeted antimicrobial peptides, bacteriocins, and quorum sensing signals] are used to balance biofilm (Ten Cate and Zaura, 2012). One novel approach among these research advances is using light as a therapeutic agent to kill *S. mutans* and other oral bacteria (Chebath-Taub et al, 2012, Feuerstein et al, 2006, Steinberg et al, 2008). The natural source of light used in conjunction with photoactive compounds like PS is called PDT. Using light alone without the presence of any PS is termed PT. Light therapy including PDT and PT and its effect on *S. mutans* are discussed in detail in this review. According to our knowledge, there are no review studies exclusively on the effect of PT/PDT on *S. mutans*.

2.2 Photodynamic Therapy

Among the various methods proposed, PDT and PT can be effective approaches to control oral biofilms **[Tables 2.1** and **2.2**, respectively**]**. PDT is a light therapy, which also includes PT. It is also called photo radiation therapy or photo chemotherapy. In the 1900's, acridine orange was used in combination with light to kill *Paramecium caudatum* (Marsh, 1994, Gold, 2011). It was successfully

used in the treatment of cancers using porphyrin-based PS such as porphyrins, chlorins, bacteriochlorins, and phthalocyanines (Soukos and Goodson, 2011). The emergence of antibiotic resistance has turned the focus towards alternative treatments such as photoactivable compounds or PS, which get activated by an appropriate wavelength, duration, and intensity thereby mediating the destruction of the bacteria through the action of reactive oxygen species [ROS] on bacterial DNA and other microbial targets. *Streptococcus sanguis, Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Aggregatibacter actinomycetemcomitans* are susceptible to toluidine blue O [TBO], methylene blue [MB], aluminum disulphonated phthalocyanine [AlS2Pc], and Hematoporphyrin HCl in the presence of a helium/neon laser or LED light (Dobson and Wilson, 1992, Wilson, 1993). Other bacteria including *Actinomyces naeslundii, Enterococcus faecalis, F. nucleatum, Pseudomonas aeruginosa, Prevotella intermedia, Proteus mirabilis, Peptostreptococcus micros, Streptococcus intermedius, S. mutans, Streptococcus pyogenes,* and *Streptococcus sobrinus* are susceptible to various light sources in the presence of different PS (Konopka and Goslinski, 2007). PDT is widely studied in *S. mutans* and in combination with other oral bacteria.

2.3 Phototherapy

PT is also called light therapy or heliotherapy and is a type of PDT. PT does not use any exogenous PS, and the endogenous PS in bacteria are used with PT to mediate bacterial destruction by the action of ROS. PDT and PT have been

investigated to control oral biofilms. Bacteria such as *Propionibacterium acnes* (Ashkenazi et al, 2003, Papageorgiou et al, 2000), *Staphylococcus aureus* Maclean M et al, 2009, *Helicobacter pylori* (Ganz et al, 2005), and *P. aeruginosa (*Guffey et al, 2006) are susceptible to light or PT. Oral bacteria including *P. gingivalis, P. intermedia, Prevotella nigrescens*, and *Prevotella melaninogenica* are susceptible to PT at a wavelength range of 380 to 520 nm (Soukos et al, 2005). Studies with PT only on *S. mutans* had been conducted to a limited extent compared to light with PS. PT without the use of exogenous PS has been effective on *P. acnes* (Ashkenazi, 2003, Papageorgiou, 2000), *Actinomyces odontolyticus, P. gingivalis* and *P. intermedia* (Soukos and Goodson, 2011, Soukos et al, 2005, Konig et al, 2007).

2.4 Photosensitizers [PS]

Chemical photosensitizing compounds can be used to kill bacteria in combination with light. They occur both naturally and in synthetic forms. Photosensitivity is the reported side effect of using these chemical compounds. However, an ideal PS should be activated only locally and be eliminated rapidly after the reaction. It should be cost effective, water soluble, produce high levels of oxygen reactive species, and exhibit storage stability. They should have low toxicity and more absorption with less transmission. (Konopka and Goslinski, 2007). Erythrosine [ER] and rose bengal [RB] belong to the xanthene group and are used as dental plaque disclosing agents, and in ophthalmological diagnosis,

respectively. PS compounds which have been approved for clinical use are Photofrin, a hematoporphyrin derivative; Visudyne, a benzoporphyrin derivative monoacid; Levulan, a 5 amino levulinic acid; Metivx, a methylaminolevulinate; and Foscan, meta-tetra hydroxyphenyl chlorine. These are mostly used in treating precancerous and cancerous oral lesions. Other PS compounds widely used in PDT in treating oral biofilms are TBO, MB, Photofrin, hematoporphyrin oligomers, and malachite green [MG]. Chemical compounds widely used for other oral bacteria are aluminum disulphonated phthalocyanine [AlS2Pc], Hematoporphyrin HCl [HP-HCl]; polyethyleneimine-chlorine 6 conjugate [PEI-Ce6]; polylysine-chlorine 6 conjugate [pL–Ce6]; Sn [IV] chlorine 6 [SnCe6]; and Zn[II]-phthalocyanine [ZnPc] (Konopka and Goslinski, 2007).

2.5 Light Sources

Light from the visible spectrum of electromagnetic radiation is mostly used in PDT and PT to mediate bacterial inhibition and killing. Visible light is sandwiched between the infrared and ultraviolet spectra with a narrow wavelength band spectrum from 400 to 700 nm. Light sources commonly used to inactivate *S. mutans* are light emitting diodes [LED] with a white light wavelength of 400 to 500 nm. (Metcalf et al, 2006, Feuerstein, 2006), CO_2 lasers and Er: YAG lasers (Krause, 2008, Feuerstein, 2012). Coherent and non-coherent light sources, monochromatic light, and low power lasers have also been used. Commonly used light sources in dentistry are curing lights, so all types of curing lights including

non-coherent blue light, plasma arc curing lamp, halogen lamp, and LED's can be used.

2.6 Mechanism of Action

A photoactive dye or photosensitizing compound, when irradiated with light of a specific wavelength or a range of wavelengths, gets activated and undergoes an energy transition from a ground state to an excited singlet state. The transfer of energy from the activated PS to available oxygen results in the formation of toxic oxygen species, such as singlet oxygen and free radicals called ROS. These very reactive chemical species can damage microbial and host proteins, lipids, nucleic acids, and other cellular components **(Figure 2.1)**. Subsequently, the PS may decay back to its ground state, with emission of fluorescence, or may undergo a transition to a higher-energy triplet state. The porphyrin-containing compounds act as exogenous substances and get activated to a ROS in the presence of light. There are some bacterial species, which are pigmented or have endogenous porphyrins that do not need exogenous compounds; however, they are more susceptible to light in the presence of PS (Soukos NS& Goodson JM 2011).

Previous literature reviews have focused only on PDT and its various applications in dentistry. There are no literature reviews focusing on PDT/PT exclusively on *S. mutans* biofilm. The aim of this review was to present an overview of light therapy and determine the effectiveness of light therapy including PDT/PT to control *S. mutans* biofilm.

2.7 Methods

Literature Search Strategy

An automated electronic literature search of databases such as PubMed was conducted using Endnote [bibliographic software] inclusive from years 1971 to 2015. The keywords used in the search were: “Blue light”, “*Streptococcus mutans*”, “phototherapy”, “photodynamic therapy”, “light therapy”, and “biofilm”. Combinations of keywords and MesH terms were used. The search yielded a total of 128 articles after removing duplicates. Studies related to planktonic cultures of *S. mutans*, periodontal oral pathogens, and use of light in wavelengths below 380 nm or above 700 nm were excluded. Our eyes can perceive wavelengths from 380 to 700 nm. Articles which use light sources in the visible light spectrum between 380 to 700 nm in the presence of light and PS and with only light in the absence of PS were included. Only *S. mutans* biofilm studies were included. Rejection of articles was based on titles, abstract, and articles in languages other than English (Costache I & Danila I 2010). The number of articles that met the inclusion criteria was 25 published between 1979 and 2015. The studies included were predominantly *in vitro*. There were 20 *in vitro* studies, 4 *in vivo/in situ* studies, and 1 clinical trial. To the best of our knowledge, there are no randomized clinical trials published in the areas of cariology and PDT/PT. In 2014, a single blinded randomized clinical trial on PT was conducted on mild to moderate chronic periodontitis patients.

2.8 Effectiveness of PT

In 1979, an *in-situ* study revealed that UV light irradiation alone reduced biofilm growth on bovine enamel fixed on the first mandibular molar of human subjects. The protein concentrations from the oral biofilm following UV light treatment [tubular ultra-pressure fluorescent lamp [TUV], 15W, Philips] for 10 minutes were less than those without UV light (Orstavik D & Ruangsri P, 1979). However, the wavelength of light used in this study was not measured. TUV primarily produces longer wavelengths of UVA ranging from 315 to 400 nm, fitting the inclusion criteria of this review. Blue light sources are commonly used in dentistry for photo curing of composites. Non-coherent blue light with a wavelength from 400 to 500 nm has a delayed antibacterial effect on *S. mutans* biofilm. It affects the reorganization and formation of new biofilm 6 hours after exposure to blue light. Live bacteria were significantly reduced after 3, 5, 7 and 10 minutes of exposure (Chebath-Taub D et al 2012). Light with wavelengths of 450 to 490 nm in conjunction with 0.3 mM hydrogen peroxide demonstrated a 96% inhibition of *S. mutans* growth after 20 seconds, and there was a 3% reduction with only light. Light alone was not effective at 20, 30, 40, 60 and 180 seconds; however, it was effective after 10 minutes of exposure (Feuerstein O et al 2006). Biofilm genes such as *brpA*; glucosyltransferase [*gtfB*]; biofilm formation hypothetical protein, *smu630*; competence stimulating peptide; and *ComDE* are up-regulated after 60 seconds of exposure to light (Steinberg D et al, 2008). This is an interesting finding suggesting increased expression of genes related to bacterial biofilm formation

and adhesion in the presence of light and H_2O_2 owing to stressful environmental conditions (Steinberg D et al, 2008).

The use of light either alone or with any chemical substance has not been reported to induce microbial resistance, which makes it eco-friendly (Feuerstein O, 2012). Bacteria in biofilm are more resistant to antibacterial agents than planktonic cells; however, light has been shown to be effective on biofilm and suspended planktonic bacteria. Diode lasers, having a monochromatic coherent continuous emission with a wavelength of 830 nm, are typically accompanied by an aiming device and a LED delivering light of 630 nm and 5mW. The investigators reported a reduction of 90.8 and 97.7% in colony-forming units at 5 and 7 Watts, respectively, through 500 um thickness of dentin. The efficiency of bacterial killing was reduced with increased thickness of dentin. This study was conducted on planktonic *S. mutans* and was not included for review (Lee BS et al 2006). Previous work in this laboratory demonstrated that Violet-Blue light within a range of 380 to 440 nm with a peak at 405 nm can inhibit *S. mutans* growth and reduce biofilm formation (Gomez GF et al 2016). Effectiveness of light alone depends on a specific wavelength, log phase of bacterial growth affecting cell multiplication and division, incubation time, light source, duration of light exposure, thickness of biofilm, presence of sucrose, intensity of the light, growth media, and many other factors. The use of visible light to rapidly provide an antibacterial effect has limitations; however, visible light lacks side effects and does not cause resistance (Feuerstein O et al 2006). The only published randomized clinical trial was conducted on mild to moderate chronic periodontitis patients treated with LED-

embedded electric toothbrushes. The duration of exposure was 3 minutes, and there was no reduction with *S. mutans.* However, there was a significant improvement in the studied clinical periodontal parameters (Jung GU et al 2014).

2.9 Effectiveness of PDT

ER, RB, TBO, MB, and MG are widely used PS in PDT. In a recent study, *S. mutans* grown for 48 hours exhibited a 0.52 $\log_{10}$ reduction with ER and a 0.62 $\log_{10}$ reduction with RB. The light source used had a wavelength of 455±20 nm and an output power of 200 mW for 180 seconds (Pereira CA et al, 2013). With light alone, there was no reduction in *S. mutans* biofilm. In another study, a dental halogen curing light with a wavelength of 400 to 520 nm in combination with ER at 20 µM affected the viability of 18-hour-old *S. mutans* biofilm. With light alone, there was a slight reduction, but this was not statistically significant. The light-treated group was compared with the group, which had no light treatment and no PS. Bacterial cell death was 75% and 55% after 8 hours of incubation, and 74% and 42% after 12 hours with 0% and 0.1% sucrose, respectively (Lee YH, 2012). Fractioned short light doses on 200 µm thick *S. mutans* biofilm exhibited increased reduction of bacterial survival over continuous irradiation for 5 minutes in the presence of 22 µM ER (Metcalf D et al 2006).

In an in-*situ* study, Wilson M (Lima JP et al, 2009) demonstrated a significant reduction of *S. mutans* with TBO and a red light-emitting diode with a wavelength of 620 to 668 nm, a peak wavelength at 638.8 nm, and energy

densities of 47 and 97 Jcm^{-2}. *S. mutans* exhibited significant reduction upon exposure to light at 97 Jcm^{-2} for 10 minutes.

As expected, *S. mutans* is more susceptible to PDT in planktonic cultures than in biofilm. A study by Paulino et al. indicated that RB [0.5 µM] using a dental hand held photopolymerizer [HHP] with a wavelength range from 400 to 500 nm and an energy range of 350 to 500 Jcm^2 for 30 seconds killed 100% of the treated *S. mutans* planktonic cells (Paulino et al 2005). Without the presence of RB, *S. mutans* did not demonstrate any reduction in viability. The duration of exposure ranged from 0 to 40 seconds with the distance of the bacterial cells at 11 cm from the light source in a 1 ml volume of the 36 hour-grown *S. mutans* planktonic culture.

S. mutans biofilm grown for 36 to 48 hours and irradiated at 630 nm showed significant killing with porfimer sodium [Photofrin] (Mang TS et al 2012). MB at 0.1 mg/ml was used as a PS with a low power InGaAlP laser of wavelength 660 nm for 98 seconds on a *S. mutans* biofilm grown for 5 days. Pereira CA et al (2011) showed that 90 seconds of treatment on dentinal samples collected from a carious lesion (Guglielmi Cde A, 2011) controlled oral biofilms. An *in vivo* clinical study among denture wearers indicated that dental plaque growth was reduced by Photogem gel [a hematoporphyrin derivative] in concentrations of 50 and 100 mg/l90% of the total microorganisms tested were eliminated from the dentures (Ribeiro DG et al, 2012). A similar study also demonstrated that Photogem and LED light affected the viability of *S. mutans* biofilm grown for 24 hours; however, the bacteria recovered from the PDT treatment after 24 hours (Silva TC et al 2012).

A comparison study of ER with MB and photofrin suggested that ER could be a potentially effective sensitizer. Fifteen minutes of irradiation from a Tungsten filament lamp with a wavelength of 500 to 650 nm resulted in log reductions of 1.5 ± 0.1 and 2.6 ± 0.2 for 48- and 288-hour biofilms, respectively (Wood S et al 2006). The effectiveness of various PS including MB, TBO, and MG in combination with LED at a wavelength of 636 nm and Eosin, ER, and RB in combination with a curing light at 570 nm was studied. TBO achieved a 99.9% reduction of *S. mutans* planktonic cultures. PDT was effective in the presence of TBO and MG compared to other PS and demonstrated 3 and 1.4 log reductions, respectively (Rolim et al, 2012). A similar result was reported in an *in vitro* study with 99.99% reduction in viability of *S. mutans* biofilms treated with TBO and LED or a helium neon laser (Zanin IC et al, 2005). The same group studied the effect of TBO on 5-day old *S. mutans* biofilm irradiated with an LED demonstrating a 95% reduction in bacterial viability (Zanin IC et al 2006). Light at a dose of 48 J/cm^2 in association with TBO at 0.1 mg/ml had the greatest effect on *S. mutans* biofilm on carious dentin (Giusti et al 2008). A laser diode and LED in combination with MB irradiated for 120 and 88 seconds, respectively, provided significant reductions in bacterial viability (Ricatto LG et al 2014). MB in combination with a red LED of 630 nm had a significant reduction in viability (de Freitas Pontes, et al 2014).

Curcumin, a natural spice, and PS used in conjunction with blue light exhibited a significant reduction of *S. mutans* (Manoil D et al 2014, Araujo NC et al 2014*)*. Other adjunct compounds such as hydrogen peroxide were used to

inactivate *S. mutans* with light and caused a 98.9% reduction in viability (Unosson E et al 2013).

Phenathiazine chloride used as a PS in combination with light at 660 nm irradiated for 2 minutes decreased the live/dead ratio of *S. mutans*. This artificial biofilm model study indicated that PDT was effective on a bacterial biofilm with the thickness of 10 µm (Schneider M, et al 2012). The addition of nanoparticles such as chitosan [CS] with ER augmented the bactericidal effect of light with a 10-minute exposure (Chen CP et al 2012). UV-A in conjunction with Titanium dioxide [TiO_2] exhibited reduction in bacterial viability (Cai Y et al 2014). All of the studies reviewed here showed that PDT had potent efficacy against *S. mutans*. It is clear that PDT in conjunction with any PS compound is effective. However, a one-size-fits-all approach cannot be applied, and the PS with the lowest toxicity, most rapid effect, commercial availability, and low cost should be chosen to be used with a portable light source that provides non-harmful effects.

2.10 Conclusion

A common consensus based on this review is that PDT with PS mediates killing of *S. mutans* more effectively than PS or light alone. However, there are limited studies focusing on light alone without PS. Appropriate light parameters such as wavelength, intensity, and duration can elicit the photo inactivation process. Various light sources with a different range of wavelengths, duration of exposure, incubation time which affects the thickness of the biofilm, distance

between the light source and biofilm, methodology, and pre-irradiation time with PS determine the effectiveness of light therapy. ER is commonly used as a dental disclosing agent and demonstrates potent PDT effects, and RB used in diagnosis of cataracts may provide alternate treatment approaches to control biofilms. Studies related to gene expression, endogenous fluorophores in bacteria, extracellular matrix, and structure of the biofilm after light therapy should be given importance. PS compounds that are easily available, inexpensive with low toxicity and reduced photosensitivity after activation by harmless visible light, are required. Future studies focusing on the best potential PDT and PT should be conducted; however, more time is required to kill biofilm cells with light alone. Since there are limited studies on light therapy without PS, we chose to conduct experiments without the presence of exogenous photosensitizer with a Violet-Blue light coming from an early caries detection device called Quantitative light induced fluorescence (QLF).

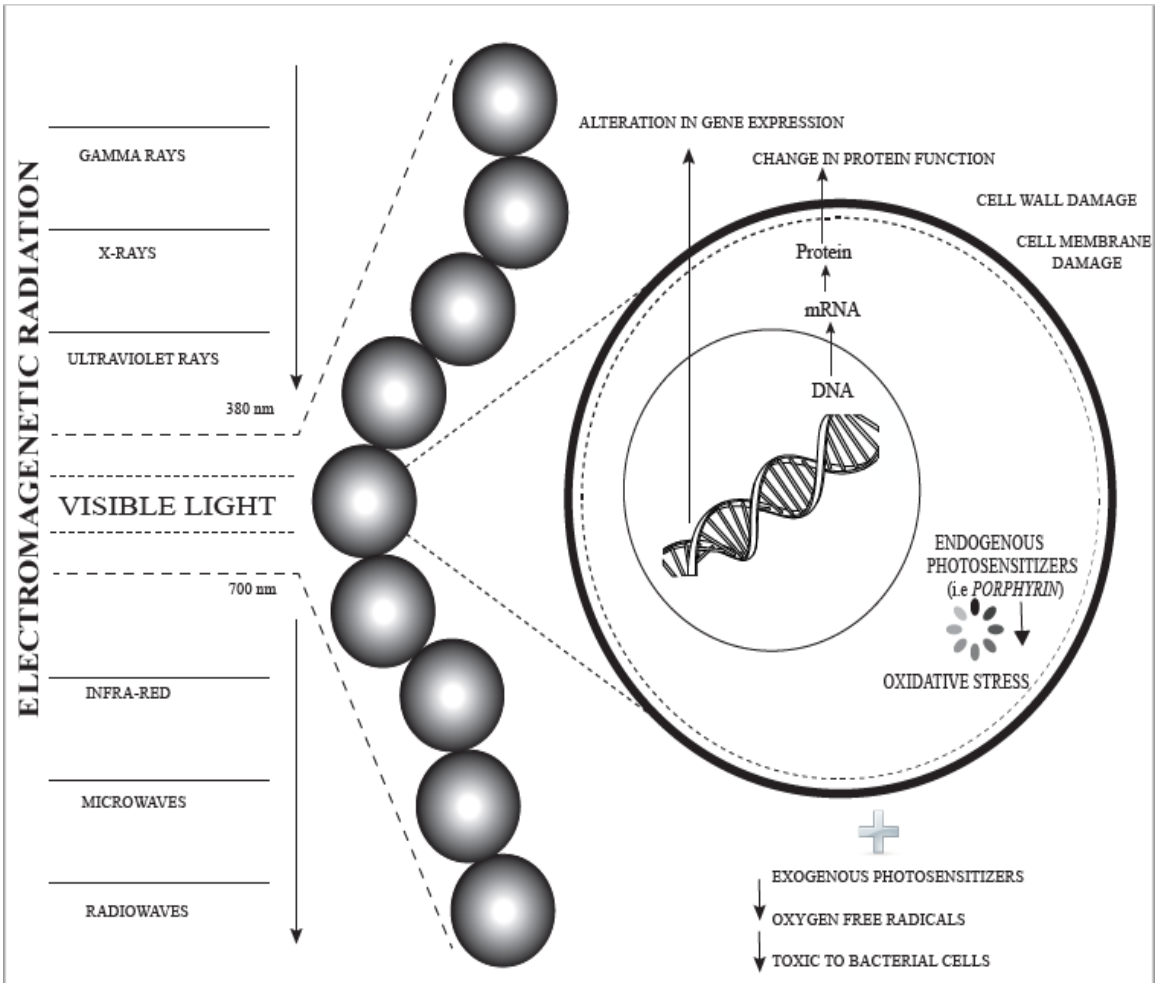

Figure 2.1 Possible mechanism of action of visible light on *S. mutans.*

Violet-Blue visible light with a range of wavelengths from 380 to 500nm irradiated on *Streptococcus mutans* biofilm is absorbed by endogenous or exogenous photosensitizers. The activation of photosensitizer in combination with molecular oxygen releases free oxygen radicals such as hydroxyl and superoxide anions causing bacterial toxicity. The cytotoxic free radicals react with protein, lipids and nucleic acids causing altered protein and gene expression.

Table 2.1 Light therapy studies without the use of photosensitizers (PS)

Table 2.1. Light therapy studies without the use of photosensitizers

Author	Year	Light Source/ Wavelength (nm)	Photosensitizer	Incubation Time	Exposure Time	Distance	Outcome	Results	Study Design
Jung, et al.	2014	Electric toothbrushes with light emitting diodes (LEDs) with wavelength (635 nm) and power density ($13mW/cm^2$)	No	Subgingival plaque samples from mild to moderate periodontitis patients	3 min	N/A	Clinical parameters (plaque and gingival index; probing pocket depth and clinical attachment level) and microbiological outcomes	*S. mutans* did not show statistical reduction with phototherapy. However, improvements in clinical outcomes and periodontal bacterial reduction were seen.	Randomized controlled clinical trial
Chebath –Taub, et al.	2012	Plasma arc lamp (400-500 nm) and power density (1.14 W/cm^2)	No	Biofilm formation for 24 hrs (40% sucrose). Regrowth of biofilm at 2, 4 and 6 hrs after light exposure	0, 1, 3, 5, 7,10 min equal to 68, 204, 340, 476, 680 J/cm^2	1.5 cm	Bacterial viability and live/dead ratio	Significant decrease in CFU at 7 and 10 min for regrown biofilm at 6 hrs. Significant decrease in live/dead bacterial ratio ($p < 0.029$) for 3, 5, 7, 10 min. Affects reorganization of biofilm after 6 hrs of reincubation.	*In vitro*
Sol, et al.	2011	Opus 20 CO_2 laser 10.6(μm)	No	Biofilm formation for 24 hrs (4% sucrose)	Energy densities of 31, 70 and $144J/cm^2$	2.5 cm	Bacterial viability, biofilm architecture and gene expression	Significant killing effect with 70 and 144 J/cm^2. Extracellular matrix is affected in irradiated biofilm. Genes related to energy metabolism, protein function, transport and binding were upregulated.	*In vitro*
Orstavik & Ruangsri	1979	TUV (tubular ultrapressure fluorescent lamps), 15 Watts, UV light source; Philips	No	Plaque formation on bovine enamel on appliances placed in molars of human volunteers	10 min	3 cm	Bacterial adherence and plaque formation	Significant reduction in plaque accumulation. Treatment was done twice on consecutive days/3 times/week.	*In vivo/In situ*

Table 2.2 Light therapy studies with commonly used photosensitizers (PS)

Table 2.2 Light therapy studies with commonly used photosensitizers (PS)

Author	Year	Light Source/ Wavelength (nm)	Photosensitizer	Incubation Time	Exposure Time	Distance	Outcome	Results	Study Design
Wood, et al.	2006	400W tungsten filament lamp (500-550 nm; intensity 22.7 mW/cm^2) and (600-650 nm; intensity 22.5mW/cm^2)	Erythrosine (ER), Methylene blue (MB), photofrin (22 µM)	48, 120, 168, 216 and 288 hrs of biofilm formation	15 min	30 cm	Bacterial viability	ER was 1-2 $\log_{10}$ more effective than photofrin, which was 0.5-1 $\log_{10}$ more effective than MB.	*In vitro*
Metcalf, et al.	2006	400 W white light source (500 to 550 nm)	ER (22 µM)	48, 120, 168, 216 and 288 hrs	Continuous irradiation for 0, 1, 2, 5, 10, 15 or 30 min/ fractions of 30 sec and 1 min pulses	N/A	Bacterial viability	98% killing occurred in the first 5 min (2 ± 0.2 $\log_{10}$). 1 min pulse five times with recovery period in between for 5 min (3.0 ± 0.3 $\log_{10}$) and 30 sec for 10 times with 2 min recovery (3.7 ± 0.3 $\log_{10}$). Fractionating light doses were more effective compared to continuous irradiation.	*In-vitro*
Chen, et al.	2012	LED 540 ± 5 nm/22 mW/cm^2	ER with Chitosan (CS)	24 hrs	25 J/cm^2 and 50 J/cm^2	N/A	Microbial cell survival and binding of ER with bacteria	2.5- and 4.5-log reduction in viable cell count was observed with 10 min and 2 hrs incubation time. Eradication with ER/CS and light at 50J/cm^2. Addition of CS with ER augments killing effect.	*In vitro*
Zanin, et al.	2006	LED (620 to 660 nm) power 32 mW/Energy density 85.7 J/ cm^2	TBO	3, 5, 7 days (5% sucrose)	7 min	N/A	Bacterial viability and microhardness	Significant reduction (95%) in viability with TBO and light. No statistical reduction in the presence of PT	*In vitro*
Cristiane Aparecida Pereira, et al.	2013	Blue LED 455±20 nm/ Output power - 200 mW fluence of 95 J/cm^2 / fluence rate of 526 mW/cm^2	5 µM of RB /ER	48 hrs	3 min	N/A	Bacterial viability	0.52 $\log_{10}$ CFU reduction with Erythrosine and 0.62 $\log_{10}$ CFU reduction with RB. No reduction with PT.	*In vitro*
Pereira, et al.	2011	Low-power laser irradiation/InGa AlP laser (660 nm)	MB (0.1 mg/ml) for 5 min	5 days/BHI with 5% sucrose	98 sec	N/A	Bacterial viability and association of bacteria with the acrylic resin	2.32-3.29 $\log_{10}$ reduction in PDI mediated by MB.	*In vitro*
Guglielmi Cde, et al.	2011	InGaAlP (indium gallium aluminum phosphide) /660 nm; 100 mW/320 J/cm^2	0.01% MB	dentinal samples from deep carious lesion	90 sec	N/A	Bacterial viability	1.38 log reduction after PACT (photodynamic antimicrobial chemotherapy)	*Ex vivo/In vivo*
Zanin, et al.	2005	HeNe laser (gas laser, 632.8 nm or LED-620 to 660 nm	TBO	Hydroxyapatite discs with artificial saliva in 2% sucrose 4 times a day (5 days)-constant depth (300 µm)	5, 15 or 30 min to HeNe laser or LED light. Power output 32 mW	N/A	Bacterial viability	Viability reduced up to 99.99% with both light sources & TBO Bactericidal effect was dependent with time. No significant reduction only in the presence of light	*In vitro*
Lima, et al.	2009	LED (620 to 660 nm)	TBO	Intra oral device (palatal with human dentin slabs) worn for 14 days	5 min and 10 min	2 mm	Significant reductions in the bacterial count (3.45 and 5.18)	*In situ*/ Without PS irradiation for 10 min had a significant reduction.	*In situ*

Silva, et al.	2012	Biotable with 40 red LED's 610 to 650 nm/3 mW	0.25 mg mL-1 of Photogem/Photo dynamic antimicrobial chemotherapy(PACT)	24 hrs (0.2% sucrose) (8 hrs anaerobic incubation/transferred to fresh medium /incubated for 16 hrs	25 min (75 J/cm^2)and 50 min (150 J/cm^2)	9 cm	Bacterial viability ,integrated mineral loss (IML) and lesion depth (LD)	3 log reduction with Photogem and Light.	*In vitro*
Schneider, et al.	2012	Diode laser (wavelength 660 nm), output power 100 mW	Phenothiazine chloride	Salivary pellicle layer formation for 4 hrs chambers/Artificial biofilm model with *S. mutans* grown for 6 hrs	2 min	N/A	Bacterial viability	Fluorescence values for laser irradiation(median 2.1 U, range 0.4-3.4 U); light and photosensitizer (median 3.6 U, range 1.1-9.0).	*In vitro*
Ribeiro, et al.	2012	LED 440–460 nm (24 blue LED lights) Intensity-24 mW/cm^2	Photogem (hematoporphyrin derivative in concentrations of P50S, P100S in suspension, P50G, and P100G in gel)	6 mth denture wearers without using disinfectant soaking solution; 48 hrs incubation after treatment	26 min (37.5 J/cm^2)	N/A	Bacterial viability	Significant reduction with Photogem gel (100G and 100S) with p = 0.045 and 0.014.	*In vivo*
Mang, et al.	2012	KTP:YAG Laser (630 nm; power density 100 mW/cm^2)	porfimer sodium Photofrin® (concentration 25 to 125 µg/mL)	36-48 hrs	5 min	N/A	Bacterial viability	Significant reduction with PS and light.	*In vitro*
Giusti, et al.	2008	LED 630± 10 nm (400 mW/cm^2 and output power of 200 mW)	Hematoporphyrin derivative (Photogem;1, 2, and 3 mg/mL) or TBO (0.025 and 0.1 mg/mL)	14 days on dentin fragments	60 sec (fluence of 24J/cm^2) and 120 sec (48J/cm^2)	N/A	Bacterial viability	Photogem or TBO was effective for bacterial reduction the greatest effect on *S. mutans* was obtained with TBO at 0.1 mg/mL and a dose of 48 J/cm^2.	*In vitro*
Ricatto, et al.	2014	LASER and light emitting diode (LED) with 95J/cm^2	MB	24 and 48 hrs incubation on bovine dentin specimens	120 sec and 88 sec	2 cm	Bacterial viability	Laser and LED with MB caused a significant reduction in CFU/mL when compared with laser without MB. Laser without MB showed reduction compared to the control group.	*In vitro*
anoil, et al.	2014	QTH lamp (360 – 550nm), 450mW/cm^2	Curcumin (concentrations of 5, 10, 20, 40, 60 µM)	48 hrs at 37°C	120 sec	N/A	Bacterial viability	Significant reduction with 5 min incubation with curcumin at 5and 60 µM and increased antibacterial activity at 10 min incubation.	*In vitro*
de Freitas-Pontes, et al.	2014	Red LED (630 nm) and output power of 150 mW	MB (0.05%)	48 hrs at 37°C	Irradiation time to achieve energy intensities 10 J/cm^2 and 30 J/cm^2	N/A	Bacterial viability	0.05% MB sensitized *S. mutans*; however was not effective with light.	*In vitro*
Cai, et al.	2014	Ultraviolet-A (UV-A) light	Titanium dioxide (TiO_2)	16 hrs	UV-A irradiation dose of 8.4 J/cm^2 to 43 J/cm^2	N/A	Biofilm characterization viability and metabolic activity	8.4J/cm^2 of UV-A radiation will cause one order magnitude of reduction and 43J/cm^2 had 5-6 order of magnitude in viability of bacteria in a biofilm aged 16 hr.	*In vitro*
Steinberg, et al.	2008	Xenon lamp (400-500 nm) power density 1.14W/cm^2; Average light power 440 mW	No photosensitizer; H_2O_2 concentrations of 3, 30 and 300 mM	24 hr/4% sucrose	30 sec (34J/cm^2) or 60 sec (68 J/cm^2)	1.5 cm	Bacterial viability and gene expression	Light and H_2O_2 showed significant reduction in all concentrations at 30 and 60 sec/No significant reduction with PT.	*In vitro*

Chapter 3

Photo inactivation of *Streptococcus mutans* Biofilm by Violet-Blue Light

As we stated in the literature review, non-invasive phototherapy/photodynamic therapy is one method used to control oral biofilm. Studies indicate that light at specific wavelengths has a potent antibacterial effect. The objective of this study was to determine the effectiveness of Violet-Blue light at 380-440 nm to inhibit biofilm formation of *Streptococcus mutans* or kill *S. mutans*. *S. mutans* UA159 biofilm cells were grown for 12-16 h in Tryptic Soy broth (TSB) or TSB with 1% sucrose (TSBS) in wells of 96-well flat-bottom microtiter plates. Biofilm was irradiated with Violet-Blue light for 5 min. After exposure, cultures were re-incubated at 37°C for 2 or 6 h to allow the bacteria to recover. A crystal violet biofilm assay was used to determine relative densities of the biofilm cells grown in TSB, but not in TSBS, exposed to Violet-Blue light. The results indicated a statistically significant ($p < 0.05$) decrease in biofilm cells compared to the non-treated groups after the 2 or 6h recovery period. There was a significant reduction in the growth rate of the Violet-Blue light-treated cells grown in TSB and TSBS. Biofilm viability assays confirmed a statistically significant difference between Violet-Blue light-treated and non-treated groups in TSB and TSBS. These results showed that visible Violet-Blue light has the ability to inhibit *S. mutans* growth and reduce the formation of *S. mutans* biofilm. Potential clinical applications

of light therapy in the future remain viable in preventing the development and progression of dental caries.

3.1 Introduction

The human oral cavity is a cornucopia of microbes with a symbiotic relationship to the human host (Avila M et al 2009; Ruby J and Goldner M, 2007). Commensal oral microbes share space in the oral cavity in a state of quiescence, protecting the human host from pathogenic bacteria (Liljemark WF and Bloomquist C, 1996). These non-pathogenic bacteria have the potential to become pathogenic, when factors related to changes in the oral environment disrupt their homeostasis (Williams RE,1973). Dental plaque, a common term for oral biofilm, is an aggregate of microbes found on the tooth surface embedded in a matrix of polymers of bacterial and salivary origin that interacts with the environment and host (Avila M et al, 2009; Matosevic D et al, 2010; Ogaard B, et al 1994; Papageorgiou P et al, 2000). Oral microbial biofilm has been shown to be an etiological factor for dental caries and other oral diseases (Socransky SS 1968). Conditions that create an imbalance in the oral environment such as increased number of bacteria, low pH, and an increased intake of sucrose in the diet causes “conditional oral diseases” (Liljemark WF and Bloomquist C,1996; Socransky SS and Haffajee AD, 2002). *S. mutans*, a facultative anaerobic, acidogenic and aciduric bacterium, is a major pathogen of dental caries (Bratthall D,1972; Hamada S and Slade HD 1980; Loesche ,1975).

Accumulation of dental biofilm is typically controlled and prevented by daily brushing, flossing, antiseptic rinses, and antimicrobial agents. Cleansing and maintaining the correct balance of microbial organisms is nearly impossible to achieve completely with traditional methods of oral hygiene measures as most individuals fail to remove the biofilm completely (Ogaard B ,1994). New technologies and approaches have been suggested to control the formation of biofilms (ten Cate JM and Zaura E, 2012). Bacteria in biofilms are more resistant to antimicrobial treatment than planktonic free-floating bacteria (Huang R et al,2011; Stewart PS and Costerton JW 2001). The search for alternative treatment methods to eliminate biofilm has turned to visible light of the electromagnetic spectrum. Optical properties of light are used by several caries detection devices to visualize carious lesions at their incipient stage. Quantitative light induced fluorescence (QLF), one of the earliest caries detection devices that uses a violet–blue light with a peak wavelength of 405 nm, was employed in this study.

Previous studies demonstrated that blue or orange light within a specific wavelength has a potent antibacterial effect (Ashkenazi H et al, 2003; Enwemeka CS et al, 2009; Ganz RA et al, 2005; Guffey JS and Wilborn J 2006; Maclean M et al, 2008; Maclean M et al,2009; Papageorgiou P et al 2000). In the 1990's, research focused on photodynamic therapy employing photosensitizers to enhance the killing of oral bacteria. Phototherapy without exogenous photosensitizers was used to eliminate *Porphyromonas gingivalis, Prevotella intermedia, Prevetolla nigrescens* and *Prevetolla melaninogenica*, and it is believed that endogenous porphyrins in the oral black-pigmented periodontal

bacteria are excited at 380 to 520 nm releasing reactive oxygen species (ROS) (Soukos NS, et al, 2005). Recent studies by Chebath-Taub et al, 2012 and Steinberg et al, 2008 indicated that *S. mutans* biofilm loses the ability to form new biofilm when exposed to blue light in the range of 400 to 500 nm and proposed a new concept of delayed antibacterial activity. In the present study, we hypothesized that Violet-Blue light from a QLF device with an exposure time of 5 min can kill *S. mutans* or inactivate established *S. mutans* biofilm formed during 12 to 16 h of growth without any exogenous photosensitizer. The effectiveness of the Violet-Blue light was determined by relative density of biofilm mass, viability of biofilm cells, and growth rate of *S. mutans* planktonic and biofilm bacteria.

3.2 Materials and Methods

3.2.1 Bacterial Strain and Growth Media

Cells of *S. mutans* strain UA159 (ATCC 700610) were cultivated in Tryptic Soy broth (TSB, Acumedia, Baltimore, MA) overnight in a 5% CO_2 incubator. Biofilm was formed in wells of 96-well flat bottom polystyrene microtiter plates (Fisher Scientific, Co., Newark, DE) using either TSB or TSBS. Biofilm cells were grown in triplicate, and the distance between the biofilm wells prepared from TSB and TSBS was kept at an 8-10 well distance. The distance was maintained to reduce light scatter between treated and untreated wells within and among groups. The cultures were incubated for 12 to 16 h at 37°C in a 5% CO_2 incubator.

3.2.2 Light Source

Quantitative light induced fluorescence (QLF™/CLIN Inspektor Research System BV, Amsterdam, Netherlands), which primarily uses fluorescence-based technology to detect early caries, was used in this study. The light source of this device was a 35 - Watt Xenon arc lamp, with an external light source diameter of 5 mm. The intensity of Violet-Blue light on tooth surfaces was approximately 13 mW/cm^2 as reported by the manufacturer. An optical high pass band filter was used to extract Violet-Blue light. The light was passed through a liquid filled light guide. Wavelength (nm) and radiant power (mW) of the light source were measured with a laboratory-grade spectrometer (Model USB2000, Ocean Optics Inc., Dunedin, FL). The spectrometer setup consisted of a fiber optic integrating sphere (FOIS-1, Ocean Optics Inc.) that collected the light, a fiber optic line that connected the integrating sphere to the spectrometer, which was then connected to a computer for analysis of the light using SpectraSuite software (Ocean Optics Inc.). Prior to use, all equipment was calibrated with a National Institute of Standards and Technology (NIST) traceable light source (LS-1-CAL, Ocean Optics Inc.). Biofilm at the bottom of a single well of a 96 well microtiter plate was irradiated for 5 min with a distance of 2 cm from the light source. The spectral irradiance or incident radiance of the light was approximated by measuring the radiant power (mW) of the light at a distance of 2 cm and dividing by the area of the opening of the integrating sphere. The average irradiance was calculated to be approximately 30.872 mW/cm^2, and the fluence or radiant exposure for a period of 5 min was

estimated to be 9.26J/cm^2. The wavelength detected ranged from 380 to 440 nm with a peak wavelength of 405 nm **(Figure 3.1)**. There was a spectral overlap of blue and violet light in the wavelength detected, so the terminology Violet-Blue light was used throughout the study. The heat dissipated at the end of the light guide was measured using a thermometer, and an average increase of 1.375°C was observed over a 5-min interval.

3.2.3 Microtiter Plate Biofilm Assay

The effect of Violet-Blue light on *S. mutans* biofilm mass was determined by a biofilm crystal violet staining assay. The distance between the light source tip and the biofilm was maintained at 2 cm. Before exposure, the supernatant liquid was removed and Violet-Blue light from the QLF was exposed directly to the wet biofilm continuously for 5 min. After exposure, 200 µL of fresh TSB or TSBS was replaced in their respective wells. The control group was not exposed to Violet-Blue light but was kept under room light conditions. To remain consistent, supernatant liquid was removed from the control group, and after 5 min fresh TSB or TSBS was added. After exposure, cultures in wells of the microtiter plates were incubated at 37°C in a 5% CO_2 incubator for 2 or 6 h to allow the biofilm to recover before biofilm staining. The biofilm was gently washed twice with sterile saline (0.9% NaCl), and 100 µL of 10% formaldehyde was added to fix the biofilm cells for 30 min. The biofilm cells were then carefully washed twice, and 100 µL of 0.5% crystal violet was added for a period of 30 min to stain the biofilm. The stained cells

were washed three times, and 200 µL of 2-propanol was added to extract the dye from the biofilm cells for 1 h. The extracted biofilm cell dye was diluted 1:5 with isopropanol. The absorbance was measured using a spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA) at 490 nm that provides quantitative information on the relative density of the biofilm cells exposed or not exposed to Violet-Blue light (Huang et al, 2012). The biofilm assays were repeated independently more than three times with similar results, and one representative experiment is reported.

3.2.4 Quantitative Determination of Bacterial Colony Forming Units (CFU)

In order to determine bacterial viability after exposure to Violet-Blue light, biofilm was exposed to Violet-Blue light for a period of 5 min and was immediately washed gently with sterile saline. 200 µL of sterile saline was added to each well, and the biofilm was gently scraped to remove biofilm cells. The bacterial suspension was serially diluted from 10^{-2} to 10^{-6} for both TSB and TSBS cultures and plated in duplicates. Aliquots of the diluted bacterial suspensions were spiral plated (Spiral System™ Cincinnati, Ohio) on Tryptic Soy Agar plates (TSA) and incubated for 48 h at 37°C in a 5% CO_2 incubator. The number of viable bacterial colonies was counted using an automated colony counter (Synbiosis, Inc., Fredrick, MD). The number of colonies counted was calculated as CFU/ml and then compared to the treated group and the control groups for both TSB and TSBS

cultures. The viability experiments were repeated, and the data from the two experiments were combined.

3.2.5 Growth Kinetics of Combined Planktonic and Biofilm of *S. mutans*

The growth kinetics of combined planktonic and biofilm *S. mutans* cells in every well was measured by its total absorbance at different time intervals. Biofilm cells (approximately 14 h old) were prepared as described above, but a gap of 2 wells was kept between TSB and TSBS samples, and the empty well adjacent to the sample was plugged with a black painted clear acrylic rod (Auburn Plastics, Indianapolis, IN) to prevent overlapping light. A six-well gap containing black painted acrylic rods was placed between the exposed and non-exposed samples. Before irradiating with Violet-Blue light, the planktonic supernatant bacterial culture was discarded, and only the biofilm cells were exposed to Violet-Blue light for 5 min. After exposure, 200 µL of freshly prepared TSB or TSBS was placed into each well, and the microtiter plate was covered by a clear sterile film (Seal Mate, Excel Scientific, Inc., Victorville, CA) and incubated in a kinetic spectrophotometer (SpectraMax 190) at 37ºC. Total kinetic growth of *S. mutans* cells was recorded at 595 nm at 20 min intervals over 24 h at 37°C. Kinetic results for a time period of 6 h including maximum absorbance (highest absorbance value recorded during the 6 h duration), time to max (time to maximum absorbance), lag time (time from the start of the incubation to initiation of logarithmic phase), and Vmax (maximum

velocity, slope of exponential growth) during the logarithmic phase from the time of incubation in the spectrophotometer were analyzed.

3.3 Statistical Analysis

Statistical analysis was performed using Microsoft® Excel (MS Excel 2010). Student's t-test was used to analyze the means of both control and Violet-Blue light-treated groups. A p value of 0.05 or less was considered statistically significant.

3.4 Results

3.4.1 Effect of Violet-Blue Light on *S. mutans* Biofilm Formation

Our results demonstrated that biofilms (n = 3) grown in TSB, but not TSBS, when exposed to Violet-Blue light were significantly decreased ($p < 0.05$) compared with the non-treated group. After 5 mins of uninterrupted irradiation, the treated biofilms in TSB in either a 2 or 6 h recovery period exhibited significant reductions in total biofilm mass ($p < 0.05$) compared with the non-treated group **(Figure 3.2 (A)**.

3.4.2 Effect of Violet-Blue Light on *S. mutans* Biofilm Viability

The viability of bacterial cells in *S. mutans* biofilms grown in both TSB (n = 11 for Violet-Blue light-treated group; n = 9 for non-treated group), and TSBS (n = 7) exhibited a statistically significant difference between Violet-Blue light-treated and non-treated groups ($p < 0.05$). Logarithmic transformation was used for all the analyses. The percentages of bacteria killed by Violet-Blue light in TSB and TSBS were 70 and 50%, respectively **(Figure 3.3)**.

3.4.3 Effect of Violet-Blue Light on the Growth Rate of *S. mutans*

The kinetic growth over 6 h of the combined biofilm and planktonic *S. mutans* grown in TSB demonstrated exponential growth, whereas cells grown in TSBS had more linear growth **(Figure 3.4 (A) and (B)**. The kinetic data after 6 h following Violet-Blue light treatment representing the maximum absorbance, time to max, lag time, and Vmax clearly depicted reduced growth of *S. mutans* in the Violet-Blue light-treated TSB and TSBS groups **(Table 3.1)**. The growth kinetics of *S. mutans* in TSBS had two logarithmic phases during the 24 h period. The logarithmic phases were more pronounced in the non-treated group than in the treated group **(Figure 3.4 (C)**.

3.5 Discussion

Non-invasive phototherapy is one of the various approaches being studied to modify and control oral biofilm. Our results indicate that Violet-Blue light of wavelengths ranging from 380 to 440 nm can inactivate and kill cells in *S. mutans* biofilm without any photosensitizer. This study indicated that cells in S. *mutans* biofilms are susceptible to violet-blue light with an exposure time of 5 mins suggesting that *S. mutans* contains an endogenous photosensitizer. The combination of a specific photosensitizer with a light source of appropriate wavelength, availability of oxygen, and the type of a particular organism or a group of microorganisms plays a vital role in the application of photodynamic therapy (Gursoy H et al, 2013; Konopka K and Goslinski T 2007, Soukos NS et al, 2005). The mechanism behind photoinactivation of S. *mutans* is not known, and to our knowledge only one study has used Violet-Blue light without photosensitizer on S. *mutans* biofilms (Chebath-Taub D et al, 2012). They used a plasma arc lamp with 400 to 500 nm wavelength and a power density of 1.14 W/cm^2. Bacterial viability was affected at 3, 5, 7 and 10 min after 6 h of incubation. Another study by Feuerstein et al (Feuerstein O, et al 2006) determined the effect of light from a Xenon lamp with a wavelength ranging from 450 to 490 nm and an average power of 440 mW on *S. mutans* biofilm treated with hydrogen peroxide. They demonstrated a 3% reduction in bacterial viability at an exposure time of 10 min in the absence of hydrogen peroxide, and a 30% reduction in viability in the presence of hydrogen peroxide with 20 sec exposure time.

The potential mechanism of photoinactivation of Violet-Blue light exposure on biofilms is that the integrity of the bacterial cell membrane is affected, causing the contents to leak out and ultimately resulting in cell death. It was stated in previous studies that phototoxicity in the presence of exogenous photosensitizers such as Rose Bengal, Erythrosine, Toluidine blue, Methylene blue and many other photosensitizers increases upon light irradiation, caused by a series of energy transfers from light energy to molecular energy, thereby generating ROS and singlet oxygen that kill bacterial cells (Araujo NC, et al, 2012; Ganz RA, et al, 2005; Guffey JS and Wilborn J, 2006, Pereira CA et al, 2013). Studies also indicate that endogenous bacterial porphyrins act as photosensitizers causing bacterial cell death due to similar photochemical reactions (Aveline BM et al, 1998; Malik Z et al, 1990; van der Meulen FW et al, 1997; Wilson M, 1993). A fluorophore or a photosensitizing compound in the bacteria will absorb the light energy of the photons and undergo a cascade of reactions mediating photoinactivation. Previously studies conducted in photodynamic/phototherapy have used coherent and non-coherent light sources, such as dental curing lights with LED, halogen, or tungsten filament lamps. None of the previous studies have used a light source from an early caries detection device such as QLF, which is the uniqueness of our study. QLF works on the principle of a fluorescent-based technology. The Violet-Blue light of the QLF device, when focused on the surface of the tooth, causes the tooth to autofluoresce, presenting a green color; however, if there is bacterial accumulation associated with plaque or calculus, it will turn orange to red due to the excitation of bacterial porphyrins. It is proposed that endogenous porphyrins

become excited at 405 nm causing a cytotoxic effect (Matosevic D et al, 2010). However, several studies indicated that *S. mutans* does not exhibit red fluorescence, but appears green (Coulthwaite L et al,2006; Feuerstein O et al 2012). We have also observed (data not shown) that *S. mutans* biofilm, when captured on a QLF screen, appears green. It is noteworthy that our recent clinical findings correlate orange to red fluorescence seen on carious lesions in QLF images with lesion progression (Gomez GF et al 2016).

There was a statistically significant ($p < 0.05$) reduction in the total biofilm mass formed in TSB **(Figure 3.2 (A)**, but not in TSBS **(Figure 3.2 (B)**. There was also a statistically significant reduction in the number of viable bacterial colonies **(Figure 3.3)** and reduced growth rate in both TSB and TSBS cultures treated with Violet-Blue light **(Figure 3.4)**. Before exposure to Violet-Blue light, bacterial cultures grown in TSBS were more turbid than those in TSB. Sucrose serves as a substrate for *S. mutans* in the production of extracellular (EPS) and intracellular polysaccharides. EPS consists of glucans and fructans facilitating bacterial adherence to tooth surfaces. The dense and thick biofilm formed by *S. mutans* grown in TSBS may have limited penetration of light into the deeper layers of the biofilm. This was discussed by Feuerstein et al (Feuerstein O et al, 2006) that the use of hydrogen peroxide with Violet-Blue light causes increased light penetration into the deepest layers of the *S. mutans* biofilm. We believe that the microcolonies and cell aggregates in biofilm grown in sucrose-supplemented cultures prevent light from getting into the deeper biofilm structures. The distance of 2 cm between the light source guide and the top of the biofilm may cause energy dissipation

reducing efficiency. The increased effect of Violet-Blue light to inactivate *S. mutans* cells in biofilm in the absence of sucrose might be due to the less dense microbial biofilm formation. Light penetrates thin biofilm easier than the denser layers of biofilm grown in the presence of sucrose. A longer wavelength or a wavelength range with a higher intensity might be required for the inactivation of cells in biofilm with sucrose in the culture.

One of the limitations of our study is analyzing the growth kinetics of combined planktonic and biofilm of *S. mutans* cells rather than only biofilm cells. Another potential limitation of the study is a 5-min exposure time. Though it may be a good at home procedure, compliance of patients to a 5-min exposure to light will be challenging.

The ability of light without an exogenous photosensitizer to cause photoinactivation depends on parameters such as light source, appropriate wavelength or range of wavelengths, irradiance, fluence, duration of exposure, incubation time, thickness of the biofilm, and distance between the light source and biofilm.

3.6 Conclusions

In summary, there was a statistically significant reduction in biofilm formation grown without sucrose after 5 min of Violet-Blue light treatment followed by 2 or 6 h of recovery. The reduction in bacterial viability and the rate of kinetic growth was significant with Violet-Blue light treatment in both no sucrose and

sucrose groups. The future of light therapy in controlling biofilm formation in the oral cavity remains strong. Phototherapy in the control of oral biofilm may become an effective prophylactic procedure. However, more studies are necessary to determine the effectiveness and application of light treatment. This study[1] was succeeded by determining the metabolic activity of *S. mutans* biofilm in response to Violet-Blue light treatment.

[1] Gomez, G. F., Huang, R., MacPherson, M., Ferreira Zandona, A. G., & Gregory, R. L. (2016). Photo Inactivation of *Streptococcus mutans* Biofilm by Violet-Blue light. *Current Microbiology, 73*(3), 426-433. doi.org/10.1007/s00284-016-1075-z. © Springer US, Reproduced with permission from the publisher.

Table 3.1 Effect of Violet-Blue light on *S. mutans* grown in TSB and TSBS measured by maximum absorbance, time to max, lag time and Vmax

Treatment Group	[a]Maximum Absorbance Mean ± (SD)	[b]Time to Max (h) Mean ± (SD)	[c]Lag Time (min)	[d]Vmax (Maximum velocity) Mean ± (SD)
Violet-Blue light in TSB	0.428 (0.077) *	2.6 (0.34)	20	0.034 (0.003) **
No Violet-Blue light in TSB	0.557 (0.015)	2 (0)	0	0.06 (0.0009)
Violet-Blue light in TSBS	0.302 (0.008) *	6 (0)	20	0.004 (0.0003) **
No Violet-Blue light in TSBS	0.356 (0.028)	5.8 (0.34)	20	0.008 (0.0008)

[a]Maximum absorbance: highest absorbance measured during the 6 h period of recovery growth at 37°C. [b]Time to max: Time to maximum absorbance. [c]Lag time: The length of time from incubation until the bacteria begins logarithmic growth. [d]Vmax: slope of exponential growth in logarithmic phase. [*]Statistical significance between the Violet-Blue light treated group and the control grown in both TSB and TSBS (* $p < 0.05$; ** $p < 0.001$).

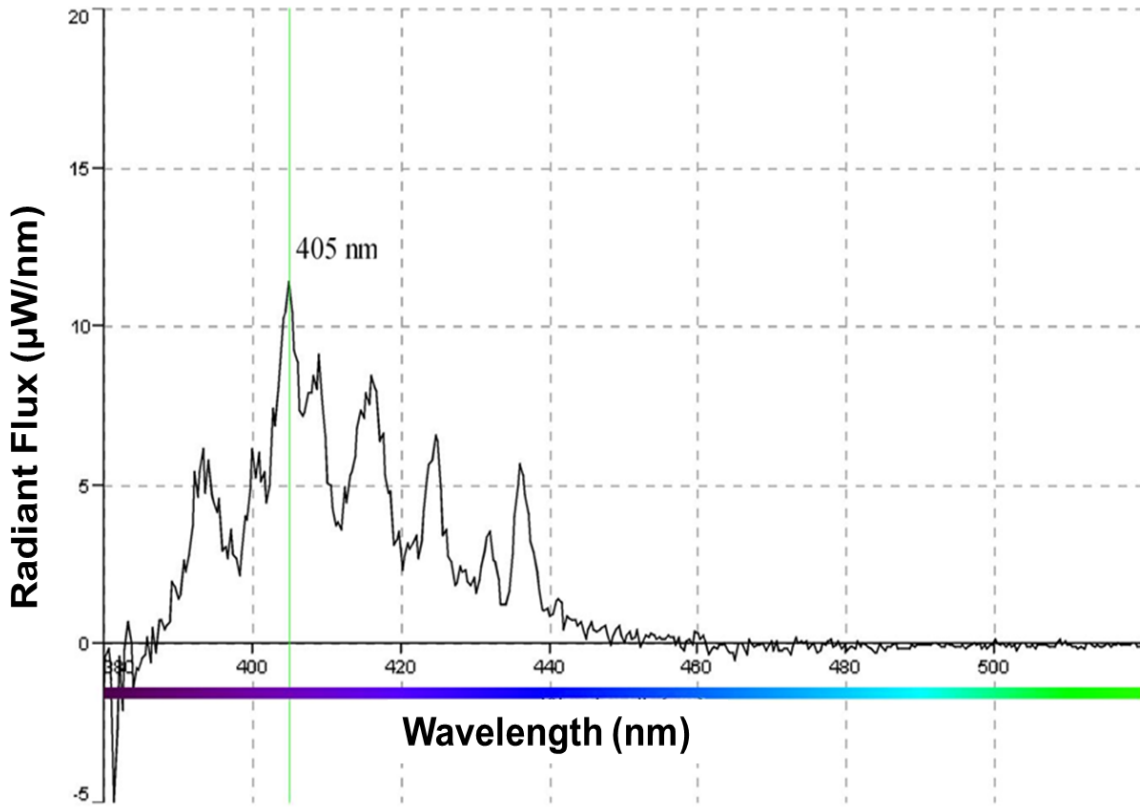

Figure 3.1 Measurement of the wavelengths emitted by the QLF light.

The wavelengths emitted from the QLF instrument (QLF™/CLIN Inspektor Research System BV, Amsterdam, Netherlands) were measured using a laboratory-grade spectrometer (Model USB2000, Ocean Optics Inc., Dunedin, FL). The peak wavelength of QLF light was at 405 nm with a spectral range from 380 to 440 nm.

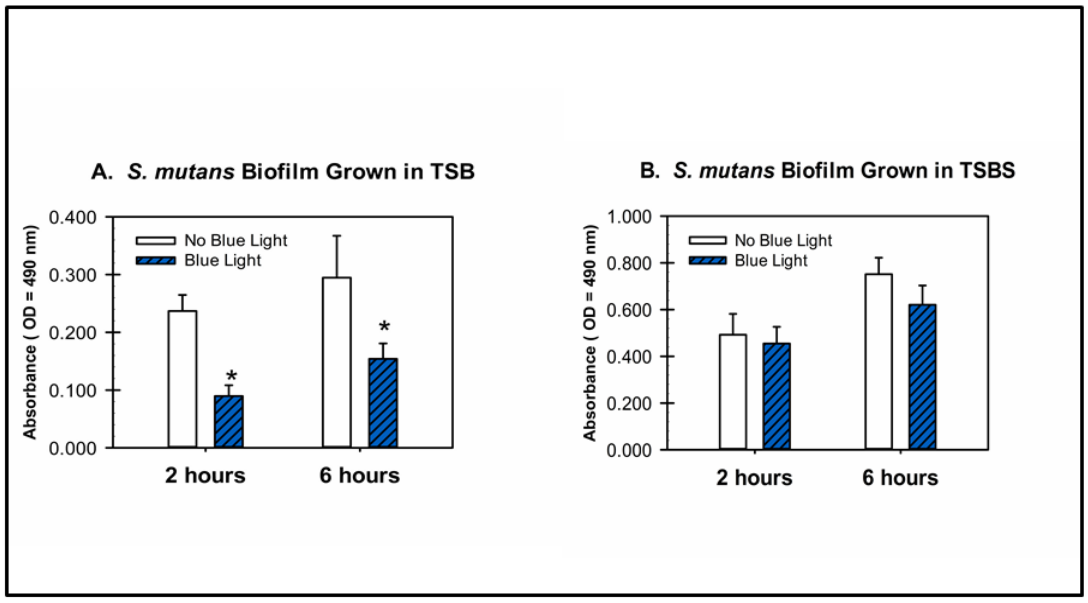

Figure 3.2 Effect of Violet-Blue light on *S. mutans* biofilm formation in **(A)** TSB and **(B)** TSBS.

(A) Absorbance values of Violet-Blue light-treated S. *mutans* biofilm (n=3) grown in TSB after staining with crystal violet and allowed to recover for 2 or 6 h. Asterisks (*) represent statistical significance between Violet-Blue light and no Violet-Blue light groups. Error bars indicate standard deviation.

(B) Absorbance values of Violet-Blue light-treated *S. mutans* biofilm (n=3) grown in TSBS after staining with crystal violet and allowed to recover for 2 or 6 h. There was no statistical significance between the Violet-Blue light and no Violet-Blue light groups. Error bars indicate standard deviation.

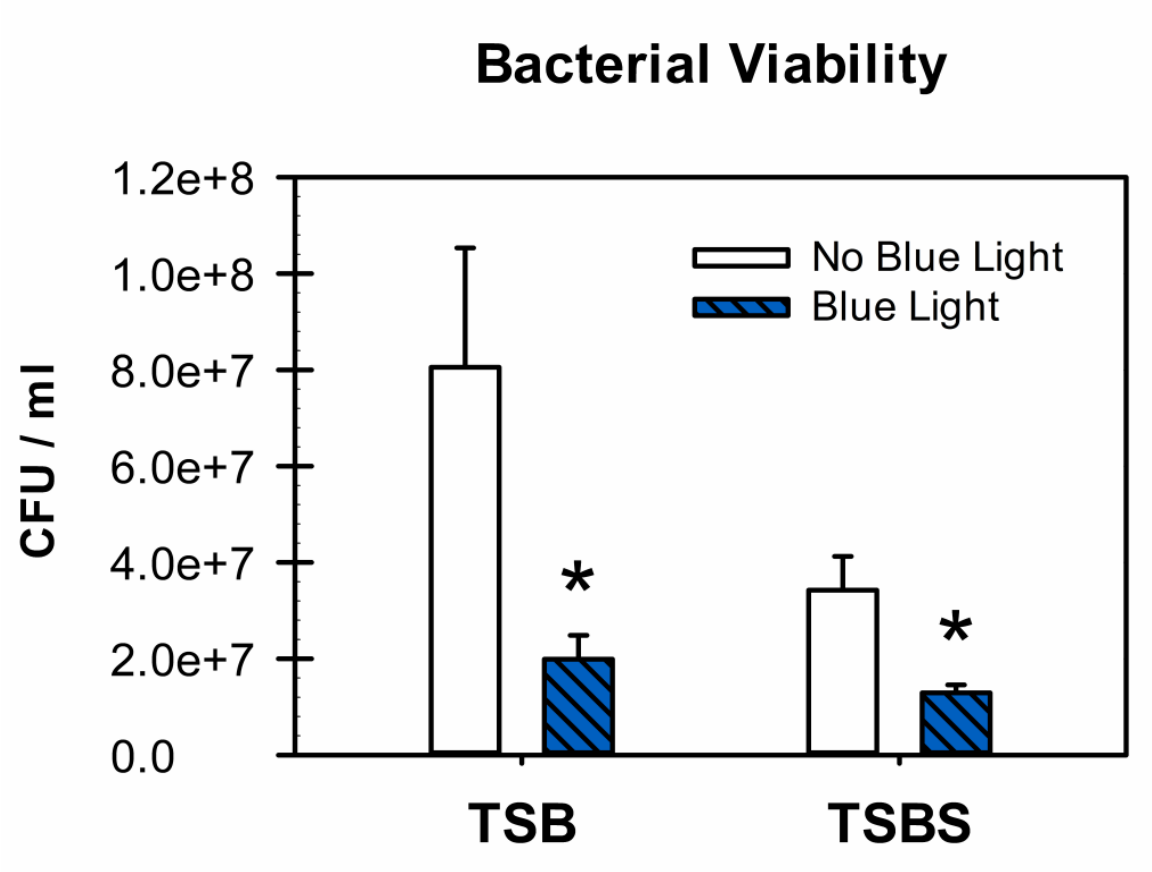

Figure 3.3 Effect of Violet-Blue light on *S. mutans* viability.

Bacterial viability of *S. mutans* grown in both TSB and TSBS and treated with Violet-Blue light compared to the no light control groups. Asterisks represent statistical significance, and error bars indicate standard deviation. n =11, TSB Violet-Blue light- treated group and n = 9 for non-treated group of TSB and n = 7 for TSBS for both treated and non-treated control groups.

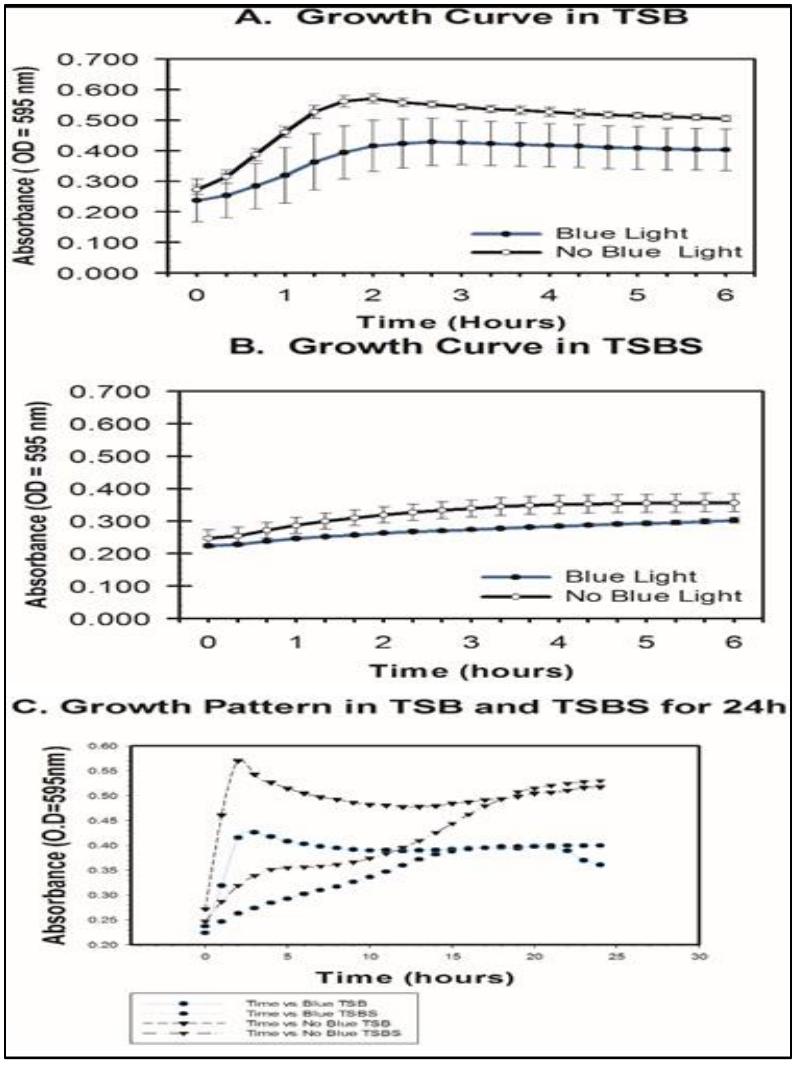

Figure 3.4 Effect of Violet-Blue light on the growth rate of biofilm/planktonic *S. mutans* in **(A)** TSB **(B)** TSBS and **(C)** growth pattern in TSB and TSBS for 24h.

(A) Kinetic growth curves of S. *mutans* cultures (n = 3) grown in TSB with no sucrose and treated with Violet-Blue light was compared with the no Violet-Blue light control group. **(B)** Growth curves of S. *mutans* cultures in TSBS and treated with Violet-Blue light was compared with the no Violet-Blue light control group. **(C)** Growth pattern of *S. mutans* in TSB and TSBS for a period of 24h**.**

Chapter 4

Effect of Phototherapy on the Metabolism of *Streptococcus mutans* Biofilm Based on a Colorimetric Tetrazolium Assay

The aim of this *in-vitro* study[2] was to determine the effect of Violet-Blue light on the metabolic activity of *Streptococcus mutans* cells in early biofilm, reincubated at 0, 2 and 6 h after 5 min of Violet-Blue light treatment. *S. mutans* UA159 biofilm cells were grown for 12-16 h in microtiter plates with Tryptic Soy broth (TSB) or TSB with 1% sucrose (TSBS). They were irradiated with Violet-Blue light for 5 min. After irradiation, plates were reincubated at 37°C for 0, 2 or 6 h in 5%CO_2. Colorimetric tetrazolium salt (XTT) reduction assay was performed to investigate bacterial metabolic activity. Mixed-model ANOVA was used to test the effect of Violet-Blue light between treated and non-treated groups at different recovery time periods of 0, 2 and 6 h in TSB and TSBS. Bacterial metabolic activity was significantly lower in the Violet-Blue light group for TSB than the non-treated ($p < 0.0001$) group regardless of recovery time. However, the metabolic activity of the treated groups without sucrose decreased as time increased. For TSBS, metabolic activity was significantly lower with Violet-Blue light at 0 and 2 h. Violet-Blue light inhibits the metabolic activity of *S. mutans* biofilm cells. This may provide a treatment method for caries active patients.

[2] Gomez G. F., Huang, R., Eckert, G., Gregory, R. L. (2018). Effect of Phototherapy on the Metabolism of *Streptococcus mutans* Biofilm Based on Colorimetric Tetrazolium Assay. *Journal of Oral Science* (In Press).

4.1 Introduction

Oral biofilm or dental plaque, composed of a group of microorganisms, is the primary causal factor of dental caries. Among them, *Streptococcus mutans* is a cariogenic bacterium with the ability to form a biofilm. Various preventive treatments have been shown to reduce, inhibit, and even eliminate oral biofilm. Non-invasive phototherapy/photodynamic therapy is an alternative therapeutic approach currently studied against microbial infections to overcome the emergence of antibiotic-resistant bacterial strains. It is one of the approaches being studied in various disciplines such as wound healing, tissue regeneration (Chaves et al, 2014; Hamblin and Demidova, 2006), cancer therapy (Gursoy et al.,2013) and skin disorders (Gursoy et al., 2013; Avci et al., 2013) as well as in the prevention of caries and oral infections (Konopka and Goslinski, 2007)). This light therapy is widely applied in the control of biofilms with and without the presence of an exogenous photosensitizer (Konopka and Goslinski, 2007; Soukos and Goodson, 2007; Mang et al 2012). Although photodynamic therapy employing exogenous photosensitizers are widely studied to control oral biofilm, investigations related to phototherapy with only light exploiting the presence of endogenous photosensitizers in the bacterial cells are developing rapidly (Fontana et al.,2015; Wang et al., 2014; Dai et al., 2012).

Visible light in the spectral range from 380 to 700 nm is commonly used to inhibit or kill bacteria (Konopka and Goslinski, 2007; Konig et al., 2000). The synergistic effect of blue light treatment for 20 sec with hydrogen peroxide

demonstrated 96% reduction in bacterial growth (Feuerstein et al., 2006). However, with 10 min of blue light exposure, there was a statistically significant reduction only within the wavelength range of 400 to 500 nm (Feuerstein et al., 2006). A delayed antibacterial effect (Steinberg et al., 2008; Feuerstein, 2012) was reported by Feuerstein et al. (Feuerstein et al., 200612) in a 6 h grown *S. mutans* biofilm, whereas Chebath et al. (Chebath et al., 2012) observed increased numbers of dead bacteria after treatment for 3, 5, 7 and 10 min (Feuerstein, 2012). Photoinactivation of *S. mutans* biofilm has been demonstrated earlier with Violet-Blue light of peak wavelength at 405 nm for 5 min (Gomez et al., 2016). This study reported the effect of violet to blue light on biofilm formation, colony forming units, and growth kinetics of *S. mutans*. The effect of Violet-Blue light treatment on the metabolic activity of *S. mutans* biofilm has not been examined. Determining the metabolic activity of *S. mutans* will establish the overall vitality of *S. mutans* after exposure to Violet-Blue light treatment and indirectly assess the viability of the bacterial cells. Although we have quantified bacterial cell numbers after treatment with Violet-Blue light, the metabolic activity assay used provides a measure of the respiratory activity of the inactivated bacterial cells. Metabolic activities such as biological oxidation of sugars or carbohydrates are vital for the survival of *S. mutans* (Ajdic et al., 2002), including respiration and fermentation (Jurtshuk P Jr, 1996. Bacterial Metabolism, Baron S, editor. Medical Microbiology. 4th edition. Galveston), resulting in the generation of high energy in the form of adenosine triphosphate (ATP) and adenosine diphosphate (ADP).

S. mutans being a saccharolytic organism has a heterotrophic bacterial metabolism to generate energy. End products such as lactic acid, acetate, formate, and ethanol are formed during fermentation of sugars by oxidizing NADH to ATP through the conversion of pyruvate. Various dehydrogenases are involved in *S. mutans* metabolism **(Figure 4.1)**. The chemical reactions in the metabolism of a bacterial cell are vital for the division, replication, viability, and growth of *S. mutans* (14). The aim of this *in vitro* study was to determine the effect of Violet-Blue light on metabolic activity using a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay on early *S. mutans* biofilm, which was reincubated for 0, 2 or 6 h after 5 min of Violet-Blue light treatment.

4.2 Materials and Methods

4.2.1 Bacterial Strain, Media and Culture Conditions

Bratthall serotype *c* strain *S. mutans* UA159 (ATCC 700610) obtained from American Type Culture Collection (Rockville, MD) was used in this study. The cells were stored at −80°C with 20% glycerol and grown on Mitis-Salivaris Sucrose Bacitracin (MSSB, Anaerobe Systems, CA) agar plates. Liquid broth cultures were prepared in 5 ml of Tryptic Soy Broth (TSB, Acumedia, Baltimore, MA) and grown for 24 h in a 5% CO_2 incubator. *S. mutans* was grown in TSB without sucrose, and in TSB supplemented with 1% sucrose (TSBS) as a biofilm in wells of sterile 96-well flat bottom microtiter plates (Fisher Scientific, Co., Newark, DE). The biofilm

cells were incubated for 12 to 16 h in a 5% CO_2 incubator to reach the logarithmic phase of growth.

4.2.2 Light Source

Quantitative light-induced fluorescence (QLF, Inspektor Research Systems, Amsterdam, The Netherlands) is an early caries detection light device with a peak excitation wavelength of 405 nm and was used in this study. It has a spectral wavelength ranging from 380 to 450 nm. It is equipped with a 35-watt Xenon arc lamp and Violet-Blue light that is filtered through a blue filter at 370 nm.

4.2.3 Reagents

XTT (2, 3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide inner salt, Sigma-Aldrich) sodium salt was used to detect metabolically active cells. Menadione (also known as 2-Methyl-1, 4-naphthoquinone or Vitamin K_3) is an electron transporter and was used as an electron coupling activator to reduce XTT in metabolically active cells to an orange colored formazan 1-phenyl-2-[phenyl(2-phenylhydrazinylidene) methyl] diazene compound.

4.2.4 Colorimetric Tetrazolium Assay for Metabolic Activity

S. mutans was grown in 200 ul of either TSB or TSBS by pipetting 190 ul of TSB or TSBS and 10 ul of an overnight culture into each well. *S. mutans* biofilm cells were grown in wells of 96-well microtiter plates with a one-well gap between the samples so that the light targeting a particular well does not impact surrounding wells. Before irradiation with Violet-Blue light, the supernatant or planktonic media was removed. The wet biofilm was continuously irradiated with Violet-Blue light for 5 minutes at a fixed distance of 2 cm between the light source and the biofilm at the bottom of the plate. After irradiation, 0.9% saline was added to some wells. To other wells, TSB or TSBS were added and the plates were reincubated for 2 or 6 h. The metabolic activity of the treated biofilm cells was determined using an adaptation (Huang et al, 2012) of the method developed originally by Pierce et al (Pierce et al., 2008). XTT sodium salt solution was freshly prepared and activated with menadione. The *S. mutans* biofilm cells were gently washed twice with 0.9% saline and then 200 ul of the XTT solution was added and incubated for 2 h in a dark environment covered with aluminum foil at 37ºC. The supernatant fluid was removed and placed into another 96-well microtiter plate followed by measuring the absorbance at 490 nm.

4.3 Statistical Analysis

The metabolic activity determined by the XTT assay and the effect of Violet-Blue light on *S. mutans* at various recovery time periods of 0, 2 and 6 h in growth media without sucrose (TSB) and with 1% sucrose (TSBS) were analyzed using a mixed–model ANOVA. Pair-wise comparisons were made using Fisher's Protected Least Significant Differences to control the significance level at 5%. Analyses were performed on the log-transformed data.

4.4 Results

The interaction between the effect of light and recovery time was significant both in the absence (TSB) and in the presence of sucrose (TSBS) ($p < 0.0001$). For TSB-grown *S. mutans* cells, metabolic activity measured through an XTT assay was significantly lower when treated with Violet-Blue light than the non-treated group regardless of recovery time ($p < 0.0001$; 0 h – **Figure 4.2,** 2 h – **Figure 4.3,** 6 h – **Figure 4.4**). The effect of Violet-Blue light decreased as recovery time increased as illustrated by decreasing differences between XTT with and without light. The metabolic activity was significantly decreased at 0 h compared with a 2 h recovery period ($p = 0.0021$; **Figure 4.3**) and at 6 h of recovery time ($p = 0.0012$; **Figure 4.4**). Metabolic activity after 2 and 6 h of recovery were not significantly different from each other ($p = 0.90$).

The metabolic activity of *S. mutans* cells in biofilm in the presence of sucrose was significantly lower with Violet-Blue light at 0 h of recovery time ($p < 0.0001$) than the non-treated control group **(Figure 4.2)**. For TSBS, XTT activity was significantly lower following blue light treatment than without blue light at 0 h ($p < 0.0001$; Fig. 2) and at 2 h ($p < 0.0001$; **Figure 4.3**) of recovery time, but there was no significant difference in XTT activity with and without blue light at 6 h of recovery time ($p = 0.30$; **Figure 4.4**). For TSBS, regardless of light treatment, there was no significant effect due to the recovery time ($p = 0.66$).

4.5 Discussion

In our previous studies, we demonstrated photoinactivation of *S. mutans* with 5 min of Violet-Blue light treatment by quantitatively determining the viability (colony forming units) of biofilm cells (Gomez et al., 2016). In this study, *S. mutans* metabolic activity was found to be decreased after 0 and 2 h of recovery time in the Violet-Blue light treated groups. Metabolic activity was unaffected after 6 h of recovery with sucrose and was increased compared to 0 h of reincubation in the light-treated group. We reported previously that there was no difference in biofilm formation after 6 h of reincubation in TSBS after Violet-Blue light treatment, which suggests that the bacterial cells are actively replicating (Gomez et al., 2016). However, XTT may be incompletely reduced in bacterial cells which are encompassed within extracellular glucans. This may explain the significant XTT reduction in bacterial cells grown in TSB compared to those grown in TSBS. The

respiring bacterial cells produce NADH, and the hydrogen ions are accepted by tetrazolium salt (XTT), which is further converted to colored formazan compound (Berridge et al., 2005). Compared to 0 h, cells with both 2 and 6 h of recovery time had increased metabolic activity. The biofilm formed at baseline of 0 h regrew at 2 and 6 h. However, cells in the Violet-Blue light-treated groups had reduced metabolic activity compared to the non-treated group.

There were some impairments in the chemical reactions responsible for metabolizing carbohydrate sources following Violet-Blue light treatment contributing to the inhibition of metabolic activities in *S. mutans* biofilm cells. The pathogenic nature of cariogenic biofilm is determined by metabolic activity (Ishiguro et al., 2015). Dehydrogenases in bacterial cells play a vital role in metabolic activities. Hydrogen ions from NADPH are accepted by artificial electron transporting reagents such as menadione, and the menadione transfers electrons to the resulting tetrazolium salt. The colorless XTT compound through a redox process can enter bacterial cells and be converted into orange colored formazan derivatives. Formazan is formed in the culture supernatant, is a water-soluble compound, and can be measured by absorbance measurements. Quantification of bacterial cell number and correlation with the colored signal may be a better option. However, photoinactivated bacterial cells that have lost their viability, but remain metabolically active, cannot be determined through counting colony forming units. The efficacy of Violet-Blue light determined through the use of a colorimetric method for assessing metabolic activity helps to design future studies such as determining the expression of virulence factors associated with biofilm

formation and efficacy of Violet-Blue light on *S. mutans* biofilm grown on human enamel or dentin specimens.

Five min of irradiation time is longer than that of an at-home procedure. However, the irradiation could be done as a part of prophylactic treatment in a dental office, but non-compliance of the patients to the procedure will be a potential disadvantage. Determination of the minimum irradiation time necessary to inhibit *S. mutans* biofilm formation should be conducted in future studies. This will potentially help to reduce the irradiation time. Effectiveness of blue light after 20, 30, and 60 sec as seen in the presence of exogenous photosensitizers cannot be achieved with only Violet-Blue light. There is also recovery of biofilm after 2 and 6 h, suggesting that repeated treatments may be necessary to prevent the regrowth of biofilm. Within the limitations of the current study, more innovative approaches are needed.

4.6 Conclusion

The metabolic activity of *S. mutans* was significantly reduced in the Violet-Blue light-treated group compared to the non-treated group immediately after treatment. However, there was some recovery of the biofilm cells after treatment with Violet-Blue light. Although the bacterial cells were inactivated immediately, the respiratory capacity of the bacteria was not affected 6 h after treatment in the presence of sucrose, suggesting that, repeated Violet-Blue light treatment is needed to inhibit biofilm formation and prevent dental caries. The use of Violet-

Blue light at home and in clinical settings could serve as an adjunct prophylactic treatment. Violet- Blue light from QLF has been shown to photo inactivate *S. mutans* biofilm which is detailed in chapters 3 and 4. Previously, we have observed orange-red fluorescence in QLF images on a longitudinal observational study by Dr. Andrea Zandona, MSD, PhD. We chose to do a retrospective study on the orange-fluorescence in carious lesions that progressed compared to non-progressive lesions. The reasons behind this study was to interlink the concept of fluorescence and photoinactivation.

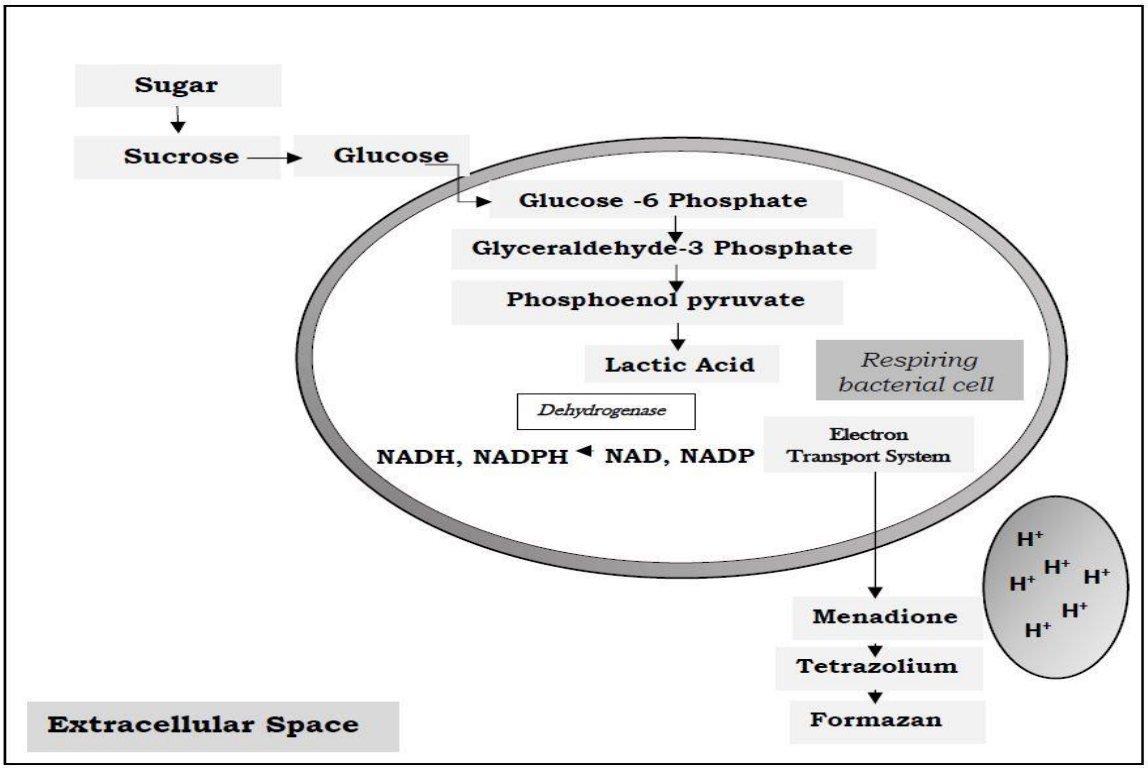

Figure 4.1 Metabolism of *S. mutans* of carbohydrates to generate energy.

S. mutans metabolizes carbohydrates including sucrose, glucose, lactose fructose and other intermediates of sugars to generate energy. The glycolytic metabolic pathway includes various intermediates such as glucose-6-phosphate, glyceraldehyde 3-phosphate, phosphoenol pyruvate, and pyruvate. Pyruvate is converted to end products such as lactic acid, acetate, formate, and ethanol. Enzymes such as various dehydrogenases are involved in *S. mutans* metabolism. The respiring bacterial cells produce NADPH during fermentation which gets oxidized with a conversion of pyruvate to acetate, and the hydrogen ions are accepted by tetrazolium salt (XTT), which is converted to a colored formazan compound.

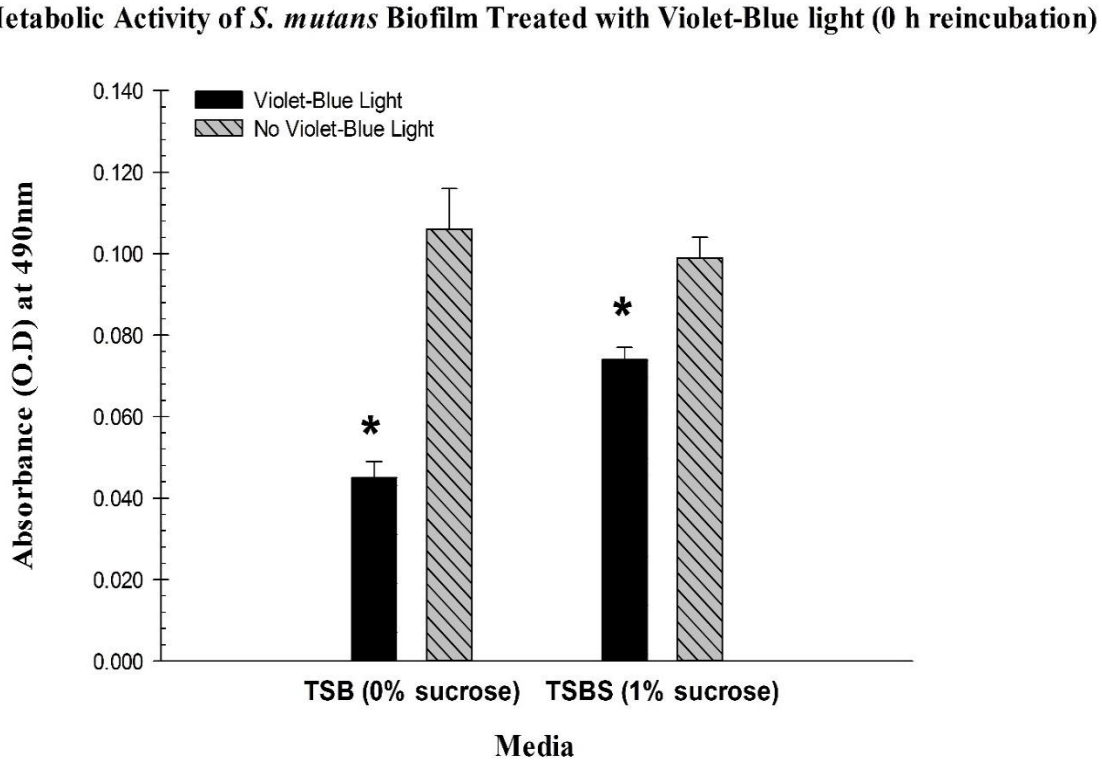

Figure 4.2 The metabolic activity of *S. mutans* biofilm at zero hours.

The effect of Violet-Blue light on TSB-grown *S. mutans.* At 0 h, *S. mutans* cells had significantly lower metabolic activity in the light-treated group compared with the non-treated group ($p < 0.0001$). Metabolic activity of *S. mutans* grown in TSBS (1% sucrose) was significantly lower in the treated than the non-treated group ($p < 0.0001$). Significance level was kept at 5%. Asterisks represent significant differences ($p < 0.05$) between the light and no light groups. Mean $\pm$ SE.

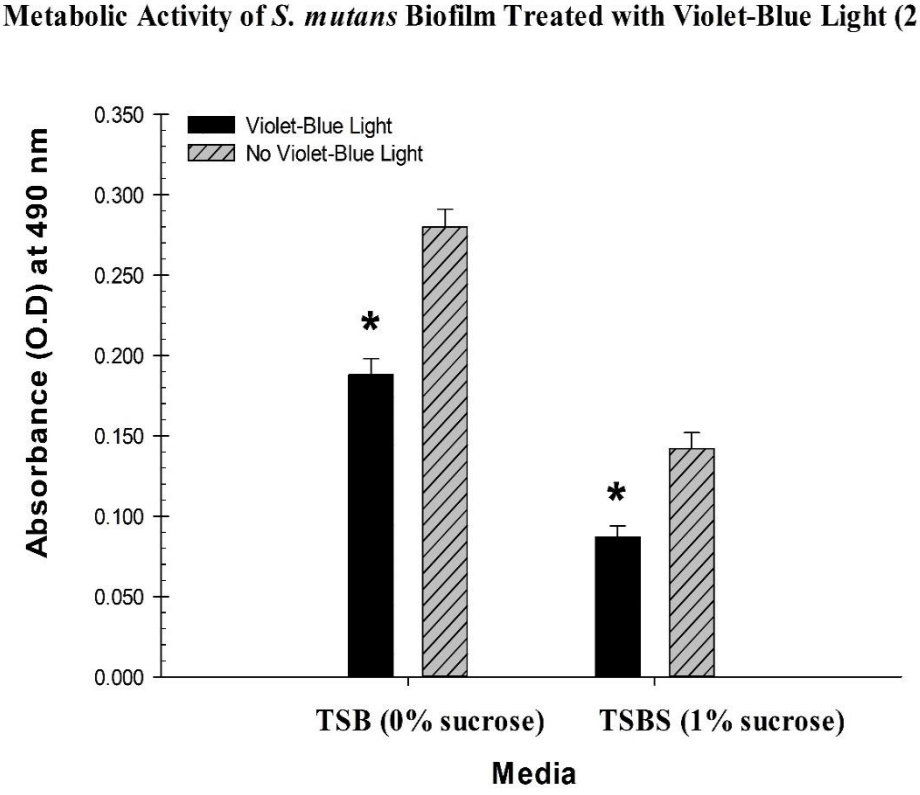

Figure 4.3 The metabolic activity of *S. mutans* biofilm at two hours.

The effect of Violet-Blue light for TSB-grown *S. mutans*. At 2 h, *S. mutans* cells had significantly lower metabolic activity in the light-treated group compared with the non-treated group ($p < 0.0001$). Metabolic activity of *S. mutans* grown in TSBS (1% sucrose) was significantly lower in the treated than the non-treated group ($p < 0.0001$). Significance level was kept at 5%. Asterisks represent significant differences ($p < 0.05$) between the light and no light groups. Mean $\pm$ SE.

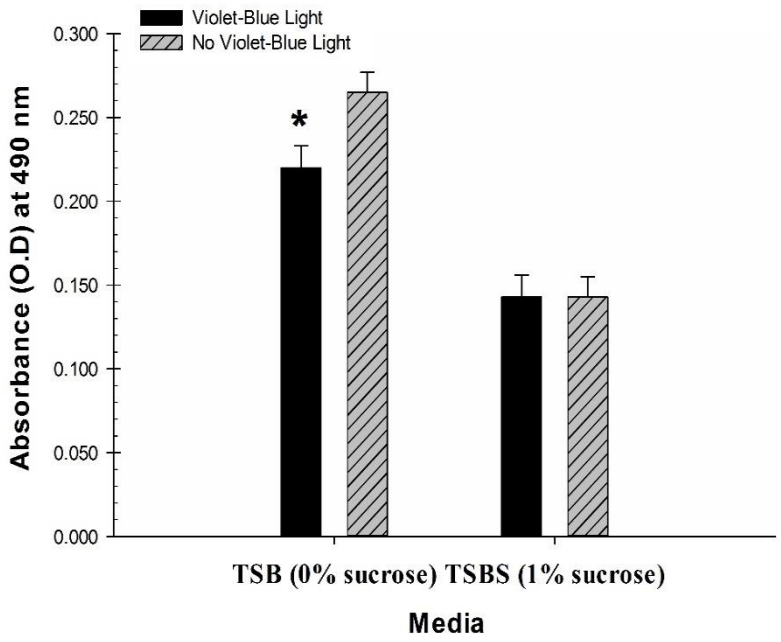

Figure 4.4 The metabolic activity of *S. mutans* biofilm at six hours.

The effect of Violet-Blue light for TSB-grown *S. mutans.* At 6 h, *S. mutans* cells had significantly lower metabolic activity in the light-treated group compared with the non-treated group ($p < 0.0001$). Metabolic activity of *S. mutans* grown in TSBS (1% sucrose) was not significantly different in the treated than the non-treated group at 6 h ($p = 0.30$). Significance level was kept at 5%. Asterisks represent significant differences ($p < 0.05$) between the light and no light groups. Mean $\pm$ SE.

Chapter 5

Orange/Red Fluorescence of Active Caries by Retrospective QLF Image Analyses

This retrospective clinical study determined the association between caries activity and orange/red fluorescence on QLF images of surfaces that progressed to cavitation as determined by clinical visual examination. A random sample of QLF images from 565 children (5-13years) previously enrolled in a longitudinal study was selected. Buccal, lingual and occlusal surface images obtained after professional brushing at baseline and every 4 months over a 4-year period were analyzed for Red Fluorescence (RF). Surfaces that progressed (N=224) to cavitation according to International Caries Detection and Assessment System (ICDAS 0/1/2/3/4 to 5/6/filling) and surfaces that did not progress (N=486) were included. QA2 image analysis software outputs the percent increase of the red/green components as ΔR and Area of ΔR (Area△R) at different thresholds. Mixed-model ANOVA was used to compare progressive and non-progressive surfaces to account for correlations of RF (ΔR and AreaΔR) between surfaces within a subject. The first analysis used the first observation for each surface or the first available visit if the surface was unerupted (baseline), while the second analysis used the last observation prior to cavitation for surfaces that progressed and last observation for surfaces that did not progress (final). There was a significant ($p<0.05$) association between RF and progression to cavitation at

thresholds ΔR0, ΔR10, ΔR20, ΔR60, ΔR70, ΔR80, ΔR90 and ΔRMax at baseline and for ΔR0 and ΔR10 at final observation. Quantification of orange/red fluorescence may help to identify lesions that progress to cavitation. Future studies to identify microbiological factors causing orange/red fluorescence and its caries activity are needed.

5.1 Introduction

The optical phenomenon of tooth autofluorescing on illumination with ultraviolet light was first observed by Hans Stubel (Buchalla, 2005; Stubel, 1911). This property has been applied in caries detection devices as means of detection of incipient carious lesions (Alfano and Yao, 1981; Benedict, 1928; Bjelkhagen et al., 1982; de Josselin de Jong et al., 1995; Sundstrom et al., 1985; Zandona and Zero, 2006). The concept held previously was that carious enamel lesions do not fluoresce (Benedict, 1928), and deep dentinal caries appeared dark brown, orange brown, or pinkish orange, when caries was debrided. It was shown later that carious lesions fluoresce in the red region of the spectrum (Buchalla, 2005; Konig et al., 1999; Slimani et al., 2014). Several studies have demonstrated heterogeneous emission spectral bands in the carious region compared to the sound surface of the tooth (Buchalla, 2005;Buchalla et al., 2004a, b; Zezell et al., 2007). The long-standing question is whether the chromogenic auto fluorescence originates from the tooth structure or from bacteria. It has been shown that

macromolecular porphyrin might be responsible for the autofluorescence (Buchalla et al., 2008).

Quantitative Light-Induced Fluorescence (QLF-clin, Inspektor Research Systems BV, Amsterdam, Netherlands) employs the optical property of fluorescence for caries detection. Inspektor™ QLF Pro employs an intra-oral camera that illuminates the tooth with light in the Violet-Blue wavelength (290-450nm) and captures fluorescence above 520 nm by a high pass filter. Under these conditions, the sound areas of the tooth fluoresce green; however, an orange to red fluorescence can also be observed on these images. A new device, the QLF-D Biluminator ™ 2, has been introduced using a Single Lens Reflex (SLR) camera with violet LED lights with a peak wavelength of 405±20nm (violet). White LED's are used for standard white-light images, while the violet LED's provide excitation light for fluorescent imaging. The filter in this device allows the images of sound tooth surfaces to have a whitish appearance instead of green, while demineralized areas look darker, and bacterially infected areas show a bright red fluorescence. The orange/red fluorescence is assumed to originate from metabolic byproducts of oral bacteria within the dental biofilm called porphyrins (Konig et al., 1998; van der Veen et al., 2006; Volgenant et al., 2013). In vitro oral biofilm studies have shown correlation between the red fluorescence captured through QLF-D Biluminator™ and caries risk (Kim et al., 2014; Lee et al., 2013). A proprietary software (Inspektor Pro System) allows analyses of the images reporting three parameters: average loss of fluorescence denoting lesion depth (ΔF [%]); lesion area (A in px^2), and lesion volume (ΔQ [$\%px^2$]). In the QLF-D Biluminator™, the

ratio of red to green fluorescence in percentage (ΔR [%]) and the area of red fluorescence ($A_{\Delta R}$) are reported. Red or orange fluorescence (△R) is represented as the percentage ratio increase of the red to green components in comparison to sound surface. RF Area (A△R) in px^2 is equal or higher than a specific threshold of △R **(Table 5.1)** (Waller et al., 2012). Red or orange fluorescence is believed to emanate from the excitation of fluorophores from bacterial byproducts on illumination with blue–violet light (van der Veen MH, 2003). A preliminary in vitro study (Alammari MR, 2010) showed the relationship between △R and △F to ICDAS and histology scores, requiring clinical validation.

The relationship between the QLF parameters (△F, △A and △Q), the visual appearance of the lesions, and its clinical behavior longitudinally has been demonstrated previously by our group (Ferreira Zandona et al., 2010; Ferreira Zandoná A, 2003). During capture and analyses of these clinical images, the red fluorescence observed by others (Bittar et al., 2014; Coulthwaite et al., 2006) was also seen on the images captured in these longitudinal studies. Thus, the objective of this study was to determine if there is an association between the intrinsic orange/red fluorescence seen in QLF images of surfaces and progression to cavitation over a period of time as compared to surfaces with non-cavitated lesions that did not progress to cavitation.

5.2 Study Participants and Methods

This is a retrospective study based on the images captured during a longitudinal study previously published (Ferreira Zandona et al., 2013). Detailed information of participant selection, sample size calculation, examinations conducted and the analyses of fluorescence in QLF images were previously published (Ferreira Zandona et al., 2013; Ferreira Zandona et al., 2010; Ferreira Zandona et al., 2012; Fontana et al., 2011). In summary, in January 2007 children (N = 565) aged 5 to 13 years from kindergarten to 9th grade enrolled in public schools from Aguas Buenas, Puerto Rico were recruited into the study. Consent and assent, if the child was over 7 years old, were obtained. The study was approved by the Institutional Review Board (IRB) of Indiana University (IU-IRB #0608-15) and the University of Puerto Rico (UPR-IRB#A1340107). The inclusion criteria included having at least one permanent molar with at least one unrestored surface and no medical problems.

5.3 Comprehensive Oral Examinations

The children underwent examinations at baseline, 8, 12, 18, 24, 28, 32, 36, 40, 44, and 48 months. It is important to note that the exams were conducted after a professional tooth brushing, thus most, if not all biofilm was removed from the surfaces of interest. A visual examination based on ICDAS, which ranges from 0 to 6 (Pitts, 2004), was performed at each time point as well as an examination of

the teeth with QLF. Fluorescent images of occlusal and buccal surfaces of all permanent molars and lingual surfaces of upper molars were obtained at each exam. At the end of the four-year study, fluorescent images of surfaces that progressed to cavitation (ICDAS 0/1/2/3/4 to 5/6 or filling) and a random sample of images of surfaces that did not progress to cavitation were analyzed longitudinally using proprietary software (QLF 2.00g).

5.4 Orange/Red Fluorescence Analyses

The same images previously analyzed were analyzed for Orange/Red Fluorescence by RF Analysis (QA2 software, Inspector Research Systems, The Netherlands). Surfaces (N=224) that progressed to cavitation according to ICDAS (ICDAS 0/1/2/3/4 to 5/6 or filling) and surfaces that did not progress (N=486) were included. (Ferreira Zandona et al., 2013). The Plaque Wizard patch of QA2 of QLF D Biluminator was used to analyze the images obtained from Inspektor ^{TM} QLF Pro. For RF analysis, the plaque wizard was applied on the same area of the carious lesion surface which was analyzed previously to monitor the progression of caries. It was applied on the last visit for sites that did not progress, or if there was a filling or a cavitation with ICDAS 5 or 6, it was applied on the visit prior to the filling/cavitation. A similar plaque wizard was applied to all other visits including the first observation, which was at baseline, or the first visit after eruption if the tooth was unerupted at baseline. QA2 outputs the percent increase of red to green components as $\triangle$R and Area$\triangle$R at different thresholds. For intra-examiner

reliability, 60 surfaces were chosen randomly including surfaces that progressed to cavitation and those that did not progress for repeat analyses by a single, experienced examiner.

5.5 Statistical Analyses

Statistical analysis was performed with SAS 9.3 (SAS Institute Inc., Cary, NC). Mixed-model ANOVA was used to compare surfaces that progressed to cavitation and those that did not progress to account for correlations of RF (△R and Area △R) between surfaces within a subject. The ranks of the data were used in the analysis because of non-normal distributions. Two sets of analyses were performed. The first analysis used the first observation for each surface, which was at baseline or the first available visit if the surface was unerupted at baseline. The second analysis used the last observation before progression for surfaces that did progress to cavitation and used the last observation for sites that did not progress. The area under the receiver operating curve (ROC) was calculated to determine the sensitivity and specificity of the threshold for the RF areas. Intraclass Correlation Coefficients (ICCs) were calculated to determine the level of intra-examiner repeatability.

5.6 Results

A statistically significant association ($p < 0.05$) was seen between RF and surfaces that progressed to cavitation. Analysis of the initial or first observation of the tooth surfaces showed that sites that progressed to cavitation had a significantly higher percentage (%) of red fluorescence for specific thresholds of ΔR0, ΔR10, ΔR20, ΔR60, ΔR70, ΔR100, ΔRMax, and Total than sites that did not progress. RF Area (A△R), which is the Area at △70 in px^2, correlated with the percentage of RF observed in the first observation ($p=0.0191$). Simple plaque score (SPS ^{TM}) was borderline significant ($p=0.0522$) at the initial observation **(Table 5.2)**.

At the first observation, the mean value (M) and standard error (SE) of RF values were 4955 (285) for surfaces that progressed to cavitation while the mean value and standard error was 3038 (166) for surfaces that did not progress. As shown on **Table 5.2**, the sites that progressed to cavitation had significantly higher ΔR0, ΔR10, △R20, △R60, △R70, △R100, ΔRMax, and Total at the first observation than sites that did not progress. Even at the thresholds where there were no significant differences between the sites, there was a tendency for the sites that did not progress to have lower values than those that did progress to cavitation. The area at threshold △R70 (Area △R70) was significant for lesions that progressed to cavitation compared to area at △R30 and area at △R120. Analysis of the final observation showed that sites that progressed to cavitation had significantly higher ΔR0, ΔR10, ΔRMax, and Total than sites that did not

progress ($p<0.0001$). SPS^{TM} scores were not significant at the last visit. The mean RF values at various thresholds during the final observation are included in **Table 5.2**. Area of thresholds at △R30, △R70 and △R120 were not significantly different.

Comparisons of RF values at △RMax and △R10 during the first and final observations area are illustrated in **Figure 5.1**. For both first and final observations, there was a significant difference for △RMax and △R10 for surfaces that progressed to cavitation compared to non-progressive surfaces. Carious surfaces that progressed to cavitation showed greater RF values during final observation at △R10. Surfaces that did not progress showed higher RF values at first observation at △RMax. Therefore, the threshold kept at maximum level of cutoff was greater compared to RF value at △R10. RF is detected during first observation with the maximum level of cutoff. Also, during final observation of non-progressive surfaces, △RMax showed higher RF values.

Dot plots are shown comparing surfaces that progressed to cavitation and non-progressive surfaces based on total values of RF during the baseline or the first observation **(Figure 5.2 (a)** and during the final observation **(Figure 5.2 (b)**. The surfaces that progressed to cavitation and non-progressive surfaces were significantly different in both cases ($p<0.0001$).

The area under the ROC curve was calculated based on the continuous variables in the RF analysis data. Sensitivity and specificity were calculated based on the ROC curve. ROC curve was plotted and was evaluated to determine the appropriate tradeoff between sensitivity and specificity. **Figure 5.3** illustrates the

ROC curve for the final observation (not progressed) or last observation before progression to cavitation for 'Total' to predict progression. As seen in **Figure 5.3**, the best combination was a sensitivity of 65% with a specificity of 63%.

The intra class correlation coefficients (ICCs) indicated good repeatability for all RF measurements: 0.99 for △RMax, 0.97 for RF total, 0.97 for △R0, 0.87 for △R10, 0.88 for △R20, 0.89 for △R30, 0.94 for △R40 and △R50, 0.92 for △R60, 0.93 for △R70, 0.96 for △R80, and 0.78 for △R90.

5.7 Discussion

Predicting caries activity is considered the "holy grail" of cariology (Ismail, 2005). To date only subjective methods are available to determine if a lesion will progress to cavitation or will arrest. It is known that not all early lesions progress to cavitation (Dirks, 1966). Our study in this population also demonstrated that lesions with rapid changes in QLF parameters like area, depth and volume of the lesion progressed to cavitation (Ferreira Zandona et al., 2013). Identifying lesions that are likely to progress to cavitation can have a great impact on how dentistry is practiced and how the caries paradigm shift towards the non-surgical management of dental caries can be implemented.

Most studies have focused on the red fluorescence of oral biofilm. The orange to red intrinsic fluorescence has been shown to be emanating from the oral biofilm (dental plaque) comprising of all the oral bacterial species. It does not come from single bacterial species (van der Veen et al., 2006). Red excited fluorescence

signal is likely derived from metabolic byproducts of oral bacteria in the dental biofilm (Koenig and Schneckenburger, 1994). Red fluorescence emission seen on QLF images is proposed to be the result of excitation of endogenous porphyrins by the Violet-Blue light at a range of wavelength from 380 to 500nm. Fluorescing porphyrins in caries detected to some extent are protoporphyrin IX, coproporphyrin and uroporphyrin (Buchalla et al., 2008). Red fluorescence from bacteria was shown to be an indicator of dentinal carious lesions (Lennon et al., 2006). Higher levels of red to orange fluorescence can be an indicator for progression of lesions as demonstrated in our study. Early detection of orange to red fluorescence may serve as a caries indicator to predict caries activity.

Our study focused on the tooth surfaces that were cleaned of visible biofilm before imaging. It showed that at specific thresholds or cutoff levels the orange /red fluorescence from lesions that progressed to cavitation compared to non-progressive carious lesions was significantly higher. At low thresholds of △R0, △R10 and △R20 at baseline or first available visit the orange/red fluorescence was significantly higher for the lesions that progressed to cavitation compared to lesions that did not progress. This indicates that the values of orange/red fluorescence have a potential to identify lesions that are likely to be active, that is, progress towards cavitation. This may be the first non-subjective means to determine caries activity at a single time point. This can have a significant impact on dental care ranging from caries risk to specific caries intervention.

There are several questions that remain to be answered. In our study[3] at every visit prior to capturing QLF images, the teeth were brushed and flossed by study personnel; however, orange to red fluorescence was not only observable in the lesions, but also an apparent indicator of caries activity. What is the origin of this red fluorescence – are these metabolic products within the body of the lesion (Buchalla et al., 2008)? Could we just use the red fluorescence from the biofilm for caries prediction as some studies seem to indicate (Buchalla et al., 2010)?

Within limitations of the study, quantification of orange to red fluorescence was able to distinguish carious lesions that progressed to cavitation from lesions that did not progress at a single observation. Though there are several factors involved in the increased contribution of orange to red fluorescence, future studies identifying the microbiological factors causing the orange/red fluorescence and its implication in caries activity are indicated. Additionally, studies are needed to understand the role of porphyrins in active and arrested carious lesions.

[3] Felix Gomez, G., Eckert, G. J., & Ferreira Zandona, A. (2016). Orange/Red Fluorescence of Active Caries by Retrospective Quantitative Light-Induced Fluorescence Image Analysis. *Caries Research, 50*(3), 295-302. doi:10.1159/000441899

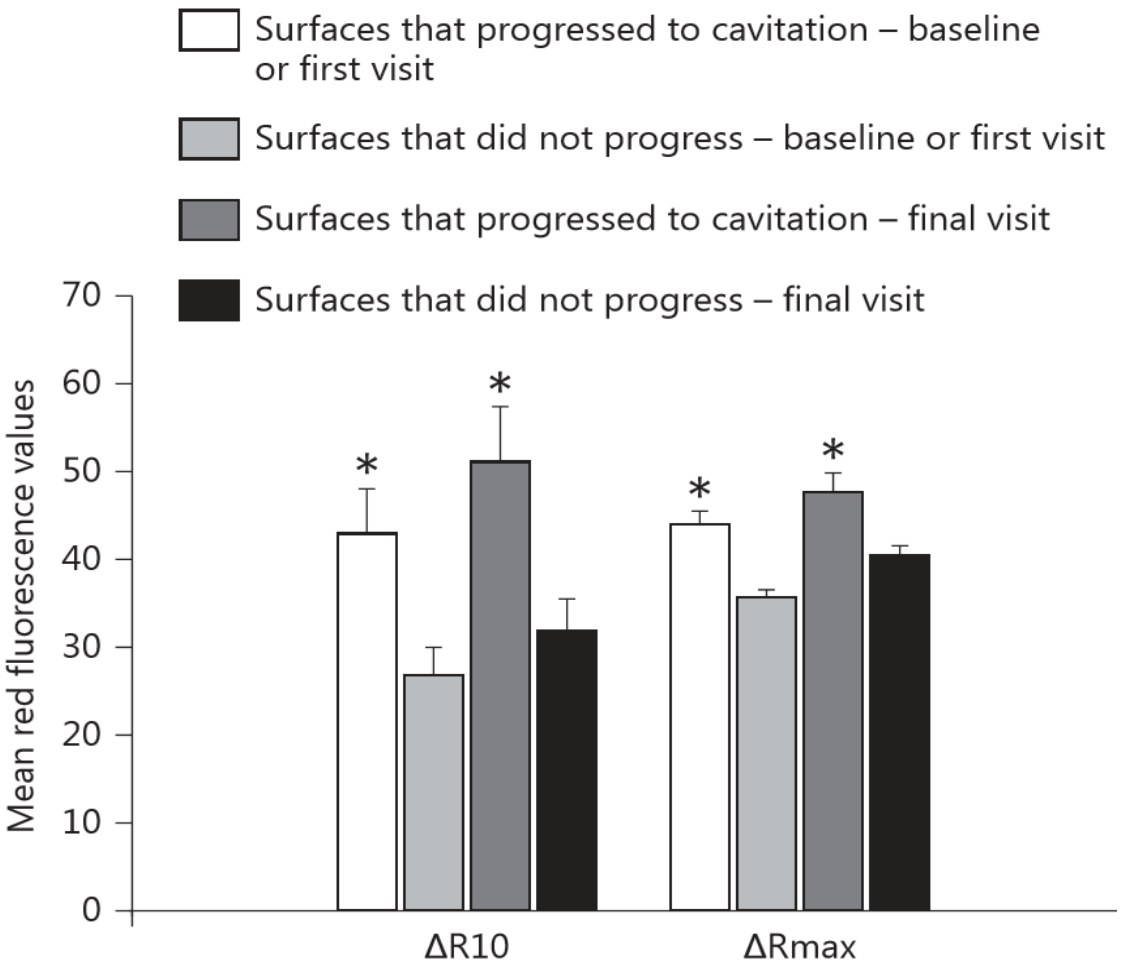

Figure 5.1 RF values at △RMax and △R10 first and final observations.

Mean Red Fluorescence values at △RMax and △R10 first and final observations. Comparison of mean red fluorescence values at first and final observation for surfaces that progressed to cavitation and those that did not progress at ΔRmax and ΔR10.

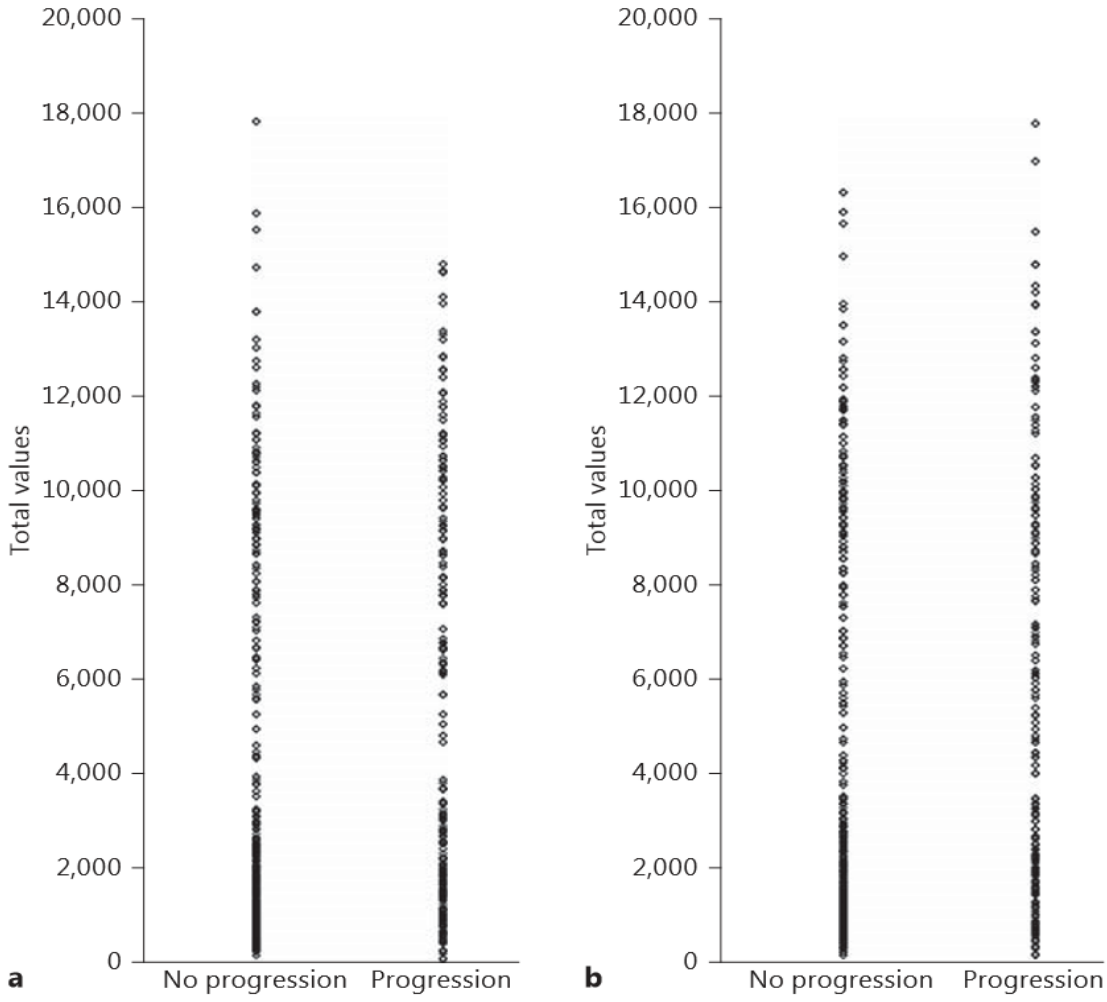

Figure 5.2 Dot plots on total values of RF during the **(a)** baseline or the first observation and the **(b)** final observation.

Dot plot comparison of surfaces that progressed to cavitation and those that did not progress, with total values of red fluorescence during the **(a)** first observation and the **(b)** final observation.

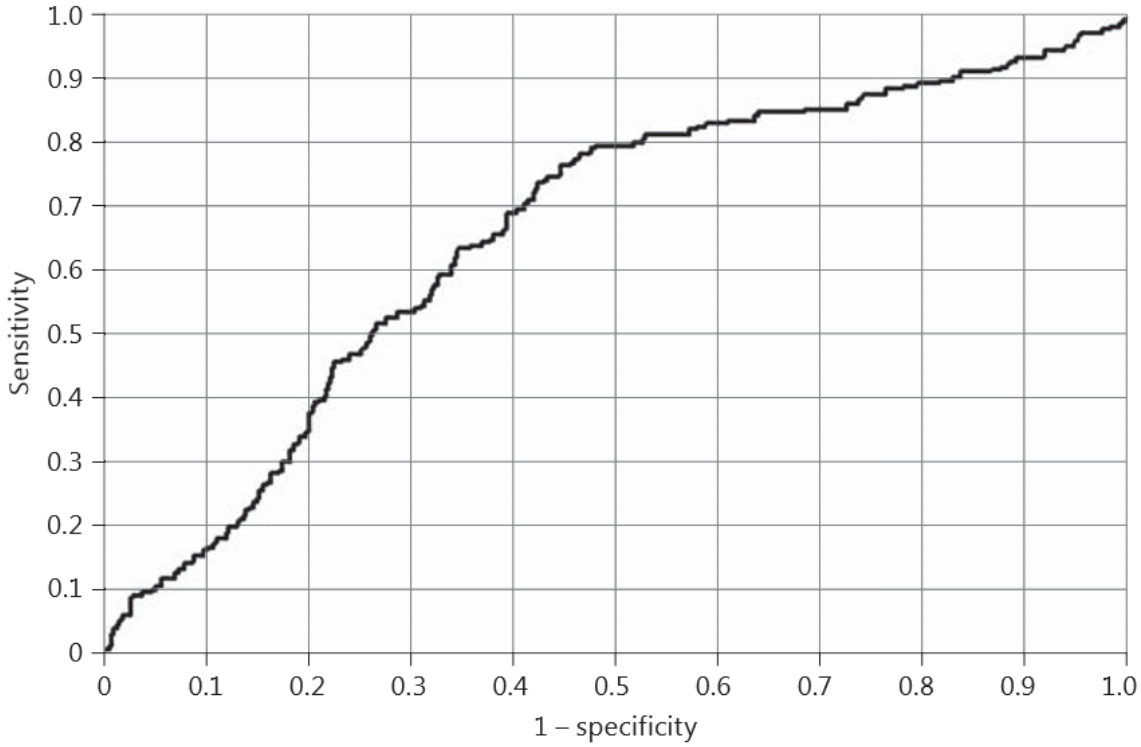

Figure 5.3 The area under the ROC curve based on RF analysis.

ROC predicting progression to cavitation from the final observation for red fluorescence total.

Table 5.1 Terminologies of QLF-D Parameters

	Terminology	Definition
1	Delta R (ΔR) $\Delta R_{Pixel} \cong \frac{(R/G)_{lesion} - (R/G)_{sound}}{(R/G)_{sound}} \times 100\%$	Ratio of red over green in the area of interest compared to a sound area of the tooth. Expressed in percentage (%). ΔR is based on thresholds at ΔR0, ΔR10, ΔR20, ΔR30, ΔR40, ΔR50, ΔR60, ΔR70, ΔR80, ΔR90, ΔR100, ΔR110, ΔR120 and Total.
2	Area of red fluorescence (AΔR)	Area of ΔR equivalent or higher to a specific threshold at ΔR30, ΔR70, ΔR120
3	Plaque wizard patch	A rectangular contour which could be adjusted to any size, and shape. Here it was placed around the lesion area, in the sound area of the tooth.
4.	Delta F(ΔF)	Average loss of green fluorescence in the carious surface compared to the green fluorescence in the sound tooth area. Expressed as percentage (%) and resembling, lesion depth.
5	Area of lesion (AΔF)	Area of the carious lesion surface expressed as px^2 equivalent to a threshold specific at ΔF.
6	Delta Q (ΔQ)	Percentage of green fluorescence loss (ΔF) times the area of the lesion (A). It is expressed as $\%px^2$ resembling, lesion volume.

Table 5.2 Comparison of RF values at the first or baseline visits and final visit of progressed surfaces compared to non-progressive surface

	First or baseline observation			Final observation or last before progression		
	Progressed to cavitation (N = 224)	Did not progress (N = 486)		Progressed to cavitation (N = 224)	Did not Progress (N = 486)	
RF Parameter	Mean (SE)	Mean (SE)	p-value	Mean (SE)	Mean (SE)	p-value
SPS™	0.045 (0.021)	0.019 (0.011)	0.0522	0.067 (0.031)	0.029 (0.009)	0.8458
Area ΔR30	0.069 (0.035)	0.039 (0.019)	0.3948	0.142 (0.086)	0.044 (0.012)	0.4164
Area ΔR70	0.016 (0.010)	0.009 (0.006)	0.0191*****	0.022 (0.017)	0.006 (0.002)	0.4774
Area ΔR120	0.000 (0.000)	0.000 (0.000)		0.003 (0.003)	0.000 (0.000)	0.1905
ΔR0	1341 (87)	752 (48)	<.0001*****	1449 (96)	867 (57)	<.0001*****
ΔR10	43.00 (5.06)	26.87 (3.14)	0.0009*****	51.17 (6.27)	32.00 (3.49)	<.0001*****
ΔR20	5.96 (1.45)	2.56 (0.48)	0.0029*****	9.55 (2.70)	3.72 (0.70)	0.3863
ΔR30	2.37 (0.83)	0.84 (0.28)	0.3770	4.43 (1.96)	1.46 (0.44)	0.3603
ΔR40	1.38 (0.58)	0.49 (0.25)	0.2837	2.76 (1.63)	1.01 (0.37)	0.2869
ΔR50	0.95 (0.44)	0.34 (0.20)	0.0538	2.04 (1.35)	0.62 (0.27)	0.4398
ΔR60	0.64 (0.29)	0.23 (0.13)	0.0167*****	1.27 (0.83)	0.37 (0.18)	0.2233
ΔR70	0.33 (0.16)	0.12 (0.07)	0.0191*****	0.71 (0.43)	0.21 (0.12)	0.4749
ΔR80	0.17 (0.09)	0.04 (0.03)	0.0511	0.42 (0.25)	0.13 (0.10)	0.7095
ΔR90	0.07 (0.05)	0.02 (0.01)	0.1440	0.33 (0.23)	0.09 (0.08)	0.3285

ΔR100	0.02 (0.01)	0.00 (0.00)	0.0373*	0.28 (0.20)	0.07 (0.07)	0.4253
ΔR110	0.01 (0.01)	0.00 (0.00)	0.1416	0.22 (0.17)	0.06 (0.06)	0.1905
ΔR120	0.00 (0.00)	0.00 (0.00)		0.18 (0.15)	0.04 (0.04)	0.1905
ΔRMax	44.08 (1.43)	35.85 (0.75)	<.0001*	47.71 (2.14)	40.51 (1.05)	0.0002*
Total	4955 (285)	3038 (166)	<.0001*	5113 (287)	3204 (169)	<.0001*

Chapter 6

Effect of Violet-Blue Light on *Streptococcus mutans* Induced Carious Lesions on Human Enamel Specimens

New and novel antimicrobial methods are evolving continuously at the primary level of preventing dental caries. The purpose of this *in-vitro* study was to determine the effectiveness of Violet-Blue light on prevention and progression of dental caries induced by *Streptococcus mutans* biofilm on human enamel specimens. *S. mutans* UA159 strain was grown on mitis-salivarius sucrose bacitracin (MSSB) agar plates, followed by a subculture with 5 ml of Tryptic Soy Broth (TSB) for 24 hrs. Biofilm was allowed to form on human enamel specimens (4x4x2 mm^2) in wells of a 96-well microtiter plate, and was incubated for 13 hrs in 5% CO_2 at 37°C in the presence or absence of 1% sucrose (TSBS). Violet-Blue light with a peak wavelength of 405 nm from an early caries detection device called Quantitative Light Induced Fluorescence (QLF) was used as the light source to irradiate the biofilm. Supernatant liquid was removed before irradiation, and biofilm was treated with QLF for 5 min, twice a day with a reincubation period of 6 hrs for 5 days, except for a single treatment and no reincubation on the 5^{th} day. Colony forming units (CFU) of the light treated biofilm were quantitated on the 5^{th} day of the experiment. QLF-D Biluminator was used to determine loss of fluorescence (ΔF), lesion Area (WS Area), lesion volume (ΔQ), and ΔF_{Max} (Maximum fluorescence loss). Lesion depth and mineral loss were determined through

transverse microradiography (TMR). The results demonstrated a significant difference ($p<0.05$) in *S. mutans* biofilm CFU between the treated and non-treated groups grown in both TSB and TSBS. QLF-D parameters were not statistically different for TSB or TSBS cultures for treated and non-treated groups. Mineral loss was significantly reduced for TSB cultures with Violet-Blue light treatment. Within limitations of the study, the results indicate that Violet-Blue light can serve as an adjunct prophylactic treatment for reducing the numbers of *S. mutans* necessary to form biofilm.

6.1 Introduction

Dental caries is a biofilm mediated disease. There are a multitude of factors involved in the formation and progression of caries (Selwitz et al, 2007). It is preventable by controlling at least few of the many factors involved in the development of the disease using a high-risk approach (Watt, 2005). Earlier, the dogma was to detect the caries at the earlier stages to prevent the progression of white spot lesion to cavitation. Currently, the prevention strategies are at the molecular and microscopic level of prevention, and to control oral biofilm. Enamel is hard and as resistant as a ceramic, composed of 95% inorganic material and 1% organic content and water (Baldassarri et al, 2010; Radlanski, 1998; Robinson et al, 2000). The first line of defense for the tooth is the enamel. It is acellular and has no capacity to regenerate (Moradian-Oldak J, 2012). Surface enamel forms an interface with the dental pellicle followed by dental plaque or oral biofilm. *S.*

mutans is considered as a primary cariogenic bacterium and has the capacity to form biofilm and resist acid conditions (Banas, 2004).

Despite fluoridation measures, dental caries remains a global public health problem (Peterson and Lennon, 2004). It can affect anytime throughout their entire life and white spot lesions usually remain unnoticed. Unfortunately, there has been no improvement in the conditions affecting oral health for the last 25 years. It is considered as a disability which affects the overall health outcome. (Kassebaum *et al*, 2017). Caries formation is a dynamic process with remineralization and demineralization. Constant chemical changes associated with fluctuations in the host oral environmental factors and acids produced by the metabolically active bacteria are responsible for the weathering of the tooth surface. White spot lesions are the first step in the ongoing caries process. Subsurface lesions may form underneath enamel with an intact surface layer (Pitts et al, 2017). The cells in bacterial biofilm formed on the surface of the tooth metabolize sugars and produces acid causing demineralization (Fejerskov O and Kidd E, 2009). Preventive methods to control plaque or biofilm includes inhibiting biofilm formation and its metabolic activity, prevention of adherence of bacteria to the tooth surface, modification of the biofilm architecture, inhibition of acid production, killing the bacteria in biofilm, and inhibition of the production of extracellular polysaccharides. One novel method is phototherapy to inactivate oral biofilm formation (Ten Cate and Zaura, 2012).

Light therapy studies have been applied for several oral microorganisms (Soukos et al, 2011). *Streptococcus mutans* had been studied widely under

photodynamic therapy with exogenous photosensitizers such as Erythrosine, Rose Bengal, Toluidine Blue, Malachite green, and much more (Wood, Metcalf, Devine, Robinson, 2006, Zanin *et al* ,2006, Pereira CA, Costa AC, Carreira, CM, Junqueira JC, Jorge ,2013, Rolim *et al*, 2012). Only a few studies have focused on phototherapy without the presence of exogenous photosensitizers (Feuerstein et al, 2006, Steinberg et al, 2008, Chebauth et al, 2012). Preliminary studies have shown the photo inhibitory effects of Violet-Blue light on *S. mutans* biofilm (Gomez et al, 2013). To further explore the effect of Violet-Blue light on carious lesions induced by *S. mutans* biofilm on human enamel specimens, we proposed a treatment with Violet-Blue light for 5 min, 2 times a day, 4 days a week, and once a day on the 5th day of the treatment.

6.2 Materials and Methods

6.2.1 Study Design

A total of 60 enamel specimens were randomized into 4 intervention groups and one baseline group. Group 1 samples (n=12) were incubated in TSB (0%) sucrose and treated with Violet-Blue light; Group 2 samples (n=12) were incubated in TSBs (1%) sucrose and treated with Violet-Blue light; Group 3 samples (n=12) were incubated in TSB (0%) sucrose and were treated with Violet-Blue light; Group 4 samples (n=12) were incubated in TSBs (1%) sucrose and were not treated with Violet-Blue light. Baseline (n=12) was randomized for both TSB and TSBS

cultures. The samples for the intervention groups (N=48) were randomized into Violet-Blue light treated and non-treated groups (n = 24). These groups were further subdivided (n=12) into SB (0% sucrose) and TSBS (1% sucrose). In addition, baseline samples (n = 6) for both TSB and TBSS cultures were also used. Groups 1 and 2 were treated with Violet-Blue light for of 5 min, 2 times a day, 4 days a week, and once a day on the 5^{th} day of the treatment. Quantification of biofilm cells were done at 13 hours at baseline and on the final day of the treatment period. Further, all 60 enamel specimens were imaged for fluorescence loss using QLF-D and sectioned to determine mineral loss and lesion depth through TMR **(Figure 6.1)**.

6.2.2 Bacterial Culture Conditions

Cells of *S. mutans* (UA159, serotype c strain) stored in -80ºC with glycerol were used in this study. The bacteria were cultured on mitis-salivaris sucrose bacitracin (MSSB, Anaerobe Systems, California) agar plates. *S. mutans* bacterial broth culture was allowed to grow in 5 ml of Tryptic Soy Broth (TSB, Acumedia, Baltimore, MA) for 24 hrs in a 5% CO_2 incubator at 37°C.

6.2.3 Selection of Tooth Specimens

Sixty enamel specimens from extracted human molars without any cracks, fractures, or caries were selected. A Lap Craft L'il Trimmer was used to decoronate

the crown portion of the tooth. Enamel specimens with a dimension of 4×4×2 mm were cut using a Buehler Isomet lathe. The specimens were ground sequentially with 500, 1200, 2400, and 4000 grit sand paper by mounting them on an acrylic block with Struers RotoPol-31/RotoForce-4 grinding machine. Specimens were ground with each sandpaper grit for 4 sec to a thickness of 2 mm. The actual thickness ranged from 1.6 to 2.10 mm. Clear nail varnish was coated on all sides and the bottom of the enamel specimen. Nearly 1/3rd of the top surface of the enamel specimen was covered with nail varnish. Quality assurance of the specimens was done with a stereomicroscope to determine cracks, fractures, or fissures.

6.2.4 Sterilization of Human Enamel Specimens

Human enamel specimens were rinsed for 3 min, sonicated for 3 min, and rinsed again for 3 min with deionized water (dH_2O). The specimens were placed in moist cotton gauze, sealed in a whirl pak bag, and sterilized with ethylene oxide gas.

6.2.5 Biofilm Formation

Bacterial broth cultures were diluted 1:100 with TSB or TSB supplemented with 1% sucrose (TSBS). *S. mutans* biofilm cells were grown in wells of sterile 96-well microtiter plates (Fisher Scientific, Co., Newark, DE) with and without 1%

sucrose (4 × 4 × 2 mm; n=24). The biofilm cells were incubated for 13 hrs in a 5% CO_2 incubator at 37°C to reach logarithmic phase of growth. A gap of one well was left in between the biofilm samples, and a 2-well distance was maintained between TSB and TSBS (1%) groups.

6.2.6 Light Source

Quantitative light induced fluorescence (QLF-clin, Inspektor Research Systems BV, Amsterdam, Netherlands) was used as a light source in this study. It is an early caries detection light device with an excitation peak wavelength of 405 nm and a spectral range of 380 to 450 nm. It is used to detect changes in the mineral content of the tooth in a noninvasive manner and identifies early lesions which will likely progress to cavitation. It employs a 35-Watt Xenon arc lamp, and Violet-Blue light is filtered through a high pass band filter. The spectral range of the Violet-Blue light is within 380 to 450 nm with a peak wavelength at 405 nm.

6.3 Treatment with Violet-Blue Light

Before irradiation, planktonic or supernatant liquid was removed, and the wet biofilm was exposed to Violet-Blue light continuously for 5 minutes. The supernatant was stored in 1.5 ml minicentrifuge tubes to measure pH. The distance between the bottom of the microtiter plate and the light source was kept at 2 cm. A black background was used to avoid scattering of light. Seal mate was placed

as a barrier between the sample and the light source opening. Immediately after exposure, freshly prepared TSB or 1% TSBS growth medium was added to each well. The treated biofilm cells were reincubated for 6 hrs in a 5% CO_2 incubator at 37°C. After 6 hours, the biofilm cells were again treated with Violet-Blue light for 5 minutes and reincubated for 13 hrs until the next treatment on the following consecutive day. The treatment was performed twice a day for four days and once on the final day without reincubation.

6.3.1 Quantification of Colony Forming Units

At the end of 5th day of the experiment, supernatant liquid was removed for pH measurements. Baseline CFU's of a 13 hr biofilm on the first day before the treatment was also obtained. Biofilm at the bottom of the plate was gently washed once with 0.9% saline. Enamel specimens were carefully removed from the microtiter plate and placed into 1 ml of saline solution in mini centrifuge tubes. The specimens were vortexed for 10 sec, sonicated on ice for 20 sec, and vortexed again for 10 sec. Serial dilution of the bacterial samples was done with 0.9% saline, and each diluted sample was plated on Tryptic Soy Agar (TSA) plates using a spiral plater (Spiral System^{TM}). TSA agar plates were incubated for 48 hrs at 37°C in a 5% CO_2 incubator, and the colony forming units (CFUs) were counted by an automated colony counter using Protocol^{TM} (Synbiosis Inc, Fredrick, MD) software.

6.3.2 Determination of Lesion Depth

A quantitative light induced fluorescence (QLF-D) biluminator was used to acquire images of the enamel specimens. A jig was prepared to secure the enamel specimen with silicone rubber (Oomo-30). The images were acquired through an illumination tube fitted on a SLR camera with white and blue light emitting diodes (LED) under dark conditions. Fluorescence images of enamel specimens were obtained using a C3 proprietary software on QLF-D. The images were digitally archived for further analysis of mineral loss or lesion depth through QA2 analysis software. QLF-D parameters including Fluorescence loss, delta F (ΔF); Maximum fluorescence loss (F_{Max}); Lesion volume, delta Q (ΔQ); and Lesion area (WS Area) were collected.

6.3.3 Transverse Micro-Radiography

Enamel specimens were treated briefly with 70% ethyl alcohol and stored under moistened conditions with deionized water (diH_2O). Enamel specimens were superglued to acrylic rods, and enamel sections of 100±20 µm thickness were prepared with a hard tissue microtome. The enamel sections were placed on an ultra-resolution flat plate sized 5×5×2 mm (Microchrome Technology Inc). Calibration of the TMR PSL Imaging System (Thermo-Kevex PXS5-928WB-LV, Tube 48934) related to its absorption coefficient was done with an aluminum step wedge for an acceptable correlation of 0.99970. Imaging of the enamel specimens

were obtained through X-ray source with a voltage (kV) and a current (µA) of 45. The obtained images were read and processed using TMRD1 5.0.01 software and finally analyzed through TMR2006 software v.3.0.0.18. Mineral loss (Vol %. µm) and lesion depth (µm) were determined.

6.3.4 pH Measurements

A pH meter (Accumet, Fisher Scientific, Pittsburgh, PA) was used to measure the pH of the pooled samples of every group. The supernatant or planktonic fluid on top of the biofilm was removed before light irradiation for pH measurement. pH was measured on the first day before the treatment and on the 5^{th} or final day of the treatment regimen.

6.4 Statistical Methods

One-way ANOVA was done separately for TSB and TSBS cultures, followed by pairwise group comparisons for baseline, Violet-Blue Light treated, and non-treated groups to determine any difference in CFUs. QLF-D parameters such as fluorescence loss (∆F), lesion area (WS Area), lesion volume (∆Q), lesion depth (µm), and mineral loss (Vol%. µm) were obtained through transverse microradiography (TMR).

6.5 Results

6.5.1 Photoinhibition of *S. mutans* Biofilm on Human Enamel Specimens

Baseline (n=6) *S. mutans* biofilm grown in the absence of sucrose with TSB had lower CFUs than the Violet-Blue light treated groups ($p<0.001$) and the non-treated groups ($p<0.0001$). Baseline *S. mutans* biofilm grown in the presence of sucrose with TSBS had higher CFU's than the Violet-Blue light treated groups ($p<0.0001$) and the non-treated groups ($p=0.0149$). Baseline CFU's obtained at 13 hrs were compared against CFU's obtained immediately after the first treatment and on the final day of the treatment without any re-incubation **(Figure 6.2; Figure 6.3)**.

Results of photoinhibition by Violet-Blue light at the end of the treatment demonstrated that Violet-Blue light treated groups (n=12) had significantly lower CFU's than the non-treated control groups (n=12) in the absence of sucrose ($p=0.0333$), and similar significantly different results were obtained in the presence of sucrose with TSBS ($p=0.0008$). There was a reduction of 28% in bacterial numbers in the absence of sucrose and 48% in the presence of sucrose **(Figure 6.2)**.

With respect to QLF-D parameters, there were no differences among the baseline, Violet-Blue light treated, and non-treated groups ($p=0.37$ for delta F, $p=0.40$ for delta Fmax, $p=0.40$ for delta Q, $p=0.41$ for WS Area, $p=0.12$) **(Figure 6.4**; **Figure 6.6)**.Enamel specimens subjected to *S. mutans* biofilm formation in the

presence of 1% sucrose demonstrated that there were significant differences among the baseline, Violet-Blue light treated groups, and non-treated control groups. ΔF, ΔF_{Max}, ΔQ were significantly lower for baseline than the Violet-Blue light and non-treated control groups ($p<0.0001$) **(Figure 6.5**; **Figure 6.6)**. The photo inhibitory effect of the Violet-Blue light treated, and non-treated groups indicated that there were no significant differences in TSBS cultures ($p>0.08$) **(Figure 6.5)**.

Photo inhibition of Violet-Blue light was effective on *S. mutans* biofilm in the absence of sucrose exhibiting less mineral loss ($p=0.0293$) **(Figure 6.7)**. However, there was no significant difference in mineral loss of samples with *S. mutans* biofilm formed in TSBS ($p=0.09$) **(Figure 6.7)**. Violet-blue light treated groups and non-treated control groups did not have significantly different lesion depths in TSB or TSBS ($p>0.14$) **(Figure 6.8)**. Results based on lesion depth indicated that there was less mineral loss and smaller lesion depth for the baseline enamel specimens compared to Violet-Blue light treated ($p<0.001$) and non-treated control groups ($p\leq0.0001$) **(Figure 6.9**; **Figure 6.10)**.

6.5.2 pH Measurements

pH values of the supernatant or planktonic fluids at the beginning of the treatment on the first day were obtained. The Violet-Blue light treated group in the absence of sucrose had a pH of 4.96 before treatment and the non-treated control groups had a pH of 4.99. The pH of planktonic or supernatant fluids in the presence

of sucrose was 4.43 for Violet-Blue light treated groups and non-treated control groups had a pH of 4.45 on the first day before the treatment. On the final day of the treatment, the pH values with TSB were 4.88 and 4.85 for the treated and the non-treated groups, respectively. With TSBS the pH value was 4.1 for both treated and non-treated groups.

6.6 Discussion

The current study[4] demonstrated that Violet-Blue light had a statistically significant photoinhibitory effect on the number of CFU's of *S. mutans* after 5 days of treatment. Irrespective of the presence or absence of sucrose (1%), treatment with Violet-Blue light reduced the numbers of *S. mutans* biofilm cells. This effectiveness was based on a 13 hr old biofilm, with two treatments on each of 4 days and one treatment on the final day of the treatment regimen. There was also a 6 hr reincubation period between the treatments with no reincubation on the final day of the treatment period. Previously, in chapter 4 we reported that the metabolic activity of *S. mutans* biofilm at 0 hr was reduced significantly compared to 2 and 6 hr of reincubation. The baseline CFU's of *S. mutans* at 13 hr was lower than the Violet-Blue light treated TSB and TSBS groups. These results demonstrated that, although Violet-Blue light was effective in reducing the numbers of the bacteria, there was regrowth of bacteria after photoinactivation. The findings correlate with

[4] Felix Gomez, G. G., Lippert, F., Ando, M., Zandona, A. F., Eckert, G.J., & Gregory, R. L. (2018). Effect of Violet-Blue Light on Streptococcus mutans-Induced Enamel Demineralization. Dent J (Basel), 6(2). doi:10.3390/dj6020006. Reproduced with permission from the publisher.

the previous findings related to significant reduction in metabolic activity at 0 hr compared to 2 and 6 hr. It would be ideal to harvest bacterial cells after each time period to determine the temporal variation in bacterial viability with Violet-Blue light treatment. The supernatant containing planktonic bacteria above the biofilm was removed and used for pH measurements. The pH on the first day before the treatment was similar to that of baseline. For the Violet-Blue Light treated and non-treated groups in the absence of sucrose, the pH averaged 4.96 and 4.99, respectively. On the 5^{th} day of treatment the pH values were 4.88 for the Violet-Blue light treated group and 4.85 for the non-treated group in the absence of sucrose. The pH value was 4.1 for both treated and non-treated TSBS groups. There was not much difference between the treated and the nontreated groups at the end of the treatment as it might have been due to the 13 hrs incubation before treatment. Measuring the pH of biofilm would be an alternative option.

In relation to the QLF-D parameters, unlike the significant reduction of CFU numbers in the Violet-Blue light treated groups in TSB, there was no difference between the delta F (ΔF), delta F_{Max} (ΔF_{Max}), Volume (ΔQ), and Area (WS Area). Though there was slight reduction in all the QLF-D parameters, it was not statistically different from the non-treated groups. This signifies that Violet-Blue light has a direct effect on the inactivation of bacteria. Since the QLF-D parameters were not much different for baseline and the experimental groups, variations of the enamel surface would have also contributed to this finding. Another possible explanation is that Violet-Blue light affects the architecture and development of the extra polysaccharide matrix of the biofilm. A recent report found that Violet-Blue

did not have an effect on soluble extracellular polysaccharides (de Souza et al, 2015). In the presence of 1% sucrose, there was no significant difference in QLF-D parameters between the Violet-Blue light treated and non-treated control groups. There was a slight reduction of all the QLF-D parameters mentioned above in the Violet-Blue light treated groups compared to non-treated groups, with minimal deviation, but this was not statistically significant. The baseline values had lower QLF-D parameters and were significantly different from violet-blue light treated and non-treated groups.

The gold standard method for determining lesion depth is to section enamel specimens, process microradiography images, and analyze them. The present findings supported that baseline values for mineral loss and lesion depth were significantly lower compared to Violet-Blue light treated groups and non-treated control groups. In the absence of sucrose with TSB, lesion depth and mineral loss were significantly lower compared to the non-treated groups; however, this was not found with TSBS-grown groups. Early caries detection devices such as QLF differ in sensitivity based on enamel or dentin. It has lower sensitivity with enamel compared to dentin. Artifacts on the surface of enamel such as sucrose-grown biofilm may have contributed to the above findings and might be one of the limitations of the study.

6.7 Conclusion

Colony forming units (CFU) and lesion depth measured as mineral loss or fluorescence loss measured through QLF-D confirmed by the gold standard of transverse microradiography were compared between Violet-Blue light treated and non-treated groups in both TSB and TSBS at the end of the treatment period. Violet-Blue light had an inhibitory effect on the bacterial viability of *S. mutans* biofilm in both TSB and TSBS. There was no statistically significant effect of Violet-Blue light on the mineral level of tooth surface or the mineral loss determined through QLF-D. There was a slight reduction in the amount of loss of fluorescence. Mineral loss obtained through TMR was statistically significant in the Violet-Blue light treated group; however, lesion depth was not statically significant. Violet-Blue light has a more effective photo inhibitory effect at the bacterial level on the surface of the tooth than at the mineral level.

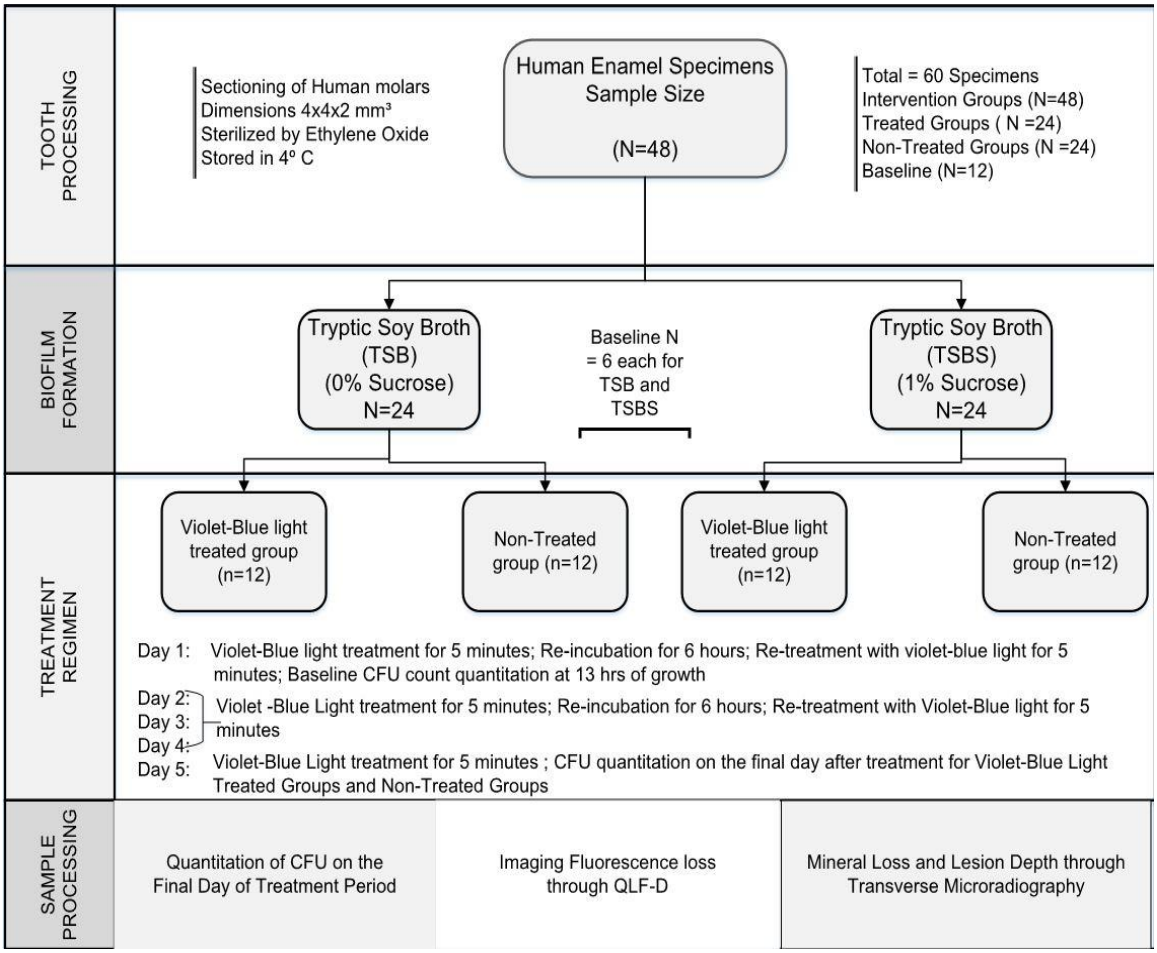

Figure 6.1 Flow chart of the study design.

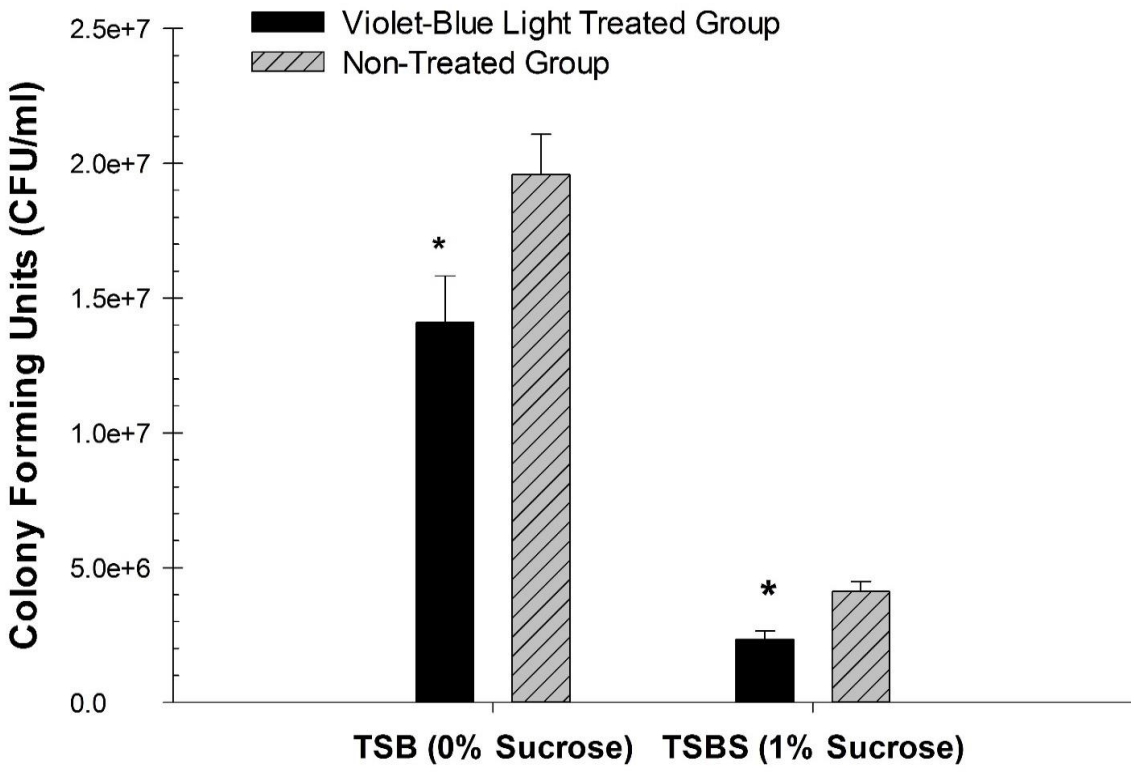

Figure 6.2 The effect of Violet-Blue light on TSB and TSBS-grown *S. mutans.*

The effect of Violet-Blue light on TSB-grown *S. mutans* at the end of the treatment period indicated significantly lower CFU's in the light-treated group than the non-treated group ($p<0.03$). The effect of Violet-Blue light for TSBS-grown *S. mutans* at the end of the treatment period had significantly lower CFU's in the light-treated group than the non-treated group ($p<0.0008$). Significance level was kept at 5%. Asterisks represent significant differences ($p<0.05$) between the light and no light groups. Mean $\pm$ SE.

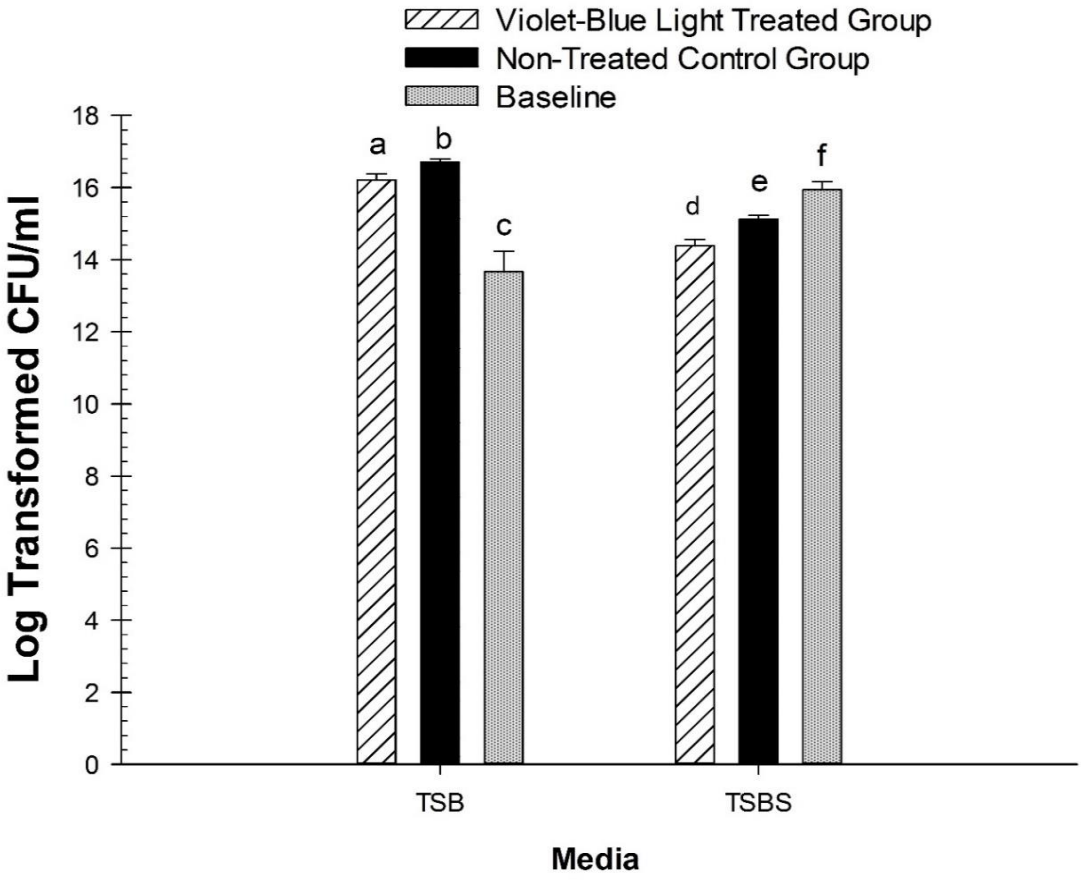

Figure 6.3 Comparison of baseline CFU with treated and non-treated groups.

The CFU's of the Violet-Blue light-treated and non-treated group on the 5th day of the treatment were compared with the baseline counts obtained at 13 hrs for TSB and TSBS. There were statistically significant differences between the Violet-Blue light treated, non-treated, and baseline groups for TSB and TSBS. CFU's of baseline in TSB were lower than those of Violet-Blue light treated and non-treated groups, and CFU's of baseline in TSBS were higher than Violet-Blue light treated and non-treated groups. Different lower-case letters represent significant differences between groups.

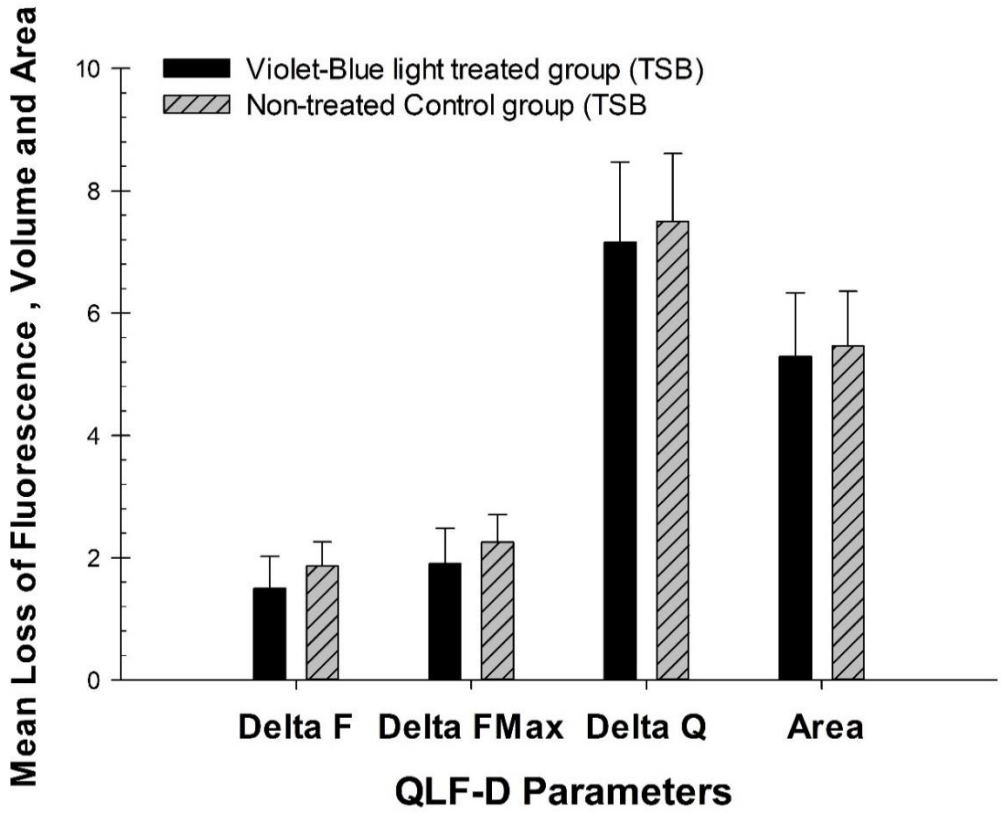

Figure 6.4 Effect of Violet-Blue light on *S. mutans* caries activity without sucrose.

QLF-D parameters such as lesion depth or loss of fluorescence (ΔF), Fluorescence Maximum (ΔF_{Max}), Volume of the lesion (ΔQ), and Area of the lesion (Area) were not significantly different in the Violet-Blue light-treated groups and non-treated groups (p=0.37 for delta F, p=0.40 for delta Fmax, p=0.40 for delta Q, p=0.41 for WS Area, p=0.12).

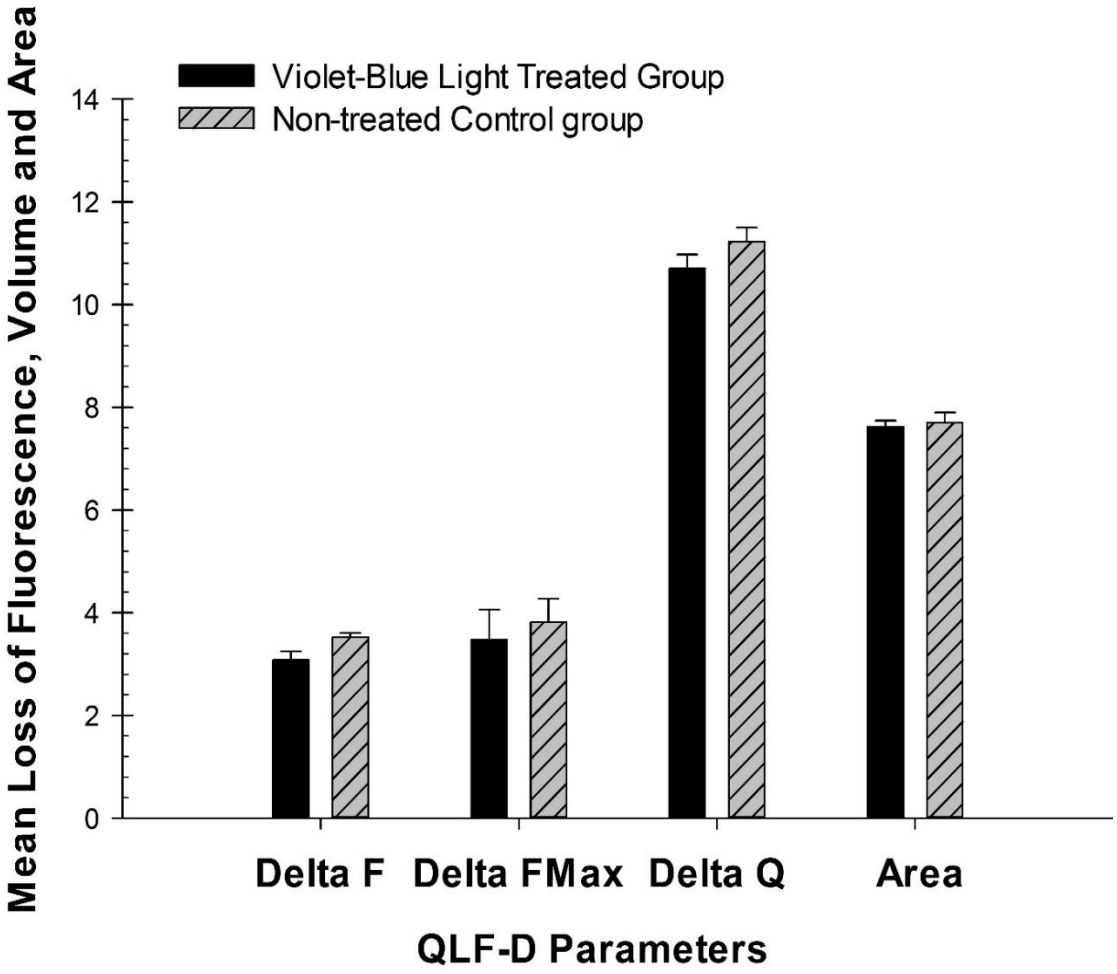

Figure 6.5 Effect of Violet-Blue light on *S. mutans* caries activity with sucrose.

QLF-D parameters such as lesion depth or loss of fluorescence (ΔF), Fluorescence Maximum (ΔF_{Max}), Volume of the lesion (ΔQ), and Area of the lesion (Area) were not significantly different between the Violet-Blue light-treated groups and non-treated groups ($p > 0.08$).

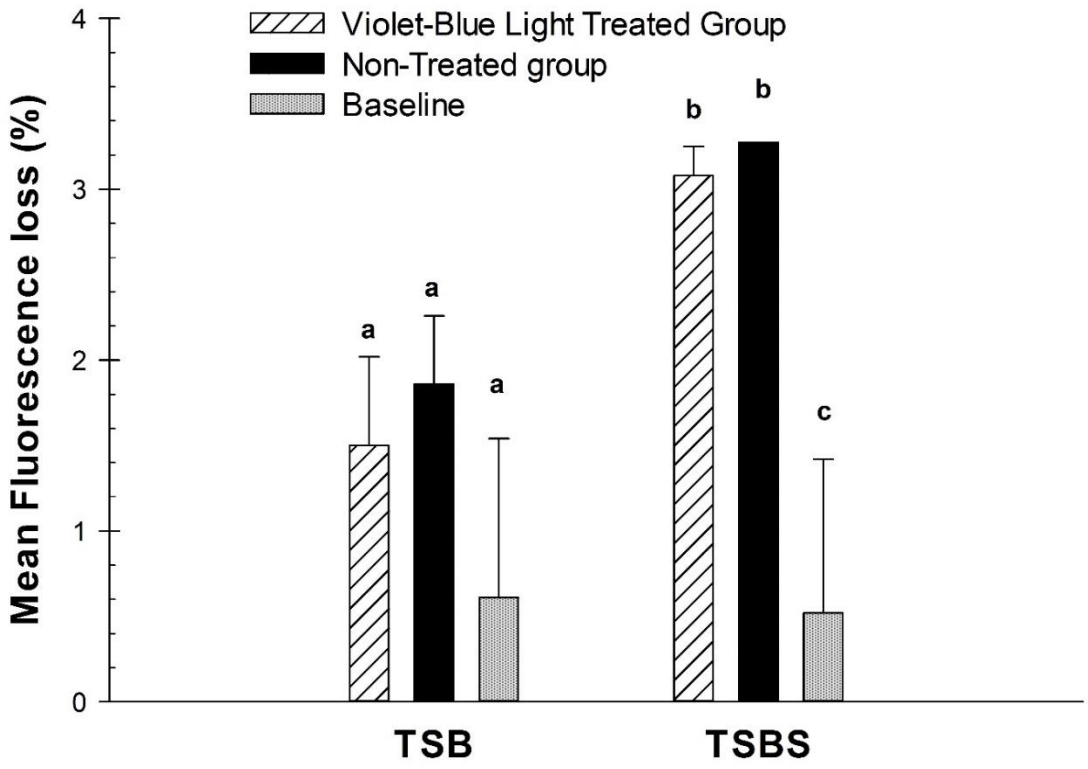

Figure 6.6 Comparison of mean fluorescence loss (%) in baseline, treated, and non-treated groups.

Lesion depth or loss of fluorescence (ΔF) were not different for baseline CFU's of TSB ($p = 0.37$). There was no significant difference among the baseline, Violet-Blue light treated, and non-treated groups with TSB. There was a significant difference in baseline CFU's compared with Violet-Blue light and non-treated groups in TSBS. Different lower-case letters represent significant differences between groups.

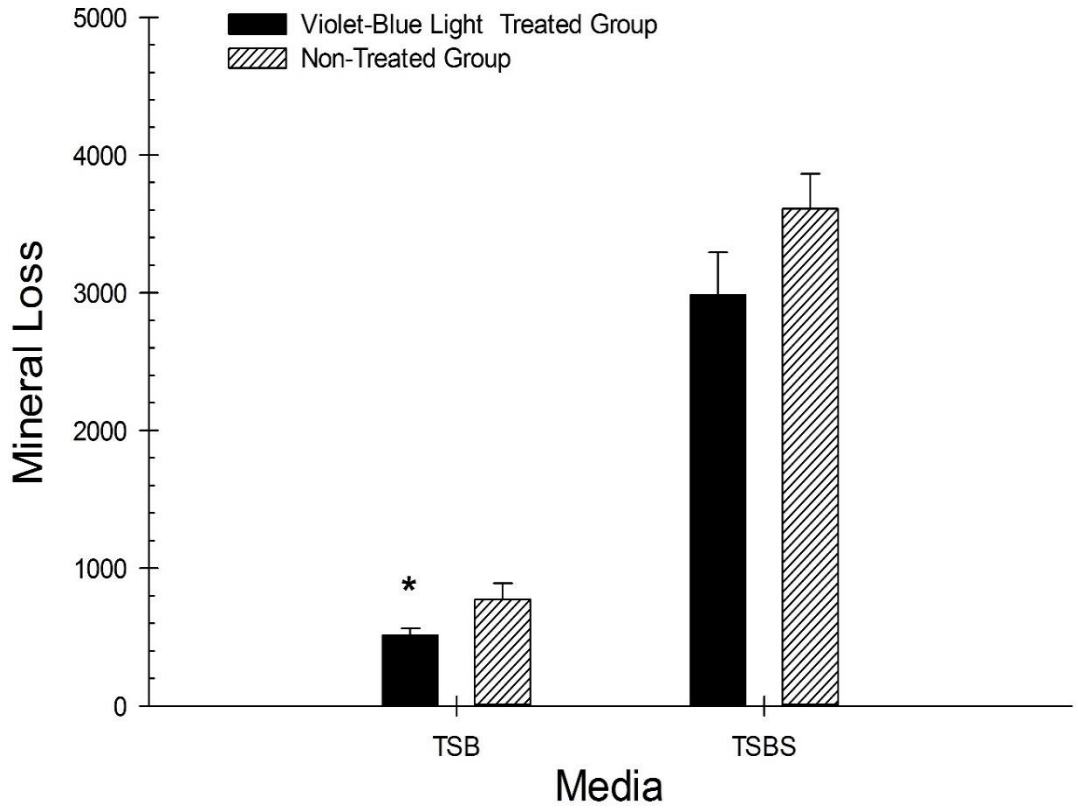

Figure 6.7 Effect of Violet-Blue light on the mineral loss (TMR) on human enamel specimens in TSB and TSBS.

Mineral loss was significantly reduced in Violet-Blue light treated TSB groups compared to non-treated groups ($p=0.0293$). There was no statistically significant difference between the treated and the non-treated groups grown in TSBS ($p=0.09$). Asterisks indicate statistical significance. Significance level was kept at $p < 0.05$.

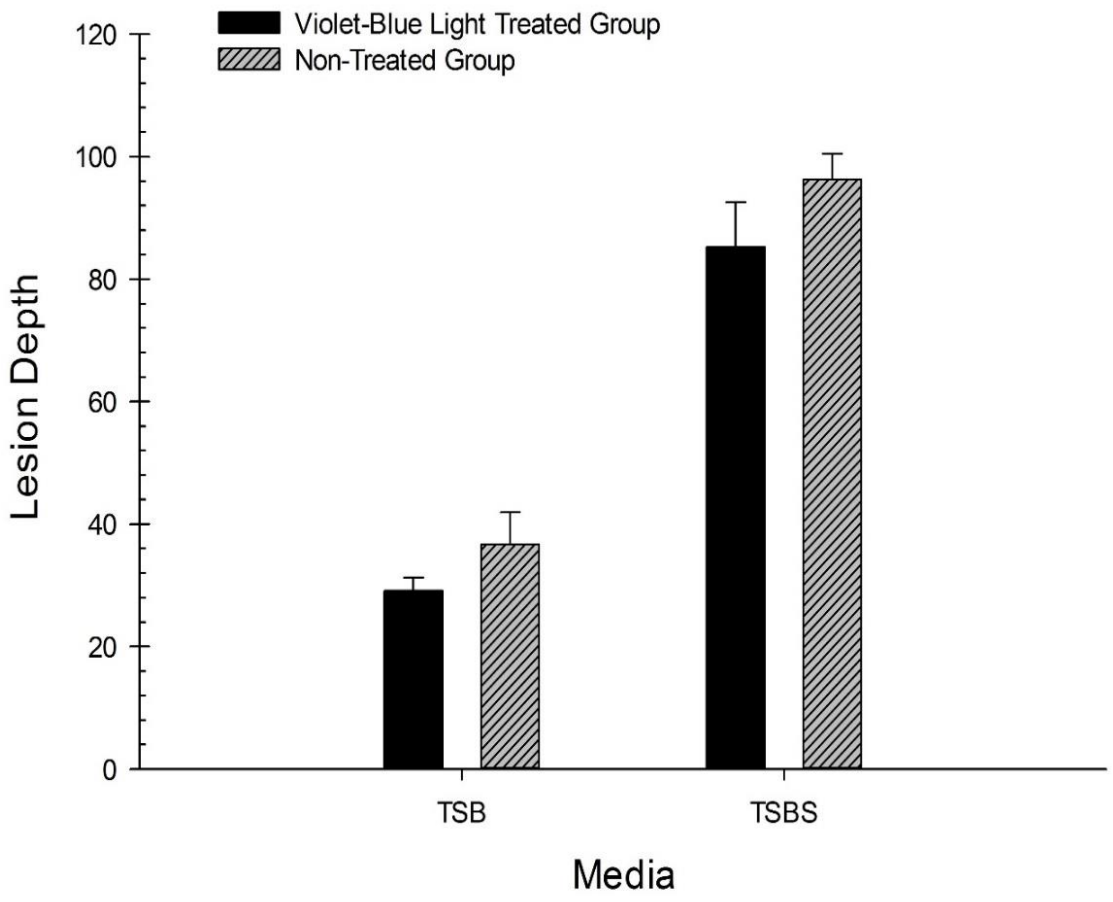

Figure 6.8 Effect of Violet-Blue light on the lesion depth (TMR) on human enamel specimens in TSB and TSBS.

The photoinhibitory effect of Violet-Blue light on the lesion depth produced by *S. mutans* biofilm on human enamel specimens was not statistically significant between TSB and TSBS ($p>0.14$) with ad without violet-blue light treatment.

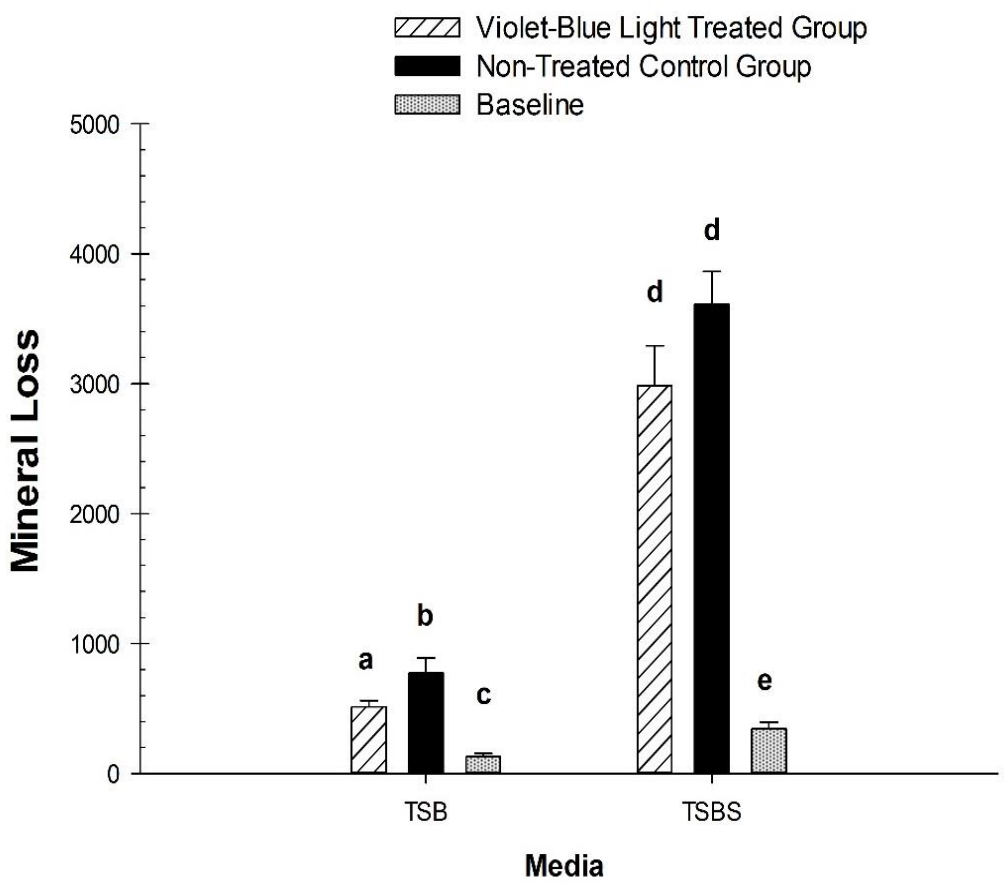

Figure 6.9 Mineral loss for baseline of biofilms grown in TSB and TSBS compared with the Violet-Blue light treated and non-treated groups.

Mineral loss for baseline of biofilms grown in TSB and TSBS was significantly reduced and compared with the Violet-Blue Light treated ($p<0.001$) and non-treated groups ($p\leq0.0001$). Mineral loss of Violet- Blue light treated groups in TSB was significantly reduced compared with that of the non-treated groups ($p=0.0293$); however, no significant mineral loss was found in TSBS ($p=0.09$). Similar lowercase letters represent no significant differences, and different lower-case letters represent significant differences.

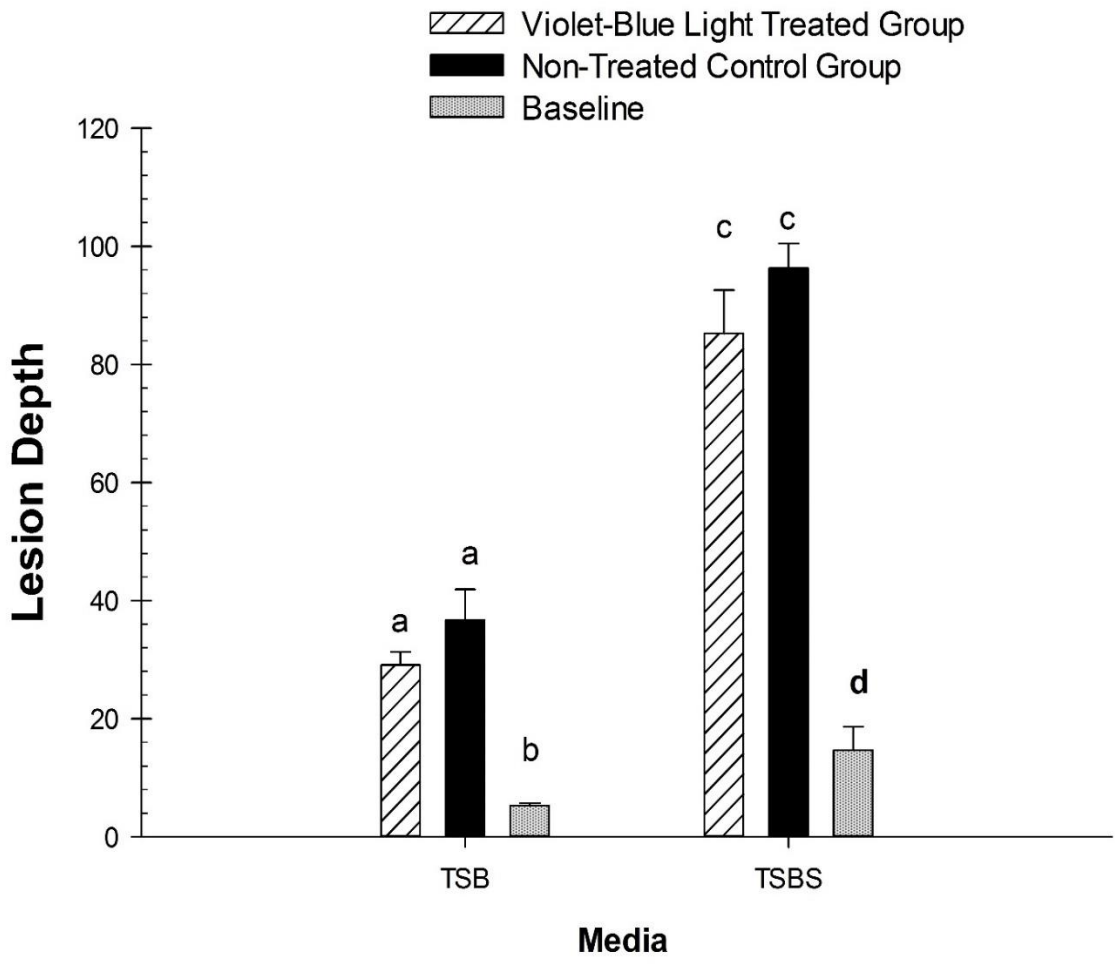

Figure 6.10 Lesion depths for baseline of biofilms grown in TSB and TSBS compared with the Violet-Blue Light treated and non-treated groups.

Lesion depths for baseline of biofilms grown in TSB and TSBS were significantly reduced compared with the Violet-Blue Light treated ($p<0.001$) and non-treated groups ($p\leq0.0001$). Similar lowercase letters represent no significant differences, and different lower-case letters represent significant differences.

Chapter 7

Photoinhibition of *Streptococcus mutans* Biofilm-Induced Caries Progression in Human Dentin by Violet-Blue Light

The purpose of this *in-vitro* translational study was to determine the effectiveness of violet-blue light on prevention and progression of dental caries produced by *Streptococcus mutans* biofilm on human dentin specimens. *S. mutans* UA159 biofilm was formed on human dentin specimens (4x4x2 mm^3) in wells of a 96-well microtiter plate and was incubated for 13 h in 5% CO_2 at 37°C in Tryptic Soy Broth (TSB) or TSB supplemented with 1% sucrose (TSBS). Violet-blue light from Quantitative Light Induced Fluorescence (QLF) with a peak wavelength of 405 nm was used to irradiate the biofilm. Before irradiation, supernatant liquid was removed, and the biofilm was treated continuously with QLF for 5 min, twice a day with an interval of 6 h for a period of 5 days. Colony forming units (CFU's) of the treated biofilm were quantitated. Lesion depth (L) and mineral loss (ΔZ) were quantified using transverse microradiography (TMR). Quantitative light-induced fluorescence Biluminator (QLF-D) was used to determine mean fluorescence loss. They were obtained at the end of the 5^{th} day of treatment and compared with the non-treated groups. The results demonstrate a significant difference ($p<0.05$) in the CFU's of *S. mutans* biofilm between the treated and non-treated groups grown in both TSB (0% sucrose) and TSBS. Lesion depth or loss of fluorescence was significantly different from the non-treated group with TSBS-grown *S. mutans*.

These results indicate that Violet-Blue light can serve as a treatment for reducing the numbers of *S. mutans* cells, thereby reducing biofilm and caries formation.

7.1 Introduction

Dental caries is a chronic and infectious disease involving multiple factors with a pandemic distribution. Caries formation is a dynamic process involving biological, social, behavioral, and environmental factors (Selwitz et al., 2007). Acid producing cariogenic bacteria, which have the capacity to develop plaque or oral biofilm, are responsible for initiation of a carious lesion. Though various factors are involved, formation and progression of dental caries do not occur in the absence of oral microorganisms. Oral microbial biofilm is a causative factor for dental caries (Socransky, 1968). *S. mutans*, a facultative anaerobic bacterium, is a major pathogen in the initiation and progression of dental caries (Bratthall, 1972; Hamada and Slade, 1980, Loesche et al., 1975). Early dental caries is reversible and preventable. There are various methods to prevent dental caries, which includes behavioral modifications related to sugar intake, increasing resistance to acid through fluoride uptake, protecting tooth surfaces through dental sealants and reducing, inhibiting or eliminating caries-causing microorganisms. Alternative novel approaches are being investigated, that range from natural products such as propolis in bee hive wax, polyphenols in tea to nanotechnological methods such as quorum sensing peptides, and interference with interspecies and interkingdom signaling. Among these methods, phototherapy with no exogenous

photosensitizers or photodynamic therapy with exogenous photosensitizers are innovative approaches being studied to control oral biofilm (Ten Cate, 2012).

Our previous published studies (Gomez et al., 2015) have demonstrated inhibitory effects of Violet-Blue light treatment on *S. mutans* biofilm cells growing in wells of a 96-well microtiter plate and irradiated for 5 min. The treated bacterial cells were allowed to regrow for 2 or 6 h after treatment at 37°C in a CO_2 incubator. The viability of *S. mutans* biofilm cells and growth rate of both planktonic and biofilm cells was determined. The bacterial viability and growth rate exhibited a statistically significant decrease in the Violet-Blue light treated groups grown in TSB (Tryptic Soy Broth with no sucrose) and TSBS (Tryptic Soy Broth with 1% sucrose) compared to the non-treated groups, and biofilm formation differences between the two groups were significant with TSB-grown *S. mutans*. Chebauth-Taub and colleagues reported that blue light has a delayed antibacterial activity with 7 to 10 min of treatment (Chebath-Taub et al., 2012). Feuerstein et al. demonstrated that 10 min of treatment decreased 3% of bacterial growth with blue light compared to the nontreated control (Feuerstein et al., 2006).

To further explore the effect of Violet-Blue light on dentinal carious lesions induced by *S. mutans*, we treated *S. mutans* biofilm grown on dentin with Violet-Blue light for 5 min, 2 times a day for 5 days. The light source that was used was a caries detection device called Quantitative Light Induced Fluorescence (QLF). This study extends the application of an early caries detection device to the field of treating the etiology of caries. The use of QLF as a source of light with a peak wavelength at 405 nm to mediate bacterial destruction was developed in this study.

The concept is based on an observation of orange to red fluorescence in carious lesions in QLF images, probably due to bacterial metabolic byproducts called porphyrins (al-Khateeb et al., 1997; Buchalla 2005; Heinrich-Weltzien et al., 2003; Konig et al., 1998). Porphyrins are fluorescent compounds or fluorophores produced by several bacteria (Fyrestam et al., 2015). Violet-Blue light is absorbed by these fluorophores, and the fluorophore undergoes a transition of energy of photons from a lower level to an excited level. At the excited state, the fluorophores react with molecular oxygen releasing free oxygen radical species that destroy proteins, lipids and biological materials (Konopka et al., 2007; Soukos and Goodson, 2007). The purpose of this phototherapy was to reduce the numbers of the biofilm bacteria, but not to achieve more than a 3-log reduction of bacterial numbers as is commonly observed with antibiotics. The aim of this study was to test the proof of concept of the inhibitory effects of Violet-Blue light to prevent the formation and progression of dental caries induced by *S. mutans* on human dentin specimens.

7.2 Materials and Methods

7.2.1 Bacterial Strain and Culture Conditions

S. mutans (UA159, serotype c, ATCC 700610) from American Type Culture Collection (Rockville, MD) stored at -80ºC with 20% glycerol was used in this study. The bacteria were cultured on mitis-salivarius sucrose bacitracin (MSSB,

Anaerobe Systems, Morgan Hill, CA) agar plates prior to use. *S. mutans* broth cultures were started by inoculating 5 ml of Tryptic Soy Broth (TSB, Acumedia, Baltimore, MA) with colonies from the MSSB plates and incubated for 24 h in a 5% CO_2 incubator.

7.2.2 Selection of Tooth Specimens

Human teeth were collected based on Institutional Review Board (IRB) approval (# NS0911-07). 144 specimens of extracted human molars without any cracks, fractures, or caries were selected. A Lap Craft L'il Trimmer was used to decoronate the crown portion of the teeth. The dentin portion from the coronal part of the teeth was obtained by cutting a circumferential section of the crowns. Dentin specimens with a dimension of 4×4×2 mm^3 were cut using a Buehler Isomet saw. The specimens were sequentially ground with 500, 1200, 2400, and 4000 grit sand paper by mounting them on an acrylic block with a grinding machine (Struers RotoPol-31/RotoForce-4). Each specimen was ground with the different sand papers for 4 sec and was reduced to a thickness of 2 mm. Clear nail varnish was coated on all sides and the bottom leaving approximately 1/3rd of the top free of varnish for growth of the biofilm. Dentin specimens were rinsed, sonicated, and rinsed again for 3 min with deionized water (dH_2O). The specimens were placed with moist cotton gauze, sealed in a whirl pak bag, and sterilized with ethylene oxide gas. They were stored at 4°C until use.

7.2.3 Quantification of Biofilm Formation

An overnight *S. mutans* TSB culture was diluted 1:100 with TSB or TSB supplemented with 1% sucrose (TSBS). 10 ul of the *S. mutans* culture was added to wells of sterile 96 well microtiter plates (Fisher Scientific, Co., Newark, DE) with 190 ul of TSB or TSBS in each well (4 × 4 × 2 mm; n=72). The cells were incubated with the dentin specimens for 13 h in a 5% CO_2 incubator for them to reach the logarithmic phase of growth and produce biofilm. A gap of one well was placed between the biofilm samples and 2 wells between TSB and TSBS groups. 9 samples with and without sucrose were simultaneously inoculated for baseline measurement of CFU's without QLF treatment at 13 h. The QLF (QLF-clin, Inspektor Research Systems BV, Amsterdam, Netherlands) used in this study was an early caries detection light device with an excitation peak wavelength of 405 nm and a spectral range of 380 to 450 nm. Visible light with a spectral range from 380 to 700 nm is commonly used to inhibit or kill bacteria. It is used clinically to detect changes in the mineral content of the tooth in a noninvasive manner, identify early lesions which will likely progress to cavitation, quantitatively measure the fluorescence, and assist clinical decision making. It employs a 35-Watt Xenon arc lamp, and Violet-Blue light is filtered through a high pass band filter. The intensity of the Violet-Blue light from the QLF device used in this study was approximately 13 mW/cm^2. Before irradiation, planktonic bacteria in the supernatant liquid were removed, and the wet biofilm was exposed to Violet-Blue light continuously for 5 minutes. The distance between the bottom of the microtiter plate and the light

source was kept at 2 cm. A black background was used to avoid scattering of light. Clear seal mate (Excel Scientific Inc., Victorville, CA) was placed as a barrier to maintain sterility between the sample and the light source opening. Immediately after exposure, freshly prepared TSB or TSBS growth media was added to each well. The treated biofilm cells were reincubated for 6 h in 5% CO_2 at 37°C. After 6 h, the biofilm cells were again treated with Violet-Blue light for 5 min and reincubated for 13 h until the next treatment on the following day. The procedure was repeated twice a day for 4 days and once on the 5^{th} day followed by a 6 h reincubation. At the end of the 5^{th} day of the experiment, supernatant liquid was removed for pH measurements. The pH of the uninoculated media, and the TSB and TSBS cultures were measured with a pH meter (Accumet, Fisher Scientific, PA). Baseline measurements of the culture supernatant on the first day before treatment were obtained with two ranges of pH indicator paper (pH 0-6 and 0-14; MColorpHast, EMD Millipore, MA). Similar pH measurements were obtained on the 5^{th} day of treatment after 6 h of reincubation. Biofilm at the bottom of the plate was gently washed once with 0.9% saline to remove unattached and loosely adherent bacteria. Dentin specimens were removed from the microtiter plate and placed in 1 ml of saline in mini-centrifuge tubes. The specimens were vortexed for 10 sec, sonicated in ice for 20 sec and vortexed again for 10 sec. Serial dilutions of the bacterial samples were prepared in saline and plated on Tryptic Soy Agar (TSA) plates using a spiral plater (Spiral System^{TM}, Cincinnati, Ohio). TSA plates were incubated for 48 h at 37°C in 5% CO_2, and the number of colony forming units

(CFUs) were enumerated by an automated colony counter (Synbiosis Inc, Fredrick, MD).

7.2.4 Determination of Lesion Depth

A QLF-D biluminator was used to acquire images of the dentin specimens. A jig was prepared to secure the dentin with silicone rubber (Oomo-30). The images were acquired through an illumination tube fitted on a SLR camera with a 60-mm macro lens and with white and blue light emitting diodes (LED) under dark conditions with a shutter speed of 1/30 second,ISO speed of 1600, and aperture value of 8. Fluorescence images of dentin specimens were obtained using a C3 proprietary software (Inspektor Research Systems BV, Amsterdam, The Netherlands). The images were digitally archived for further analysis for mineral loss or lesion depth through QA2 analysis software (Inspektor Research Systems BV, Amsterdam, The Netherlands). The QLF parameters ΔF represents fluorescence or mineral loss, ΔQ represents the volume of the lesion, ΔA represents the area of the lesion, and ΔFMax is the maximum amount of fluorescence loss or mineral loss (lesion depth).

7.2.5 Transverse Microradiography

Human dentin specimens were treated briefly with 70% ethyl alcohol and stored under moistened conditions with 0.01% thymol. Dentin specimens were

fixed to acrylic rods, and sections were prepared with a hard tissue microtome. The sections were placed on an ultra-resolution flat plate sized 5×5×2 mm^3 (Microchrome Technology Inc) for imaging. Calibration of the TMR PSL Imaging System (Thermo-Kevex PXS5-928WB-LV, Tube 48934) related to its absorption coefficient was done with an aluminum step wedge for an acceptable correlation of 0.99970. Imaging of the dentin specimens were obtained through X-ray source with a voltage (kV) and a current (μA) of 45. The obtained images were read and processed using TMRD1 5.0.01 software and finally analyzed through TMR2006 software v.3.0.0.18. Mineral loss (ΔZ) and lesion depth (L) were determined.

7.3 Statistical Methods

It was calculated that 36 dentin specimens for each TSB or TSBS group would provide 80% power to detect a 25% difference in CFUs and a 10 μm difference in lesion depth. A logarithmic transformation was used for all analyses. ANOVAs with fixed effects were used to analyze data related to groups (Violet-Blue light, no light, baseline), media (TSB, TSBS), their interactions. Each group-media combination was allowed to have a different variance.

7.4 Results

7.4.1 Photo inhibitory Effect on Colony Forming Units of Treated *S. mutans* Biofilm on Human Dentin

The quantitation of *S. mutans* biofilm reported was based on the comparison of Violet-Blue light-treated (n = 36) and non-treated groups (n = 36) at the end of the treatment period in human dentin specimens. At the end of the 5th day of the treatment, there was a statistically significant (p = 0.0006) decrease in CFUs in the treated groups with TSB (n =72). There was a 60.6 percent reduction of bacterial colonies without sucrose in the treated groups compared to the non-treated groups **(Figure 7.1)**. The results were based on two daily treatments of 5 minutes with a reincubation interval of 6 h for 4 days and one treatment on the 5th day with a 6 h reincubation.

For TSBS, there was also a significant decrease (p = 0.0337) in CFU in the Violet-Blue light-treated groups (n = 36). There was a 28.8% reduction of bacterial colonies in sucrose grown groups **(Figure 7.1)**. There were significant differences between the effect of Violet-Blue light in TSB and TSBS groups compared to the non-treated groups. The effect of Violet-Blue light on TSB-grown groups was significantly higher for Violet-Blue light treated and non-treated groups (p=0.0480) (p=0.0042) compared to sucrose-grown TSBS groups.

There were significant differences in baseline CFU's among the Violet-Blue light-treated TSBS group and the non-treated TSB and TSBS groups **(Figure**

7.1). The Violet-Blue light-treated groups were not significantly different in baseline from the TSB group (p=0.07); however, non-treated groups were significantly different in baseline from the TSB group (p=0.0001). CFU's of the Violet-Blue light-treated groups were significantly different from those of baseline of the TSBS group (p<0.0001). Non-treated groups were also significantly different than baseline for TSBS (p<0.0001). However, there were no differences in baseline CFU's between TSB and TSBS groups (p=0.06). The Violet-Blue light-treated and non-treated TSB groups were not significantly different in baseline CFU's **(Figure** 7.1**)**.

7.4.2 Photo inhibitory Effect on the Lesion Depth of *S. mutans* Induced Carious Lesions

The negative signs from the original data were removed from the parameters of QLF such as ΔF, ΔFmax, WSArea, and ΔQ measurements before the statistical analysis, so that larger values indicate larger lesions. Logartithmic transformations were done for the analysis. The lesion depths of TSB Violet-Blue light-treated groups and non-treated group were not significantly different (p=0.20 for ΔF, p=0.20 for ΔFmax, p=0.17 for ΔQ, p=0.18 for WSArea; **Figure 7.2**). There was a decrease in lesion depth in the treated group; however, this was not statistically significant compared to the non-treated group.

The lesions in the TSBS Violet-Blue light-treated group were significantly decreased compared with the TSBS non-treated group (p=0.0116 for ΔF,

p=0.0168 for ΔFmax, p=0.0152 for ΔQ, p=0.0224 for WSArea; **Figure 7.2**). The lesion of the light-treated TSB group was significantly decreased compared to the treated TSBS group (p=0.0148 for ΔF, p=0.0091 for ΔFmax, p=0.0025 for ΔQ, p=0.0015 for WSArea) and non-treated TSBS group (p=0.0021 for ΔF, p=0.0015 for ΔFmax, p=0.0002 for ΔQ, p=0.0001 for WSArea; **Figure 7.2**). Comparison of mean fluorescence or mineral loss (ΔF) imaged through QLF-D did not reveal any statistically significant differences between TSB and TSBS baselines (p = 0.55 for ΔF). However, the TSBS Violet-Blue light-treated group was significantly different from the baseline (p=0.0417) and the non-treated TSBS groups were significantly different from their baselines (p=0.0005). Baselines of Violet–Blue light treated (p=0.64 for ΔF) and non-treated TSB groups did not significantly vary (p=0.23 for ΔF) **(Figure 7.2)**.

There was a statistically significant difference between Violet-Blue light treated group and the non-treated control group in lesion depth on the dentin specimens incubated with *S. mutans* in TSBS (p<0.02) **(Figure 7.3)**. However, there was no significant difference in lesion depth for TSB groups (p=0.24). Violet-Blue light treated groups and non-treated control groups did not have significantly different mineral loss with TSB (p=0.59) or TSBS (p=0.36) **(Figure 7.4)**. There were significant differences in lesion depth and mineral loss baseline values of both TSB and TSBS Violet-Blue light treated and non-treated groups (p<0.0001) **(Figure 7.3**; **Figure 7.4)**.

7.5 Discussion

After 5 days of treatment (9 total treatments) and reincubation for 6-13 h following each treatment, there was a significant reduction in the CFU of *S. mutans* biofilm in the absence of sucrose. There was also a significant reduction in CFU of *S. mutans* biofilm in the presence of sucrose after the final treatment regimen. Biofilm bacterial numbers were about 10^7 CFU/ml in TSB cultures and about 10^6 CFU/ml in TSBS cultures. It is a well-known phenomenon that the number of TSBS-grown *S. mutans* biofilm cells is reduced due to an increase in the amount of extracellular polysaccharides (EPS), while the overall *S. mutans* biofilm mass increases in the presence of sucrose (Leme et al., 2006). Lactic acid released from biofilm cells with EPS is primarily responsible for caries formation. The effect of Violet-Blue light on EPS or glucans was not determined in this study. However, it has been shown previously that biofilm architecture is affected by blue light, and the light reduces the amount of insoluble EPS (Souza et al., 2015, Cohen-Berneron et al., 2016). EPS may play a role in providing a barrier thus reducing the effectiveness of light on the bacteria. There was also a significant difference in the effect of Violet-Blue light on the sucrose groups. This encouraged us to study in detail the photoinhibitory effect on glucans and pH variability. Increased bacterial numbers in TSB cultures compared to TSBS cultures suggest that *S. mutans* with less EPS (i.e., TSB-grown *S. mutans*) has greater ability to multiply than to metabolize sucrose (Monod, 1949).

Although pH was measured throughout the study, detailed results and analysis are not provided because different methods employed including using pH strips with a range of pH 0 to 14 or 0 to 6 and using a pH meter for pooled samples The pH of the uninoculated TSB and TSBS media was approximately 7.00. The pH of the Violet-Blue light-treated TSB group at the end of the treatment period was about 5.27, and that of the non-light treated group was 5.11. The pH of the TSBS Violet-Blue light-treated group was 4.16, and that of the TSBS non-light treated group was 4.07. The pH difference (ΔpH) between the treated and non-treated TSB groups was 0.16, and that between the treated and non-treated TSBS groups was 0.09. The pH at baseline for a 13 h biofilm was around 5.3 for TSB and 4.5 for TSBS. There was no significant difference between the values at baseline and the end of the treatment period.

The lesion depth measured with QLF as loss of fluorescence was not significantly different between the treated and non-treated groups without sucrose. There was a decrease in lesion depth, lesion volume, and area in the Violet-Blue light treated group compared to the non-treated groups; however, the differences were not statistically significant. The variability in lesion area and volume of the lesion in cultures without sucrose could have contributed to the non-significance. In contrast, the lesion depths of the Violet-Blue light-treated TSBS groups were significantly different from the non-treated groups. The lesion depths were also quantified by a gold standard procedure, Transverse Microradiography (TMR). *S. mutans* has an increased binding capacity to the collagen of the dentin compared

to enamel. These results provide more evidence for the effectiveness of Violet-Blue light as an adjunct treatment for carious lesions.

7.6 Conclusion

Based on our findings, we concluded that Violet-Blue light has photoinhibitory activity on *S. mutans* minimizing the formation of biofilm on dentin specimens. This may result in a reduction in carious lesions if used clinically. This translational *in vitro* study demonstrates a reduction in the number of bacteria that are necessary for the formation of carious lesions. Violet-Blue light reduces the counts of bacteria, thereby preventing accumulation of oral biofilm and reducing the formation of carious lesions. More translational and clinical studies are needed to explore the effect of Violet-Blue light in dental plaque on tooth surfaces with and without the use of photosensitizers or reactive oxygen species producing substances.

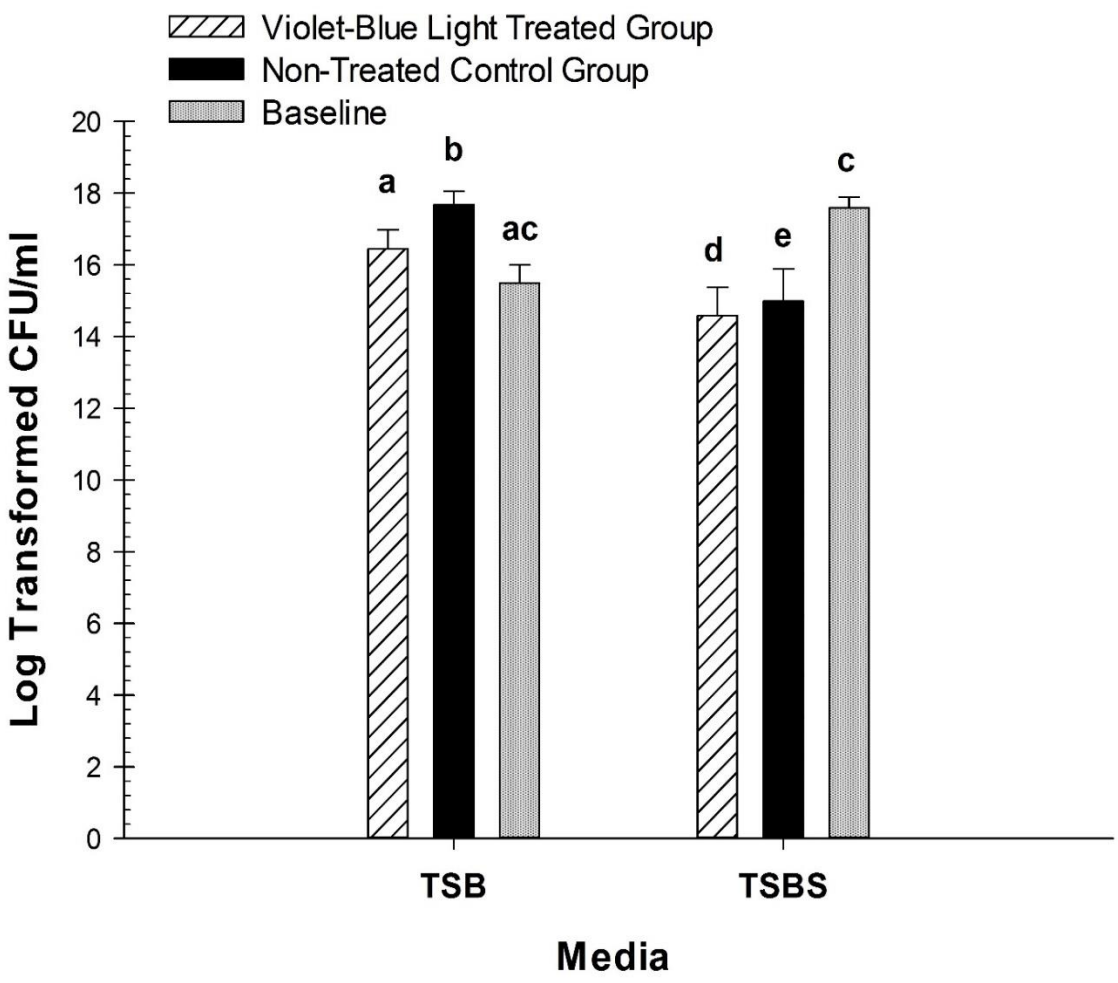

Figure 7.1 Comparison of baseline CFU, treated and non-treated groups.

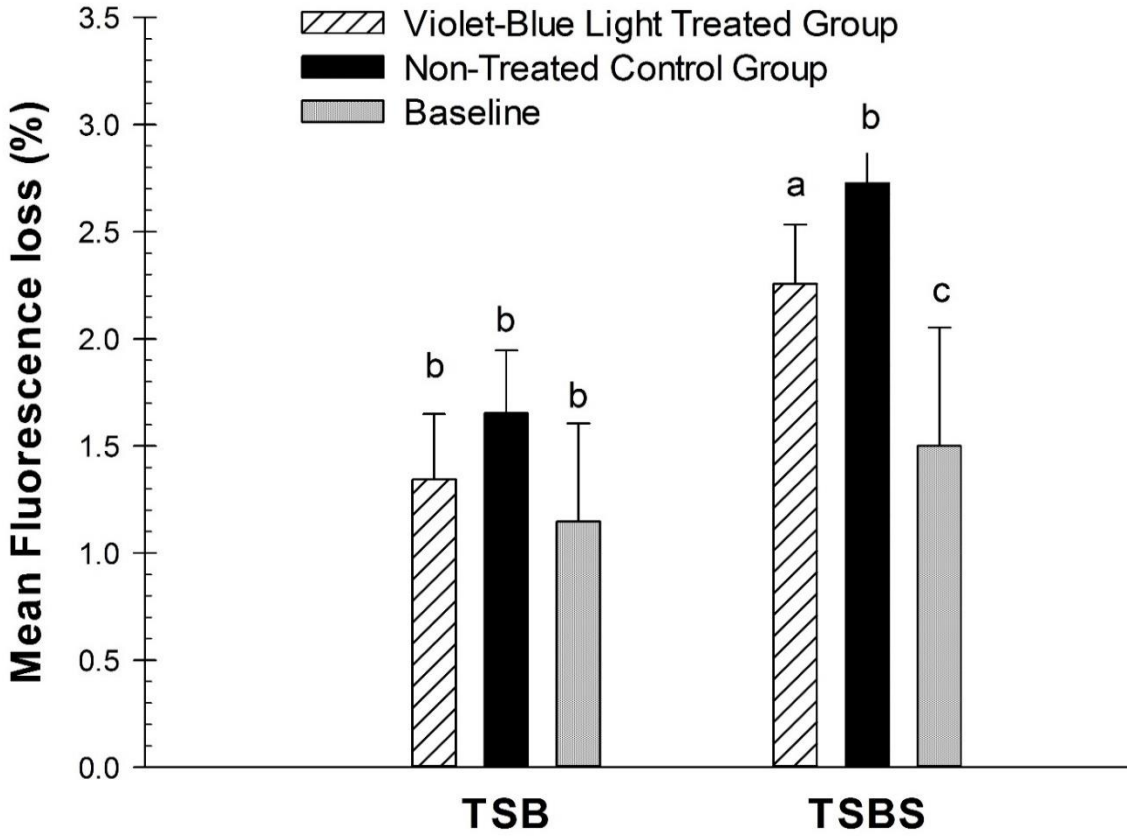

Figure 7.2 Comparison of mean fluorescence loss (∆F) in baseline, Violet-Blue light treated and non-treated TSB and TSBS groups.

∆F of TSB Violet-Blue light treated, and non-treated groups was significantly lower than that of TSBS Violet-Blue light treated and non-treated groups. TSB and TSBS groups were not different in baseline. TSBS Violet-Blue light treated groups was significantly higher than baseline. For TSB cultures, Violet-Blue light treated groups were not significantly different from baseline. ∆F were significantly higher for TSBS cultures in non-treated groups than baseline. Non-treated TSB groups were not significantly different from baseline.

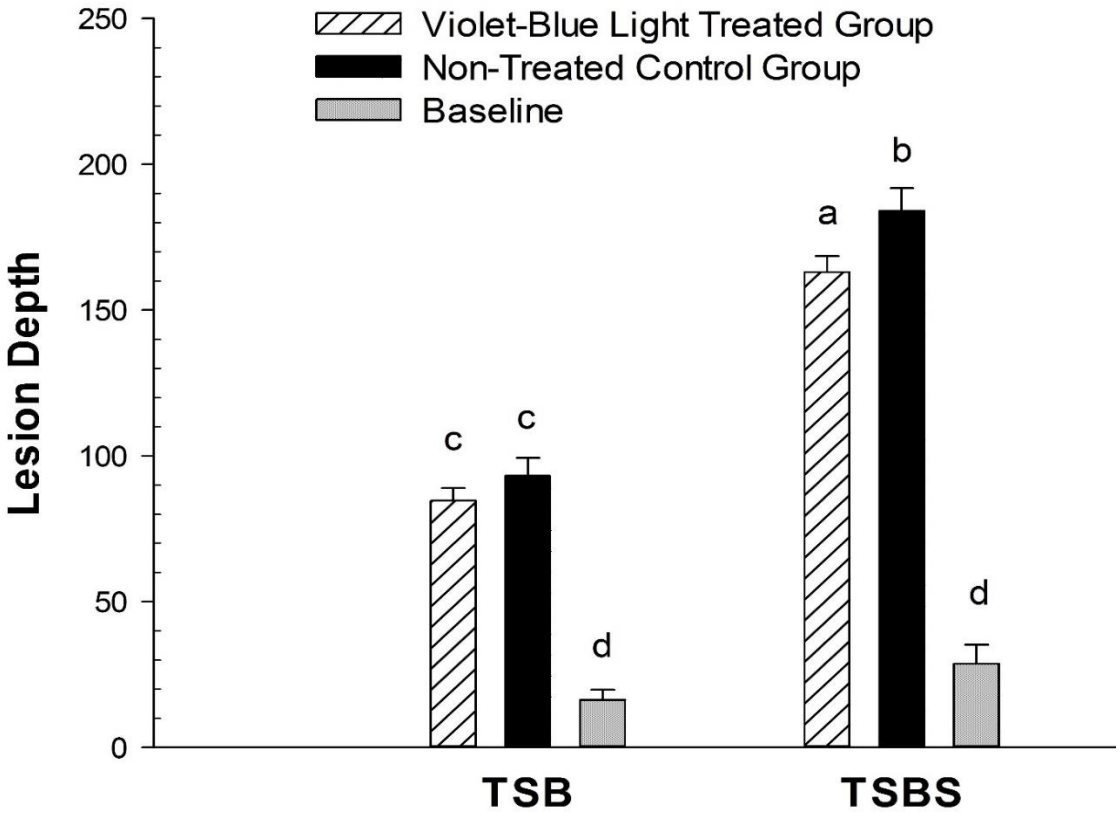

Figure 7.3 Comparison of lesion depth (L), in baseline, Violet-Blue light treated and non-treated groups of TSB and TSBS.

Treated and non-treated TSBS groups were significantly different. Similar lower-case letters represent no significant difference and different lower-case letters represent significant differences between groups.

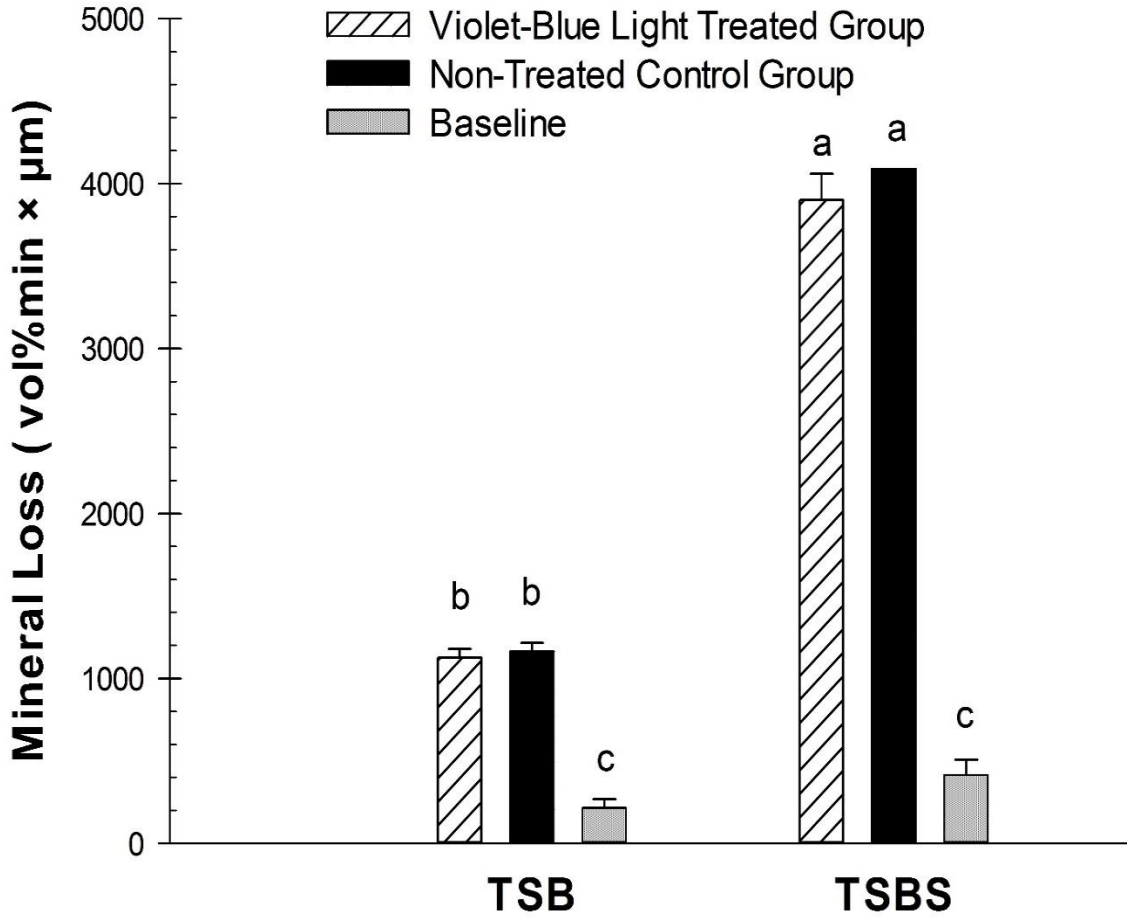

Figure 7.4 Comparison of mineral loss (ΔZ), in baseline, Violet-Blue light treated and non-treated groups of TSB and TSBS.

Treated and non-treated TSB groups were significantly different.

Chapter 8

Auto fluorescence of Biofilm of Caries-Active *Streptococcus mutans* Strains

The objective of this study was to determine whether caries active (CA) and caries resistant (CR) *Streptococcus mutans* (UA159) grown in 0, 1 and 2% sucrose had the ability to autoflouresce under biofilm growth conditions. Cells of various strains of *S. mutans* UA159 was grown for 24 h with 0, 1 and 2% sucrose at 37^{o}C in 5% CO_2. A stock solution of Protoporphyrin IX (0.01 g/ml) was prepared in 100% ethanol and diluted to a concentration from 1.6-3.1×10^{-4} g/ml. Emission spectra were measured using a Spectrofluorometer by excitation at 385 or 405 nm with 10 nm steps with planktonic fluid on top of the biofilm (total biomass) or only with biofilm. ANOVA was conducted comparing UA159 vs. no UA159 or CA vs. CF strains for total biomass based on effects of sucrose concentration, and emitted wavelengths. The analysis was performed using ranked data, allowing sucrose-group combinations to have different variances within each experiment. Emission spectra at 770 nm were observed with *S. mutans* strains UA159, CR (314 and 317), CA (192 and A32-2) and Protoporphyrin IX when excited at 385 nm, and 800 nm, and occasionally at 810 nm, when excited at 405 nm. UA159 was significantly higher in emission than other strains for all combinations ($p<.0005$) except total biomass with 2% sucrose at 770 nm (no difference) and at 800 and 810 nm (UA159 lower). CA was significantly higher than CF in emission for all combinations except biofilm only at 810 nm and 2% sucrose at 810 nm. CA was significantly different in

emission from CF for total biomass. *S. mutans* autofluoresces in the red and infrared regions of the electromagnetic spectrum.

8.1 Introduction

Biological tissues upon absorption of light of a shorter wavelength, have an inherent ability to emit light at a longer wavelength known as fluorescence. The phenomenon of fluorescence occurs due to the presence of endogenous fluorophores, which act as a timed molecular switch, which get excited at a specific shorter wavelength and undergoes a stokes shift at a different longer wavelength. Ultra-Violet (UV) spectrum of the electromagnetic radiation with a long wave band A (UVA) at a wavelength range from 315 to 400 nm becomes absorbed by molecular fluorophores or chromophores and undergoes excitation and electronic transition to high energy levels. Subsequently, the molecules emit light as fluorescence by losing energy at a longer wavelength (Soukos NS and Goodson JM, 2011; Walsh LJ and Shakibaie F, 2007; Shakibaie F, et al 2011). Hans Stubel, a German scientist in 1911 found that teeth have a bluish white fluorescence when illuminated with UV light, and when compared with other tissues, it was reported that the brilliance was only secondary to the lens of the eye (Stubel, 1911; Hartles and Leaver, 1953; Buchalla et al, 2005; Shakibaie et al, 2011). This was the first documented report on the autofluorescence of teeth. Later in 1927, Bommer observed that tooth film (now known as oral biofilm) had an orange to red fluorescence with UV light from a Woods lamp (Bommer, 1927; Shakibaie et al,

2011). Hartles, in his investigations on carious teeth found that calculus emits a reddish orange color (Hartles and Leaver, 1953). The red fluorescence from bacterial plaque was proposed to be due to bacterial byproducts called porphyrins (Koenig and Schneckenburger, 1994; Heinrich-Weltzien et al, 2003). The exact origin of fluorescence is being studied and is known to be from bacteria such as *Porphyromonas gingivalis* and A*ctinomyces actinomycetans* (Jonas Fyrestam et al 2015). Black pigmented bacteria such as *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens,* and *Prevotella melaninogenica* have also been reported to have porphyrins. However, *Streptococcus mutans*, which is a major cariogenic bacterium, remains unexamined for the presence of porphyrins and its contribution towards fluorescence. Carious lesions, plaque, and calculus are known to fluoresce; therefore, we hypothesized that *S. mutans* is a source of the fluorescence. Dental plaque is made up of a plethora of oral microorganisms, and if not all, some of them can fluoresce. Mature dental plaque or calculus emits a visible orange to red fluorescence, when illuminated with Violet-Blue light from an early detection device named Quantitative Light Induced fluorescence (QLF), and the surface of the tooth appears green. A study by Lisa Ann Coulthwaite determined the fluorescing properties of mature plaque in the polished and fitting surfaces of a maxillary complete denture. The fitting surface had an intense orange to red fluorescence with Violet-Blue light excitation. When the bacteria from this mature plaque on the dentures were cultured, *Prevetolla melaninogenica* and *Fusobacterium nucleatum* fluorescing in the green region of the electromagnetic

spectrum were observed (Coulthwaite, L et al, 2006). Based on this data, we know that bacteria fluoresce under specific wavelengths of light. Also, bacteria can be inactivated, killed, or lose their vitality and viability with specific wavelengths of blue light (Chebauth Taub et al 2012; Feuerstein et al 2006, Steinberg et al 2008).

Previous studies have shown that *S. mutans* fluoresces in the green spectrum, and anaerobes like *Porphyromonas gingivalis* fluoresce in the red region (Couthwhite et al, 2006, Lennon et al, 2006). *S. mutans* was shown to fluoresce in carious lesions on enamel and dentin surfaces (Yoshimi Shigetani et al, 2008). Red fluorescence was observed with *S. mutans* (Volgenant CMC et al, 2013). Porphyrins were reported to be at excitation wavelengths ranging from 358 to 405 nm in mildly acidic and neutral conditions. pH affects the fluorescence spectra of various porphyrins (Matosevic et al, 2010). Fluorophores found in carious lesions have an absorption range of 350 to 420 nm (Borisova et al 2006).

The primary objective of our study was to determine whether *S. mutans* grown as biofilm autofluoresces in the presence of 0%, 1% and 2% sucrose. Our secondary objective was to compare clinical isolates of caries active (CA) and caries resistant (CR) strains in autofluoresce in similar growth conditions.

8.2 Materials and Methods

8.2.1 Bacterial Strains

S. mutans strains obtained from caries-active and caries-resistant subjects with appropriate IRB approval were used in this study. The numbers of each *S. mutans* in saliva from each subject was determined by plating. Specifically, 5 *S. mutans* strains including UA159; caries resistant (CR) strains 314 (with less than $10^{\wedge 5}$ Colony Forming Units/ml of whole saliva (CFU) and 317 (with more than $10^{\wedge 5}$ CFU); caries active (CA) strains 192 (with less than $10^{\wedge 5}$ CFU) and A32- 2 (with more than $10^{\wedge 5}$ CFU) were used in this study. Cells of these strains were stored at -80°C in Tryptic Soy Broth (TSB) and 20% glycerol and were grown on Mitis Salivarius Bacitracin agar plates (MSSB) for studies. *S. mutans* broth culture was in 5 ml of Tryptic Soy Broth and incubated for 24 hrs in 5% CO_2 at 37°C.

8.2.2 Biofilm Formation

S. mutans biofilm (UA159) was formed with TSB and 1 or 2% sucrose (TSBS) by diluting 10 ul of broth culture in 1 ml of TSB or TSBS 1% or 2%. They were allowed to grow in a sterile 96-well microtiter plate for 24 hrs in a 5% CO_2 incubator at 37°C. A one well gap between the samples were maintained to avoid seepage of light.

8.2.3 Protoporphyrin Standard

Protoporphyrin IX (PP-IX; 100 mg of PP-IX (Sigma Chemical Co., St. Louis, MO) was dissolved in 10 ml of 100% ethanol. The stock solution was further diluted to a concentration ranging from 3.125×10^{-4} to 1.562×10^{-4} g/ml.

8.2.4 Fluorescence Measurement

A Spectrofluorometer (Spectra Max, Molecular Devices M3) was used to measure the fluorescence of *S. mutans* biofilm of strains UA159, Caries active strains (A32-2 and 192), and Caries resistant strains (314 and 317). Relative fluorescence units (RFU) of total biomass (biofilm and planktonic) and biofilm only were quantitated through a spectral scan by exciting at a fixed wavelength of 385 nm with a sweep of emission wavelengths ranging from 500 to 850 nm. Another excitation at 405 nm was conducted with a similar range of emission wavelengths. After the emission wavelength measurements, the supernatant fluid or planktonic culture was removed from the wells and was subjected to similar measurements.

8.3 Statistical Analysis

A mixed model ANOVA (SAS version 9.4.) was used to determine the differences between groups (UA159 vs No UA159) in autofluorescence and effects of sucrose (0%, 1%, 2%), fluid (fluid or no fluid), and wavelength (385 nm/770 nm,

405 nm/800 nm, 405 nm/810 nm) on fluorescence intensity with all main effects and interactions included in the model. The analysis was performed using the ranks of the data, with the ranks calculated within each experiment. The analysis also allowed each sucrose-group combination to have different variances within each experiment. Comparisons between Protoporphyrin concentrations 3.125×10^{-4} and 1.562×10^{-4} g/ml were made using ANOVA, with fixed effects for group, wavelength, and their interactions. A random effect for experiment and different variances were allowed for each group-wavelength-experiment combination.

8.4 Results

8.4.1 Autofluorescence of *S. mutans* (UA159) Biofilm and Total Biofilm Mass

The relative fluorescence units (RFU) of the total biomass of *S. mutans* including the biofilm at the bottom of the microtiter plate and the supernatant was measured. The excitation wavelength of 385 nm had an emission maximum of 770 nm for *S. mutans*. The RFU values of total biomass was less than the RFU of biofilm only. The RFU of *S. mutans* (UA159) was significantly higher than no UA159 in all combinations. The 4-way factor combination was significant ($p<.0001$). Autofluorescence of UA159 was significantly higher than that of other strains in the presence and absence of supernatant fluid with 1% or 2% sucrose ($p<0.0001$) at 385 nm **(Figure 8.1)**. In the absence of sucrose, the fluorescence emission at 770 nm of culture with UA159 was also statistically significantly higher

than that without UA159 **(Figure 8.1)**. Fluorescence emission of 800 and 810 nm from *S. mutans* was elicited by an excitation of 405 nm. RFU of total biomass of *S. mutans* UA159 with TSB at 0, 1 and 2% was statistically significantly higher than TSB containing 0, 1 and 2% of sucrose without UA159 with an excitation of 405 nm **(Figures 8.2** and **8.3)**.

8.4.2 Autofluorescence from Clinical Isolates of *S. mutans* Biofilm and Total Biofilm Mass

Biofilm formed with *S. mutans* isolated from CA patients had significantly higher RFU values in TSB with 1% sucrose at an excitation of 385 and 405 nm than in TSB only and in TSBS with 2% sucrose. Excitation of biofilm only with 1% sucrose, without the presence of supernatant fluid containing planktonic bacteria, had an emission maximum at 770 nm, and this was significantly ($p< 0.05$) different between CA/CS and CR/CF strains **(Figure 8.4)**. CA strains had higher RFU than CR strains in cultures with 2% sucrose at an excitation of 385 nm **(Figure 8.4)**. Emission at 800 and 810 nm with an excitation wavelength of 405 nm by CA strains was also significant higher than CR strains cultures with 1% sucrose **(Figure 8.5)**. Although, the 4-way interaction among the factors ($p=0.22$) and the fluid-wavelength-group interaction were not significant ($p=0.26$), the fluid-sucrose-group interaction ($p=0.0026$) and the wavelength-sucrose-group interaction ($p=0.0001$) were significant. There was no difference between CA and CF strains in the No Fluid-0% sucrose ($p=0.31$) and the 810 nm-2% sucrose ($p=0.94$)

comparisons. The RFU for the CA strains was significantly higher than CF for all other combinations ($p<.005$) **(Figures 8.5** and **8.6)**.

The Protoporphyrin IX (PP-IX) standard with a concentration of 3.125×10^{-4} g/ml had higher RFU values at 385 nm than at 405 nm. The RFU of PP-IX at 3.125×10^{-4} g/ml had higher values than at 1.562×10^{-4} g/ml. RFU values of PP-IX were compared to cultures with and without UA159 Although the 4-way interaction among the factors was significant, cultures with UA159 were was significantly lower than those without UA159 for all wavelength-fluid-sucrose combinations ($p<0.0001$). With regards to CA and CR strains, there were no significant effects found for group ($p=0.76$), wavelength ($p=0.26$), or the group-by-wavelength interaction ($p=0.99$), and group effects were not significant among various wavelengths ($p=0.91$ for 385 nm, $p=0.77$ for 405 nm/800 nm, $p=0.86$ for 405 nm/810 nm).

8.5 Discussion

S. mutans biofilm grown on dentin specimens and irradiated with a Violet-Blue light with a wavelength ranging from 380 to 440 nm appeared green. *S. mutans* has been reported to have green and red fluorescence (Shigetani et al, 2008, Lennon et al, 2006, Buchalla W, 2008, Volgenant et al, 2013, 2016). The emission wavelength of fluorescence in this study was 770 and 800-810 nm that are in the far red and infra-red regions of the electromagnetic spectrum, respectively. The common consensus of this study was that there is a fluorophore

in *S. mutans* that gets activated in the 380 to 500 nm wavelengths. The fluorescence emission characteristics is dependent on the structure of the molecule and its optical properties.

RFU values of biofilm only were higher than total biofilm mass in UA159, CA/CS, and CR/CF strains. This phenomenon may be due to the path of length and scattering of light in the sample with supernatant that causes reduced RFU values. The huge variability among the samples of total biofilm mass may be due to optical properties of light such as rapid diffusion and scattering of the incident light.

Future work investigating autofluorescing properties of *S. mutans* may help to determine caries susceptible individuals and aid in caries risk assessment. However, more in-depth studies are required to determine the contribution of fluorescence from substrates such as dentin, enamel, media, and various additives used for bacterial culture. Protoporphyrin concentrations in different ranges should be prepared to determine the presence and also the amount of Protoporphyrin IX in *S. mutans* if found.

8.6 Conclusion

S. mutans has the ability to autofluoresce in the far red and infra-red regions of the electromagnetic radiation. The nature of the fluorescing properties will help caries risk assessment in individuals. Colorimetry indicators related to microbial

presence on the surface of the tooth will be used to monitor the susceptible surface and plan for a prophylactic and preventative treatment.

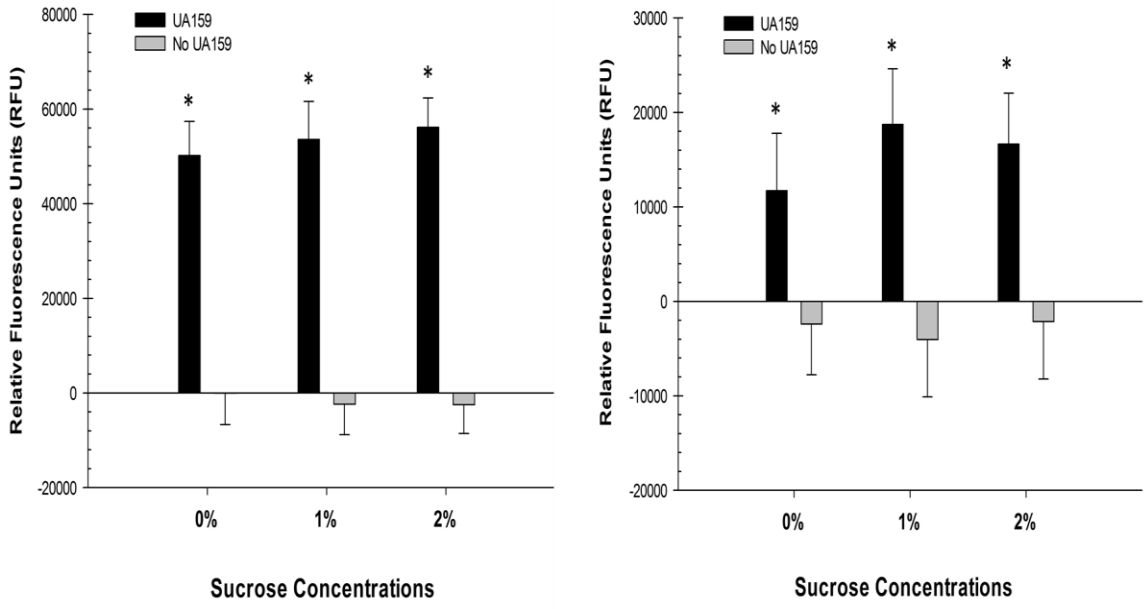

Figure 8.1 Relative fluorescence units (RFU) of *S. mutans* UA159 with an excitation of 385 nm in the (Left) absence and (Right) presence of supernatant fluid.

Asterisks (*) represent significance.

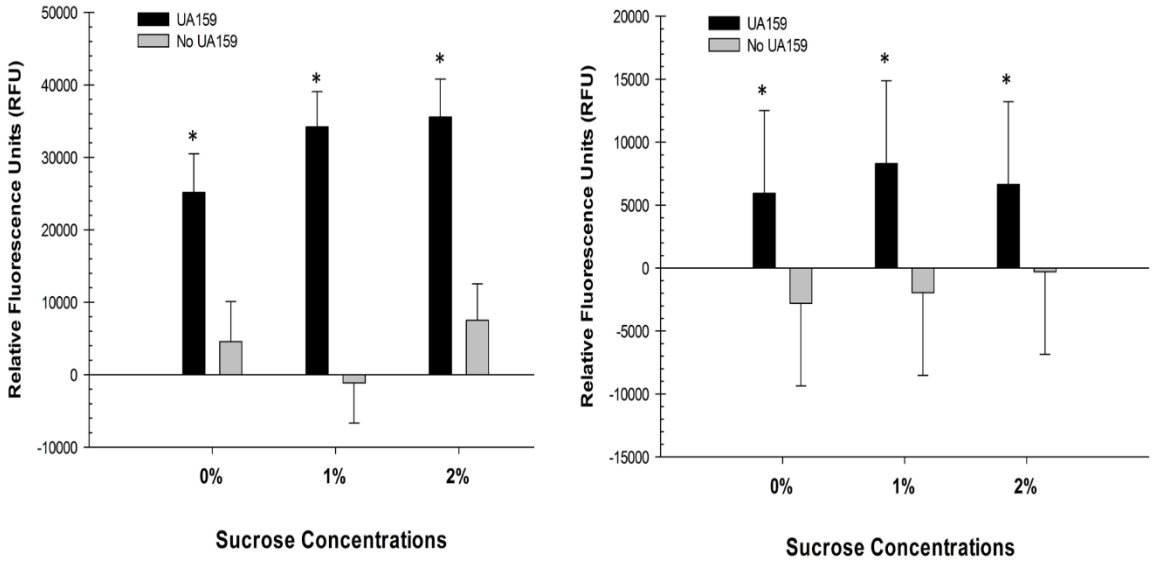

Figure 8.2 Relative Fluorescence Units (RFU) of UA159 in the (Left) absence and the (Right) presence of supernatant fluid with an excitation of 405 nm and emission of 800 nm.

Asterisks (*) represent significance.

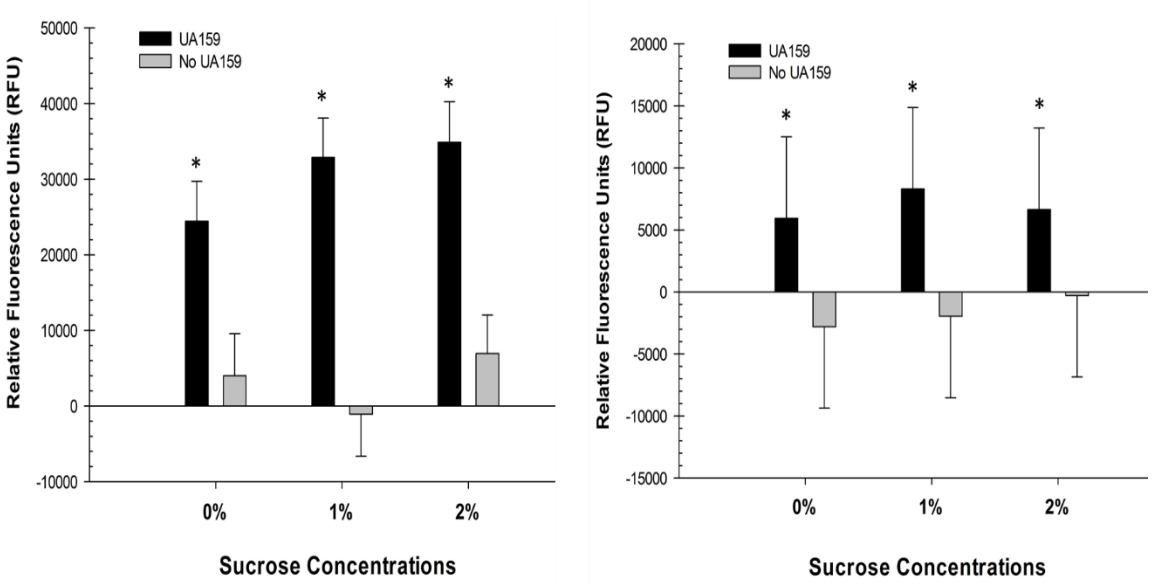

Figure 8.3 Relative Fluorescence Units (RFU) of UA159 in the (Left) absence and the (Right) presence of supernatant fluid with an excitation of 405 nm and emission of 810 nm.

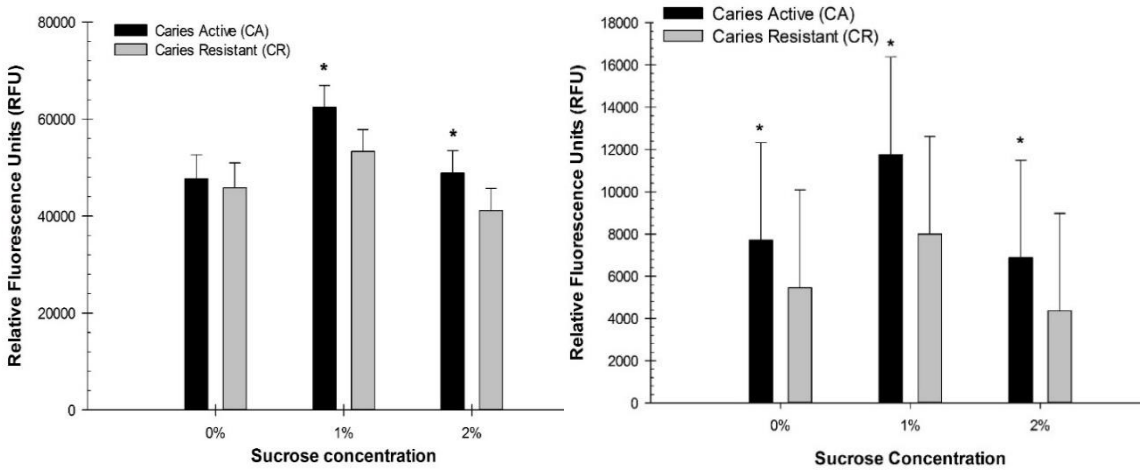

Figure 8.4 Relative Fluorescence Units (RFU) of Caries Active (CA)/Caries Resistant (CR) strains in the (Left) absence and the presence (Right) of supernatant fluid with an excitation of 385 nm and emission of 770 nm.

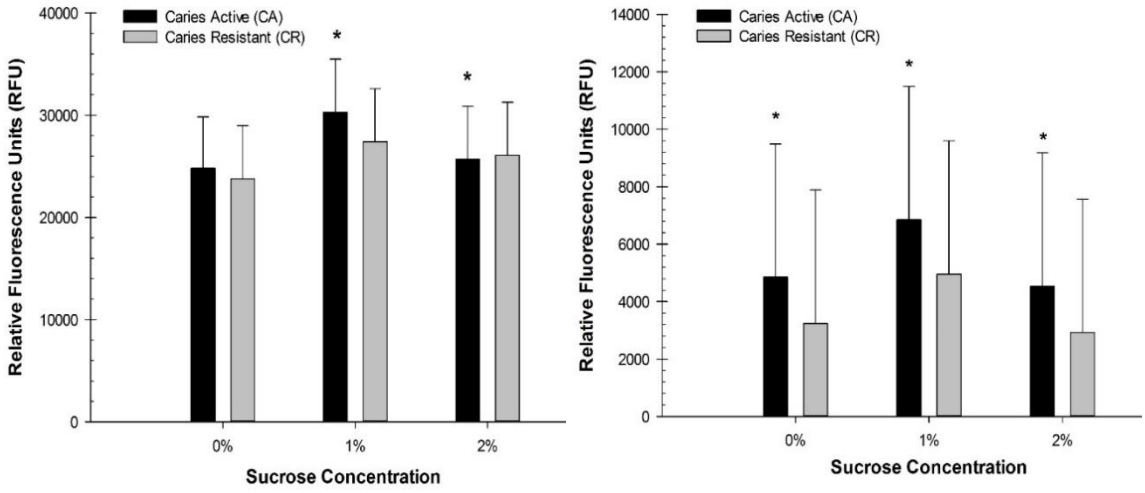

Figure 8.5 Relative Fluorescence Units (RFU) of Caries Active (CA)/Caries Resistant (CR) strains in the (Left) absence and the (Right) presence of supernatant fluid with an excitation of 405 nm and emission of 800 nm.

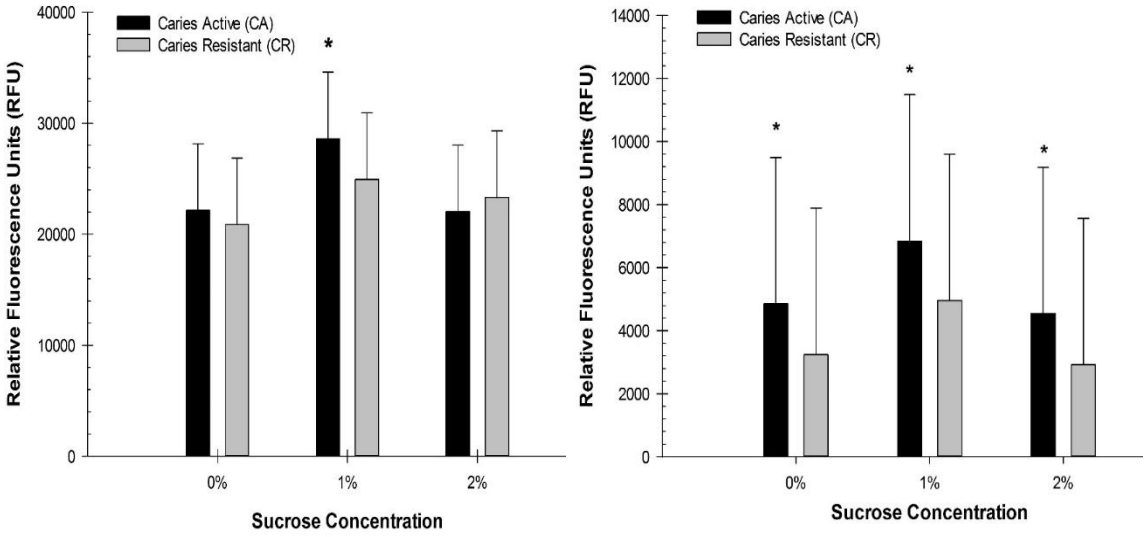

Figure 8.6 Relative Fluorescence Units (RFU) of Caries Active (CA)/Caries Resistant (CR) strains in the (Left) absence and the (Right) presence of supernatant fluid with an excitation of 405 nm and emission of 810 nm.

Chapter 9

Detection of Photosensitive Fluorophore Protoporphyrin IX in *Streptococcus mutans* Biofilm

Streptococcus mutans has autofluorescence and photodestruction properties at certain wavelengths of light. The aim of this study was to identify the presence of a potential endogenous fluorophore, namely protoporphyrin IX (PPIX) in *S. mutans*. Cells of *S. mutans* UA159 were subcultured in Tryptic Soy Broth (TSB) for 24 h from mitis salivarius-bacitracin agar plates. *S. mutans* biofilms were formed for 24 and 96 h in tissue culture flask or in wells of 24-well microplates in the presence or absence of dentin specimens with or without 1% sucrose. Biofilm pellets were collected by centrifugation. The pelleted biofilm was lysed with chloroform, methanol, and Millipore water. Total porphyrin was extracted through a two-phase method by transferring the aqueous layer (water:methanol) through 0.5 ml centrifugal filters with 3000NMWL/3K membrane. Samples and 10 µg of standard PPIX were analyzed using an Agilent 1200 SL-6520 an Accurate Mass Quadrupole Time of Flight (Q-TOF) LC/MS (Liquid Chromatography/Mass Spectrometer) with electrospray ionization. 10 µL of each sample or standard was injected into an Agilent Poroshell EC-C18 2.1x150 mm, 2.7 µm HPLC column and separated with a flow rate of 0.5 ml /min and a gradient of 95% H2O(A)/5% acetonitrile(B), both with 0.1% formic acid, 60% solution B for 14 minutes, and 100% solution B for 16 minutes followed by column cleaning and re-equilibration.

Masshunter qualitative analysis software was used to extract and integrate the chromatographic peaks. The mass of PPIX ($C_{34}H_{34}N_4O_4$) was 562.2629 at a retention time of 14.514 minutes with a molecular ion peak (m/z) at 563.2783. The mass of PPIX in samples ranged from 562.2874 – 562-3131 with m/z ranging from 563.2433 – 563.3203. These results demonstrate that endogenous PPIX was present in the gram positive cariogenic bacterium *S. mutans* when grown as a biofilm.

9.1 Introduction

Dental plaque is the main risk factor for caries. Mature dental plaque or calculus emits an orange to red fluorescence upon illumination with Violet-Blue light in the range of wavelengths from 380 to 450 nm (Dolowy et al 1995; Coulthwhite et al, 2006; Monique Vander veen et al, 2016, Kim YS et al, 2014; Nomura et al, 2017, Han S et al, 2016). Some of the oral pathogens in dental plaque responsible for causing dental caries and periodontal problems fluoresce under specific wavelengths of visible light. Anaerobic bacteria causing periodontal infections such as *Prevotella intermedia, Prevotella. melaninogenica, Porphyromonas gingivalis, Prevotella nigrescens, Aggregatibacter actinomycetocomitans,* and *Actinomyces israelii* fluoresce in the red spectrum of the electromagnetic radiation (Coulthwhite et al, 2006; Lennon et al 2006; Hope et al 2011; Volgenent C, 2011). *Fusobacterium nucleatum* fluoresces green, and *Propionibacterium* fluoresces red upon excitation with Violet-Blue light (Dobrev et

al 2010). It has been suggested that emission of red fluorescence is due to the presence of porphyrin related compounds (Koenig 1994 and Schneckenburger H; Konig et al, 1998; GF Gomez et al 2016). Porphyrins are aromatic compounds with a central porphine tetrapyrole ring. Each pyrole is made up of 4 carbons with a molecular formula of C4H4NH.These pyrrole rings are connected by methane bridges (-CH=). They are involved in the synthesis of heme and chlorophyll. Some bacteria lack the ability to synthesize heme or require the presence of heme in the growth media. The presence of porphyrins was confirmed in some of the oral anaerobic bacteria (Fyrestam et al 2015; Soukos NS et al 2000). The parent porphyrin is linked to various side chains such as alkyl, alkene, or carboxylic acid groups which determine their chemical and physical properties. These compounds fluoresce and have an intense soret band at 380-500 nm (Fyrestam et al 2015). Blue light wavelengths of the electromagnetic radiation cause the parent porphine ring to emit a red fluorescence.

The caries-causing bacterium *Streptococcus mutans* had been shown to have fluorescing properties in both the green and red regions (Shigetani et al 2008). Although dental plaque emanates red fluorescence with excitation of blue light, *S. mutans* on isolation exhibites green fluorescence. However, *S. mutans* = that induces carious lesions in enamel and dentin emits an orange fluorescence. We have previously observed that *S. mutans* biofilm grown on acrylic resin chips had a green fluorescence.

Teeth have an inherent ability to autofluoresce. They have a bluish white fluorescence on irradiation with ultraviolet light and when the excitation light is

filtered through a yellow high pass band filter, teeth fluoresce green. According to Shigetani et al., *S. mutans* fluoresces red in carious lesions which might be attributed to its interaction with organic components of the tooth. Volgenent et al. have shown that *S. mutans* fluoresces red due to presence of hemin or chlorophyll. As stated in the previous chapter related to the autofluorescing properties of *S. mutans*, there was an emission noted at a wavelength of 770 nm. According to Ajdic et al. *S. mutans* lacks heme proteins, catalases, and cytochromes. However, according to the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway for *S. mutans* UA159, there is an indication for the presence of the HemZ gene, which encodes the enzyme ferrochetalase **(Figure 9.1)**. Ferrochetalase catalyzes the insertion of ferrous iron into protoporphyrin-forming protoheme in the heme synthetic pathway (Ferreira GC). According to our knowledge, the presence of PPIX in *S. mutans* has not been reported in previous studies. Oral bacteria which have porphyrins, namely PP-IX, can be inactivated by blue light with wavelengths between 400 and 500 nm. *S. mutans* is also inactivated within wavelengths of 400-500 nm. This wavelength range activates porphyrins. Since some of these bacterial species possess metal-free porphyrin, specifically protoporphyrin IX, that gets activated with these wavelengths. The presence of protoporphyrin IX in *S. mutans* remains largely unexplored. Results of our previous experiments showed that *S. mutans* gets inactivated by blue light. As progressing carious lesions had an increase in red fluorescence, we hypothesized that protoporphyrin IX is present in *S. mutans*. Our primary objective was to determine the presence of protoporphyrin IX in *S. mutans* biofilm formed on dentin specimens.

9.2 Materials and Methods

9.2.1 Bacterial Growth

Bratthall serotype *c* strain *S. mutans* UA159 (ATCC 700610) obtained from American Type Culture Collection (Rockville, MD) was used in this study. Cells of the strain were stored at -80ºC in Tryptic Soy Broth (TSB, Acumedia, Baltimore, MA) supplemented with 20% glycerol and were routinely grown on Mitis-Salivaris Sucrose Bacitracin (MSSB, Anaerobe Systems, CA) agar plates to ensure purity. Liquid broth cultures were prepared in 5 ml of TSB and grown for 24 h at 37ºC in a 5% CO_2 incubator. *S. mutans* biofilm was formed in TSB without or with 1% sucrose (TSBS) in 25 cm^2 tissue culture flasks (Fisher Scientific, Co., Newark, DE). The biofilm cells were incubated for 24 and 96 h at 37°C in 5% CO_2. The biofilm cells were also grown in 24-well sterile polystyrene tissue culture plates in the presence or absence of dentin specimens.

9.2.2 Preparation of Dentin Specimens

Human molars were selected free of cracks, fissures, or caries. A Lap Craft L'il trimmer was used to separate the crown. A Buehler Isomet lathe was used to section the dentin from the coronal part of the tooth to a dimension of 4x4x2 mm^2. The sections were ground with grit sizes 500, 1200, 2400, and 4000 by mounting them on an acrylic block in a Struers RotoPol-31/RotoForce-4 grinding machine.

They were ground for 4 sec, followed by rinsing, sonicating, and rinsing again for 3 min with deionized water (dH_2O). The dentin specimens were carefully placed in a moist cotton gauze sealed in a whirl pak bag and sterilized with ethylene oxide gas. They were stored at 4°C until further use.

9.2.3 Sample Preparation

After 24 and 96 hrs of incubation, the supernatant containing planktonic cells above the biofilm was removed, and cell culture grade water (10 ml/tissue culture flask) was added. The biofilm cells were scraped to obtain a homogenous suspension. 30 ul of the resuspended biofilm cells were further diluted from $1:10^2$ to $1:10^4$ in sterile water for quantitating bacterial numbers by spiral plating onto blood agar plates.

The biofilm cell suspension was centrifuged at 4°C at 3750 rpm twice for 15 minutes. 16 ml of ice cold HPLC grade chloroform, 16 ml of methanol, and 10 ml of tissue culture grade water per g of pelleted wet weight of biofilm cells were added to lyse the cells (Li et al, 2016). The suspensions were vortexed to dissociate biofilm cell aggregates. A 3-phase extraction of proteins was done to separate the aqueous and organic phases. Total porphyrin was extracted by transferring the organic phase through a 0.5 ml centrifugal filter with a 3000NMWL/3K membrane.

9.2.4 Standard Protoporphyrin IX

Protoporphyrin IX (PP-1X; Frontier Scientific) stock solution was prepared in 100% ethanol at a concentration of 0.01 g/ml. Dilutions of PP-IX were made in cell culture grade water with concentrations of 0, 1, 2, 3, 5 and 10 ug/ml. The molecular formula of protoporphyrin IX is C34H34N4O4, and the molecular weight is 562.67 g/mol.

9.2.5 Quadrupole Time-of-Flight (Q-TOF) LC/MS

Samples and the PPIX standards were analyzed using an Agilent 1200 SL-6520 Accurate Mass Quadrupole Time of Flight (Q-TOF) LC/MS (Liquid Chromatography /Mass Spectrometer) and electrospray ionization (ESI). Minimal amounts of sample dissolved in solvent and the standard protoporphyrin IX were sprayed into the mass spectrometer with an electrospray ionization source for desolvation. The samples were injected onto an Agilent Poroshell EC-C18 2.1x150mm, 2.7µm column and separated using a flow rate of 0.5 ml /min and a gradient of 95% H2O(A)/5% acetonitrile(B), both with 0.1% formic acid, increasing to 60% B over 14 minutes, then to 100% B over 16 minutes followed by column cleaning and re-equilibration. The ion signal was amplified, and results were recorded as a spectrum based on the m/z value. Mass hunter qualitative analysis software was used to extract and integrate the chromatographic peaks. Accurate

mass method was used to scan the molecular ion peak of PP-1X. The area of the spectral peak was used for analysis.

9.3 Results

The mass of the PPIX standard ($C_{34}H_{34}N_4O_4$) was 562.2560 with retention times ranging from 14.484 – 14.619 minutes. The molecular ion peak of PP-IX was measured at a monoisotopic mass of 563.2797 (M_+H_+). The chromatographic and mass spectral display of the PPIX standard was at a retention time of 14.514 minutes **(Figure 9.15)**. The area of the spectral peak was at 7860404 for PP-IX.

The total number of viable CFU of *S. mutans* biofilm cells grown for 24 hrs in a 25 cm^2 tissue culture flask in TSB was approximately 1.50*10^8, and the CFU after 96 hrs of growth was 1.77*10^6. The CFU of TSBS-grown cells after 24 and 96 hrs of incubation were 4.52*10^7 and 2.72*10^7, respectively. There was a statistically significant difference ($p<0.05$) in CFU between the TSB- and TSBS-grown biofilms for 24 and 96 hrs **(Figure 9.2)**. The areas of the spectral peak for the PP-1X standard obtained through QTOF were analyzed. There were no substantial differences in the spectral areas for the mass ranges from 562.2874 – 562.3093 with TSB- and TSBS-grown biofilm for 24 and 96 hrs for PP-IX **(Figure 9.3** and **9.4)** for TSB at 24 and 96 hrs respectively; **Figure 9.5** and **9.6** for TSBS at 24 and 96 hrs respectively.

In addition to the determination of PP-IX in 24 and 96 hr-grown *S. mutans* biofilms in the presence or absence of sucrose, PP-IX was also measured in *S.*

mutans co-cultured with human dentin specimens. The chromatogram and mass spectrum of PP-IX are shown in the figures. The results obtained for TSB- and TSBS-grown biofilms after 24 and 96 hrs in 24 well tissue culture plates were similar to those obtained with *S. mutans* biofilms grown in tissue culture flasks. Quantitative comparison of the data obtained through spectral peaks with *S. mutans* co-cultured with dentin specimens was not done because of limited sample size. The spectral peaks of PP-1X obtained are provided in the following figures **Figure 9.7** (without dentin) and **9.8** (with dentin) for TSB grown for 24hrs; and **Figure 9.9** (without dentin) and **Figure 9.10** (with dentin) for TSBS-grown biofilm grown for 24hrs) with retention times ranging from 11.683 to 11.844. The spectral and chromatographic peaks of PP-IX in *S. mutans* grown for 96hrs **Figure 9.11** (without dentin) and **Figure 9.12** (with dentin) in TSB (0%sucrose) and **Figure 9.13** (without dentin) and **Figure 9.14** (with dentin) in TSBS (1% sucrose).

5 and 10 ug of PP1X had a mass spectral peak at 563.278; however, 1, 2, and 3 ug did not demonstrate a spectral peak at 563 m/z. The chromatogram revealed a mass peak at 579 m/z, which is a protoporphyrin IX-modified derivative. Lower concentrations of the PP-IX compound in the stock solution had a spectral mass peak ranging from 579.2842 – 579.2868 m/z **(Figure 9.15)**. However, a 579 m/z peak was not detected with sterile H_2O (0 mg of PP-IX).

9.4 Discussion

The presence of endogenous protoporphyrin IX was detected in *S. mutans* in this project. The amount of protoporphyrin in *S. mutans* grown for 96 hrs in TSB was increased compared to that grown for 24 hrs. Studies have previously shown increased red fluorescence in mature biofilms. In this study, the area of the spectral peak of protoporphyrin IX with TSB-grown biofilm was increased at 96 hrs compared to 24 hrs; however, the number of bacteria was reduced at 96 hrs. One of the possible explanations for this is that the biosynthesis of protoporphyrin IX in the logarithmic and stationary phases of growth and its release and accumulation may be different in the two phases. During 96 hrs of growth, the media was not replenished, which might have contributed to the decrease in cell number but an increase in metabolic byproducts. Suspending the bacterial cells in sterile water instead of 0.9% saline could have also contributed to the low yield of CFU during spiral plating. There was a statistically significant difference between 24 and 96 hrs of growth in TSBS with increased CFU at 24 hrs. Furthermore, both TSB- and TSBS-grown biofilm had an increase in CFU at 24 hrs over 96 hrs. The area of the spectral peak of protoporphyrin with TSBS-grown biofilm of 24 hrs was increased compared to that after 96 hrs of growth. The results indicate that protoporphyrin IX is biosynthesized from *S. mutans*. The area of the spectral peak of protoporphyrin in dentin-grown bacteria was reduced with *S. mutans* grown for 24 hrs in TSB on a dentin specimen compared to *S. mutans* grown in a microtiter plate. Quantitating the biofilm cells in the presence and absence of dentin at 24 and 96hr should be

considered. Larger sample size is also required for quantitative analysis of the spectral peak of PP-IX in the presence and absence of dentin at 24 and 96 hrs with the presence and absence of sucrose.

9.5 Conclusion

The area of the PPIX peak in *S. mutans* grown without sucrose on a microtiter plate for 24 hrs was relatively higher than biofilm grown for 96 hrs and on dentin specimens with and without sucrose for 24 and 96 hrs. The retention time ranged from 11.27 to 15.86 min with masses of 562.2529-562.2631. Endogenous PPIX was detected in the gram positive cariogenic bacterium *S. mutans*.

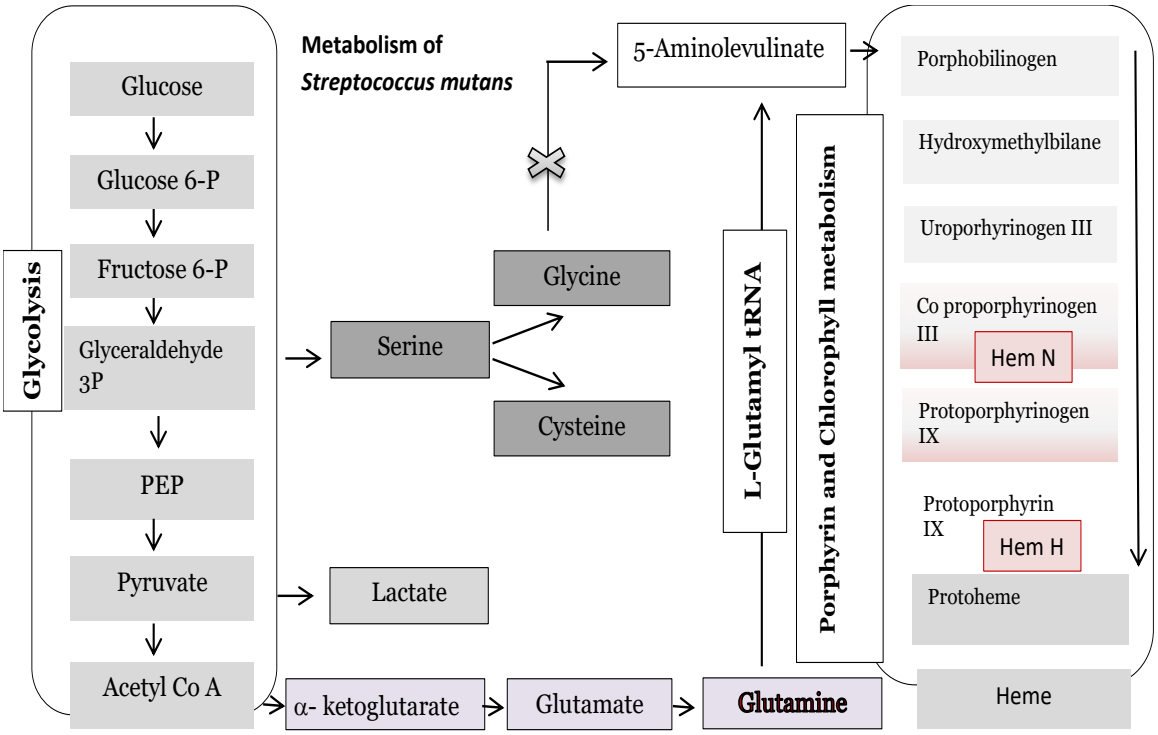

Figure 9.1 Porphyrin metabolism of *S. mutans* UA159

The tricarboxylic acid (TCA) cycle is incomplete in *S. mutans*. There is formation of glutamine and glycine. Porphyrins are synthesized from 5-aminolevulinate by combination of succinyl Co A and glycine. Glutamine is also used as an alternate pathway to synthesize porphyrins. *S. mutans* synthesizes glutamine in large amounts, and there are 5 glutamine ABC transporters which may play a role in the synthesis of porphyrins.

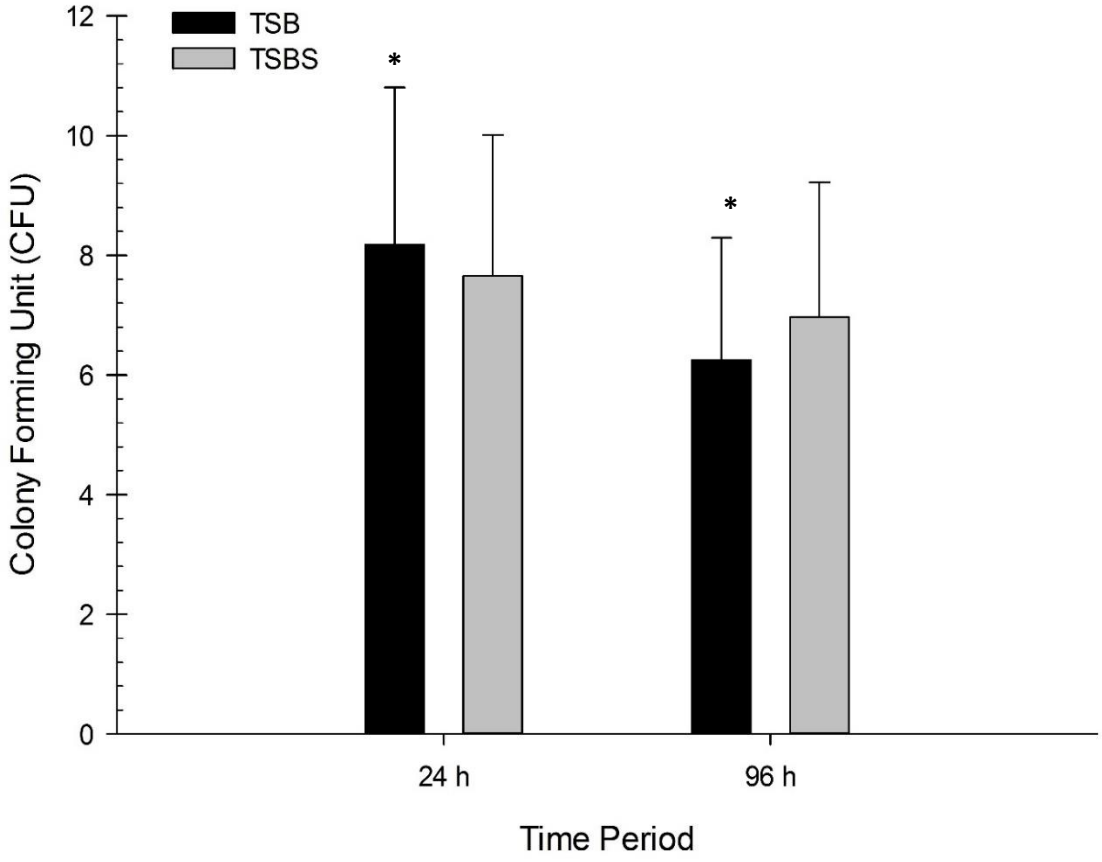

Figure 9.2 Colony forming units (CFU) of *S. mutans* biofilm cells grown for 24 and 96 hrs in TSB (0% sucrose) and TSBS (1% sucrose)

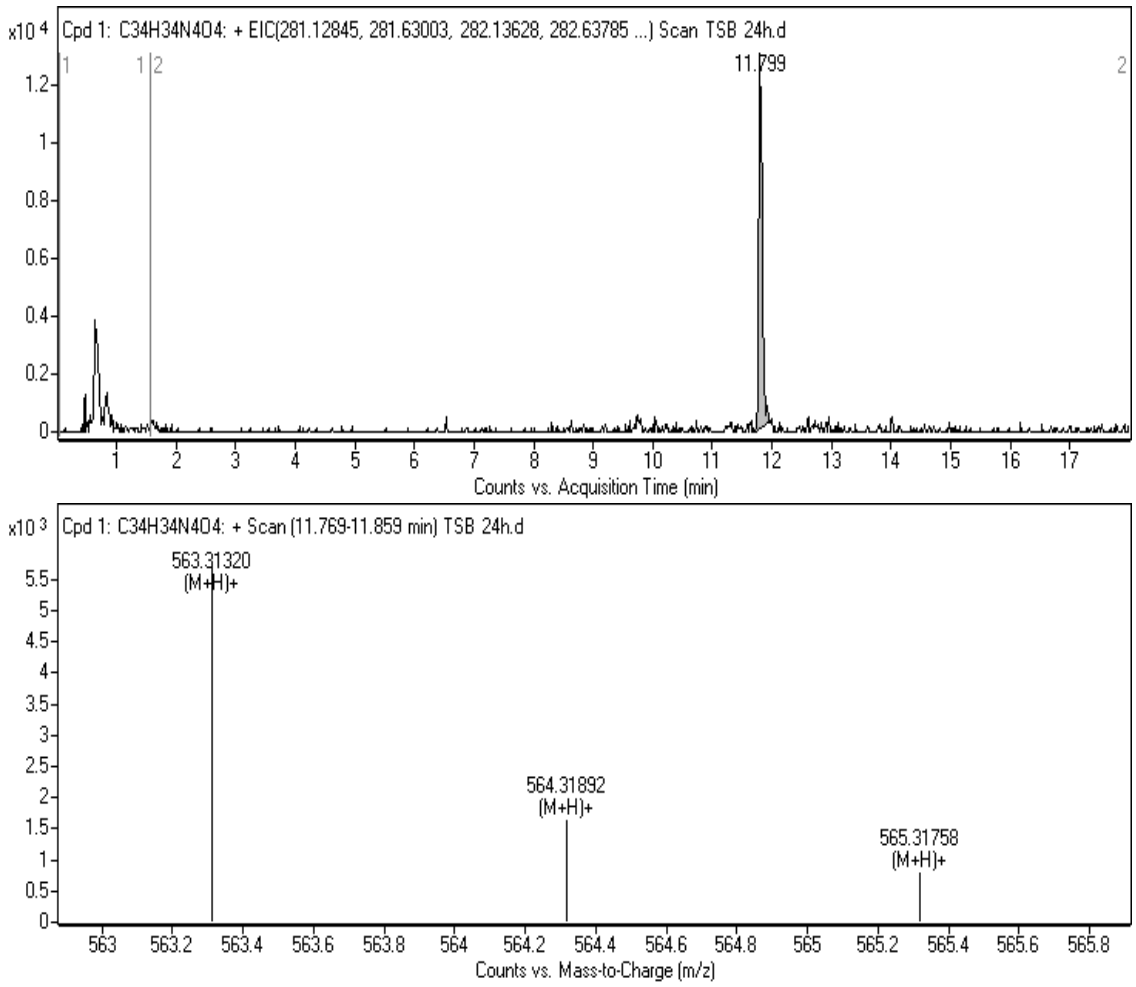

Figure 9.3 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 24 hrs with TSB (0% sucrose).

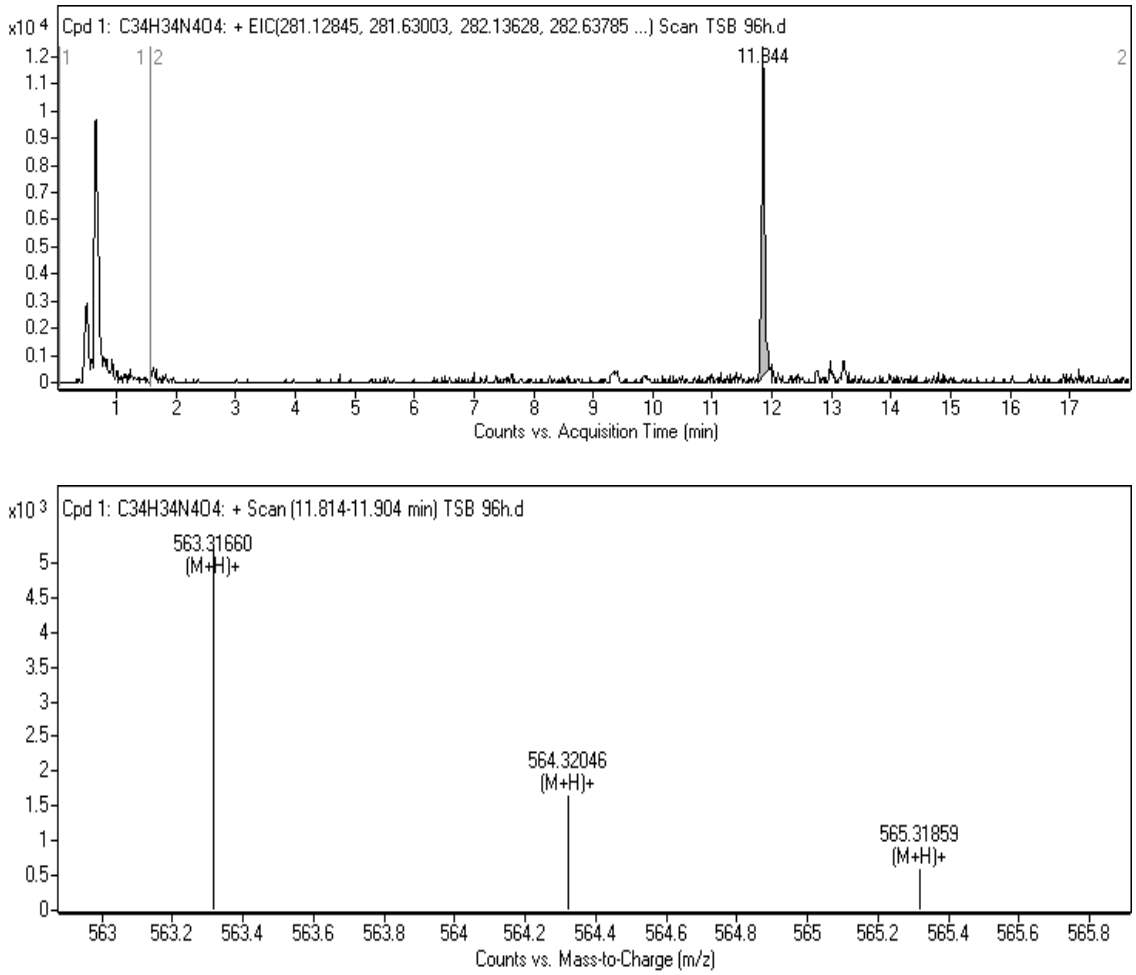

Figure 9.4 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 96 hrs with TSB (0% sucrose).

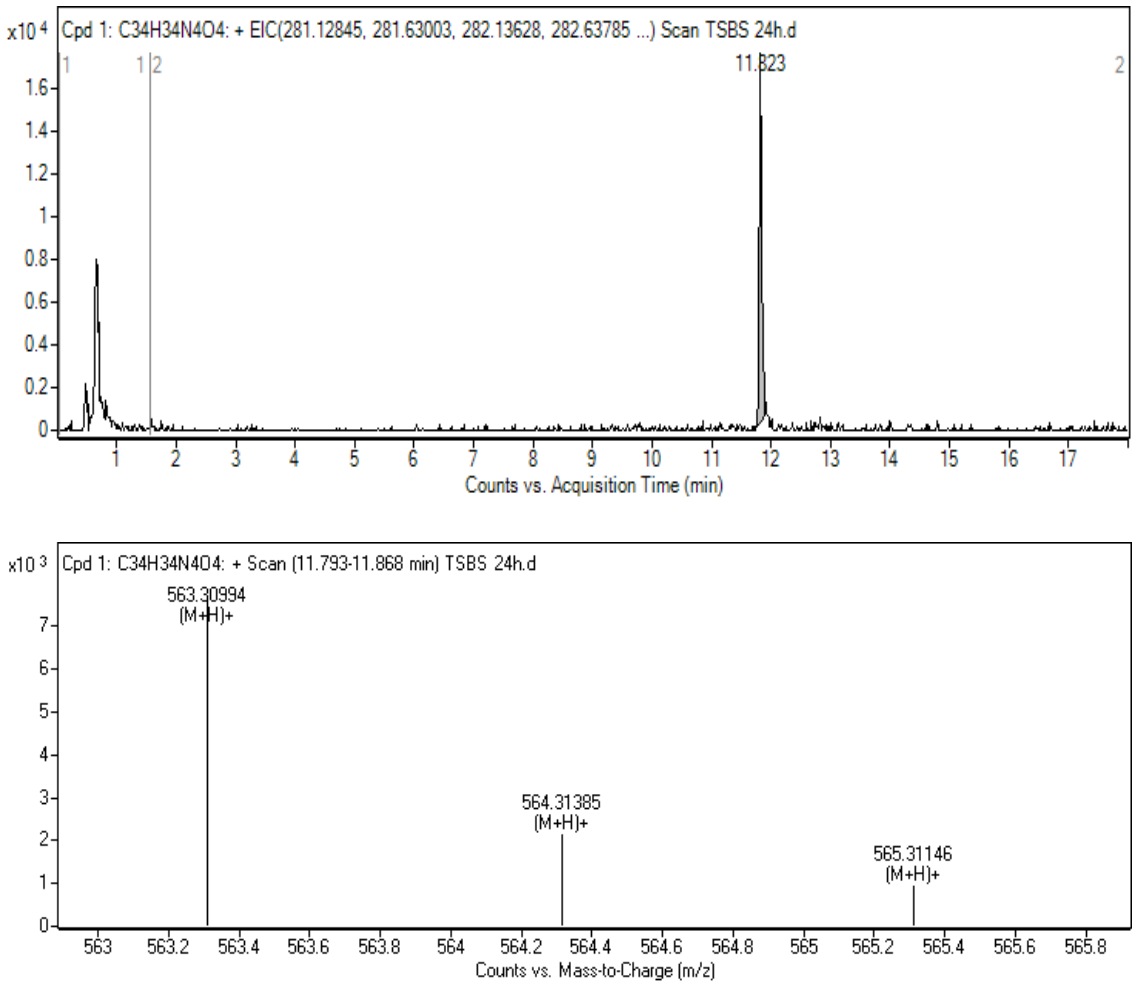

Figure 9.5 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 24 hrs with TSBS (1% sucrose).

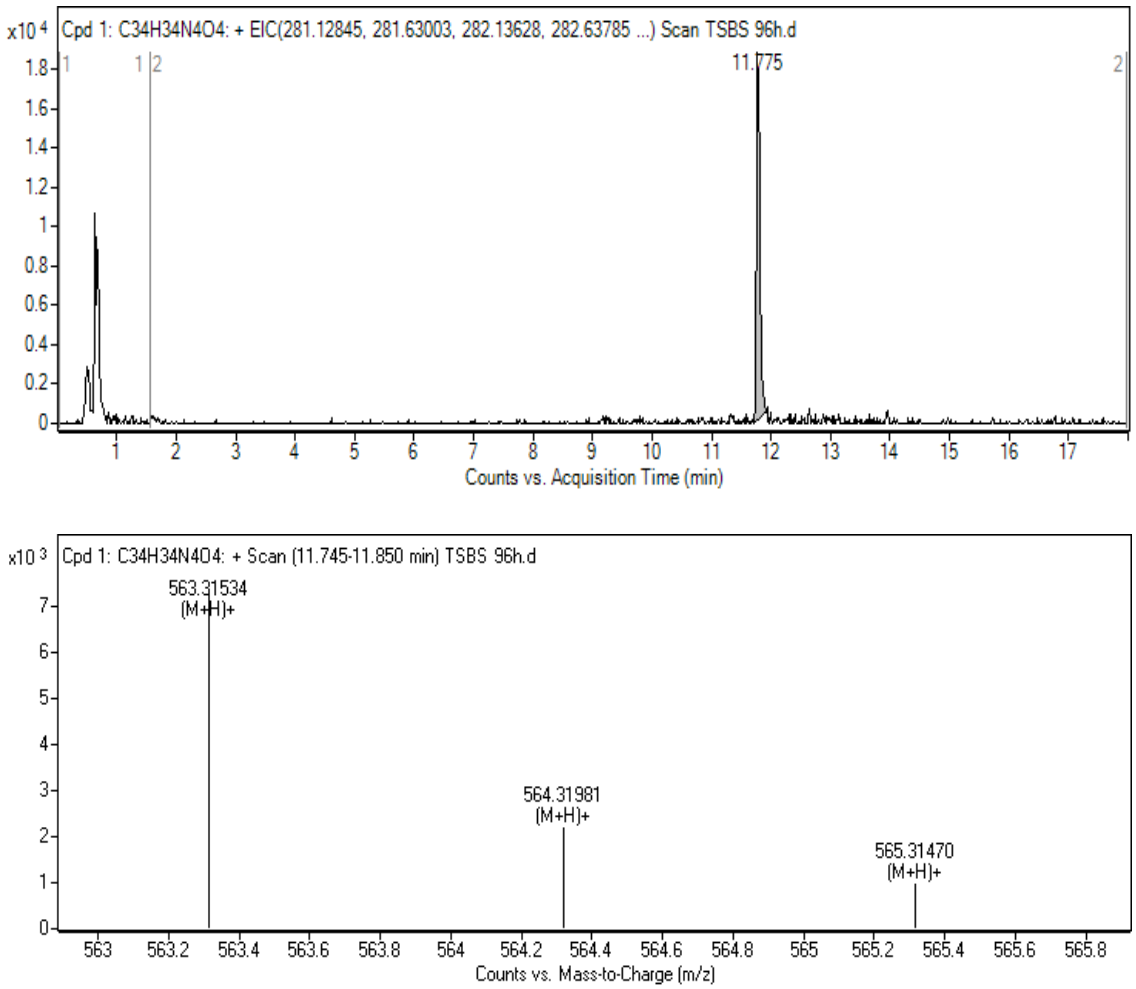

Figure 9.6 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 96 hrs with TSBS (1% sucrose).

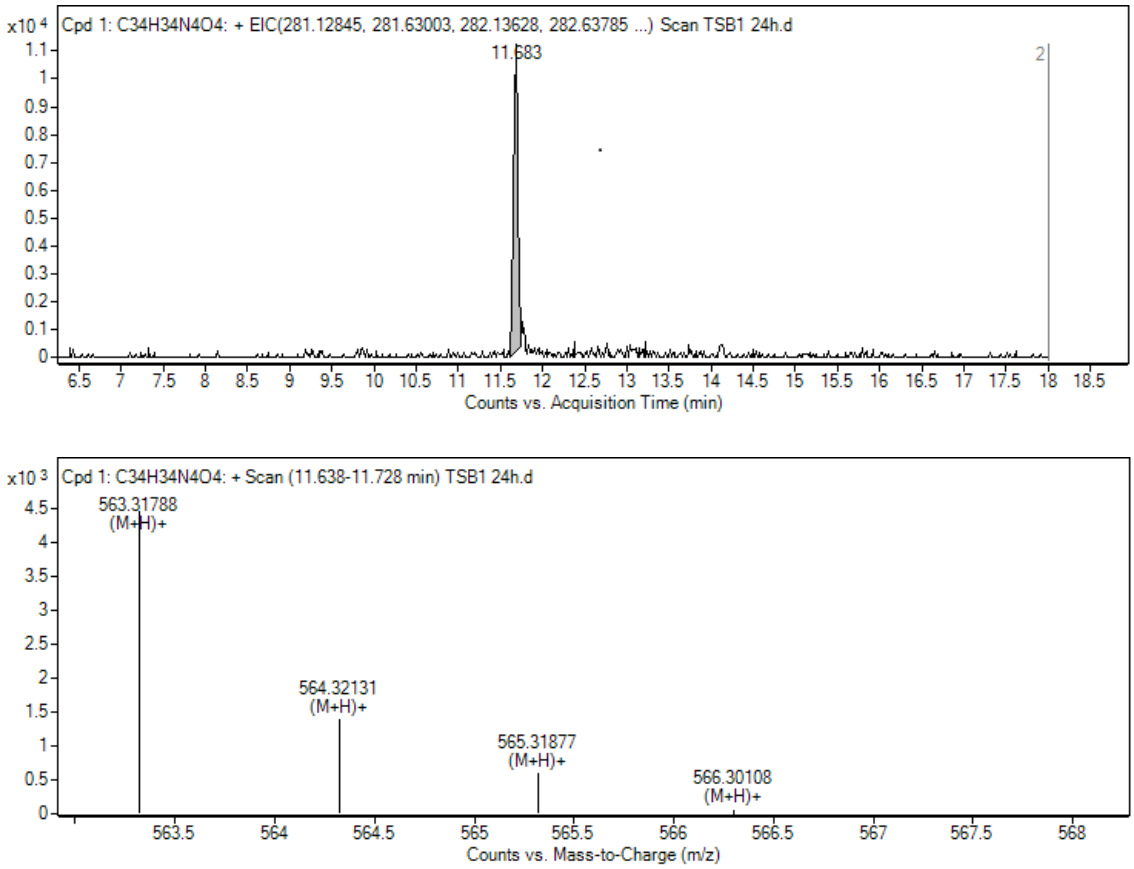

Figure 9.7 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 24 hrs with TSB (0% sucrose) in a microplate.

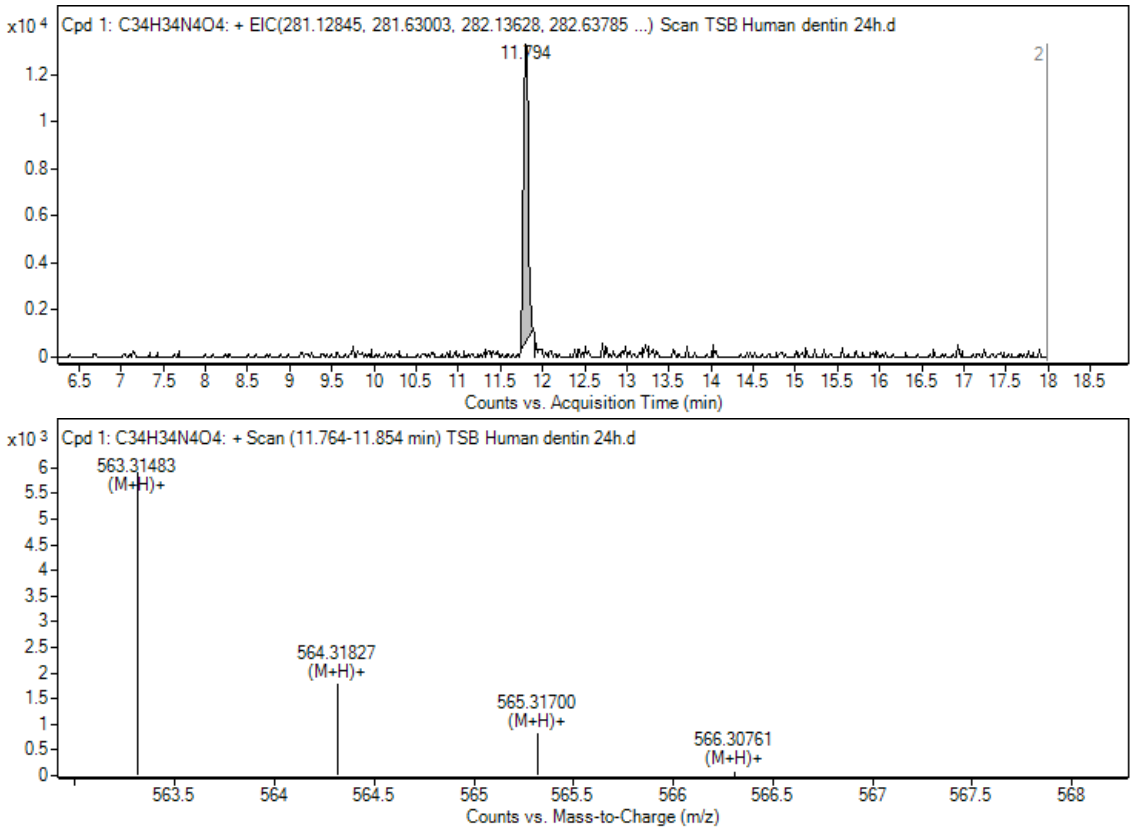

Figure 9.8 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 24 hrs with TSB (0% sucrose) in a microplate co-cultured with human dentin specimens.

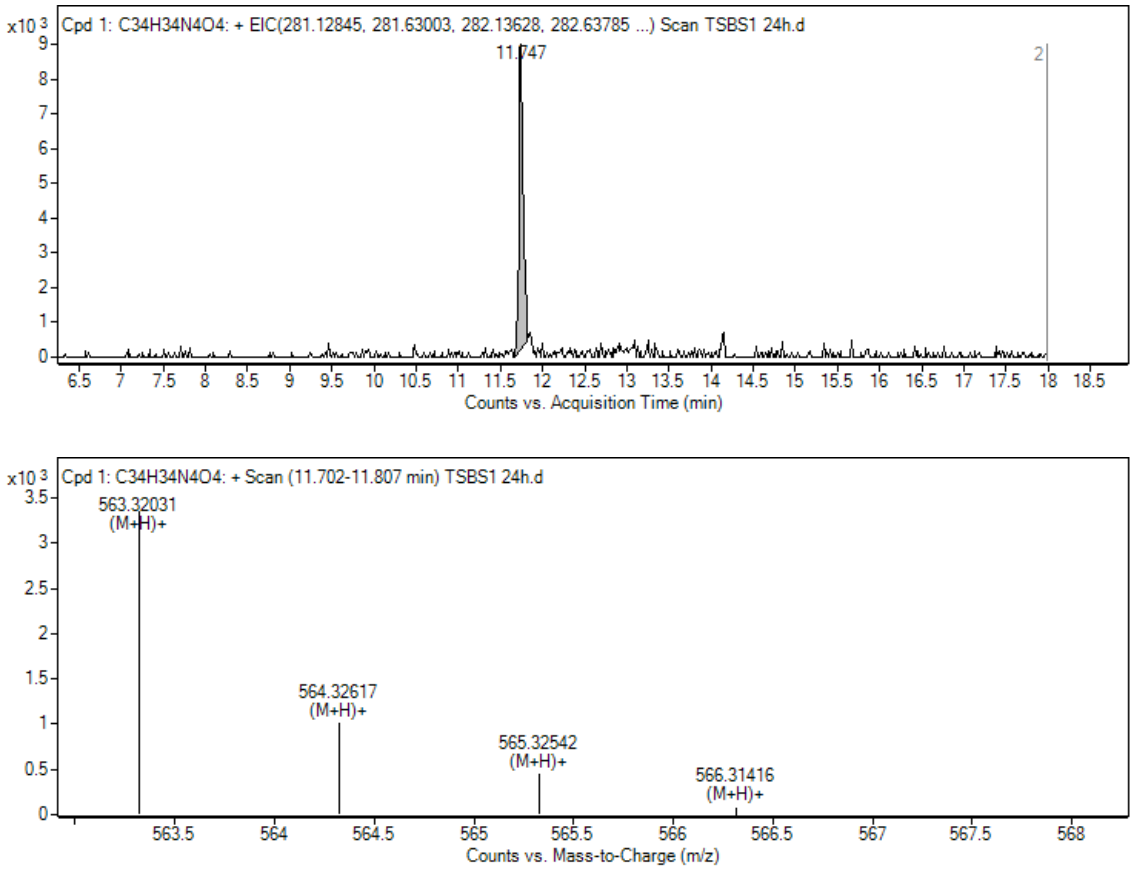

Figure 9.9 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 24 hrs with TSBS (1% sucrose) in a microplate.

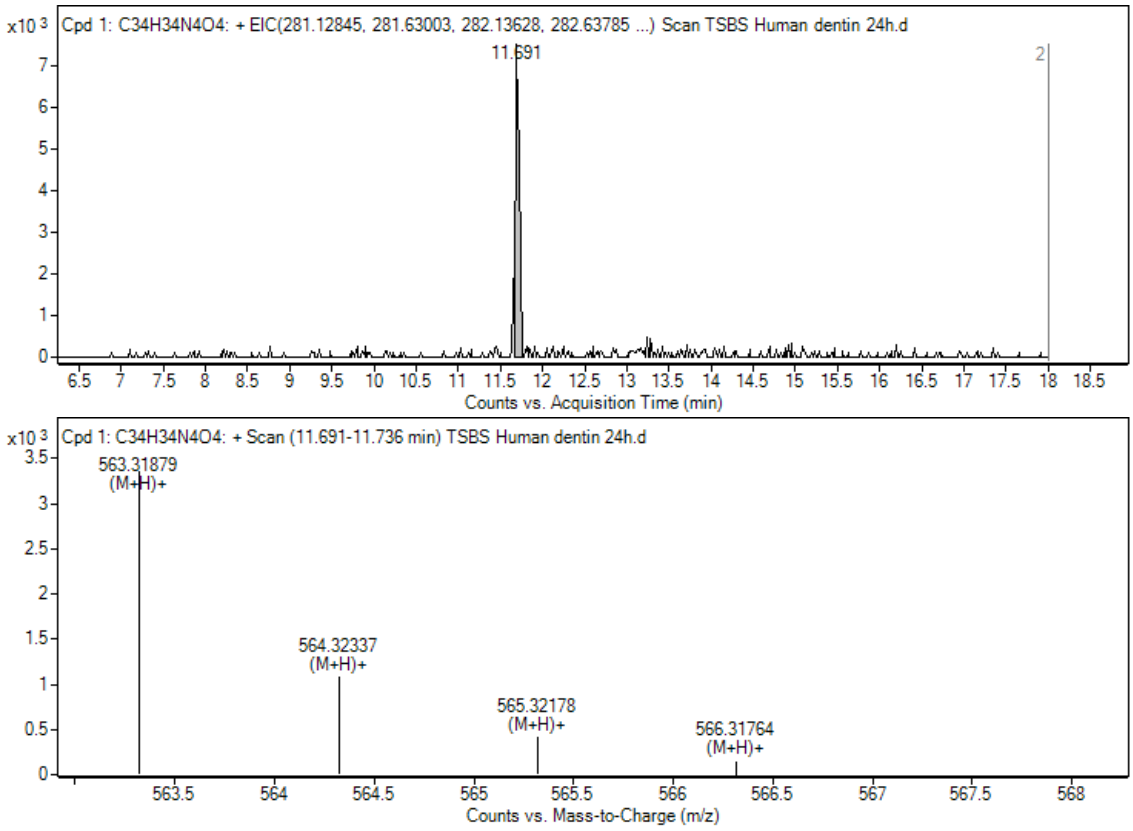

Figure 9.10 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 24 hrs with TSBS (1% sucrose) co-cultured with human dentin specimens in a microplate.

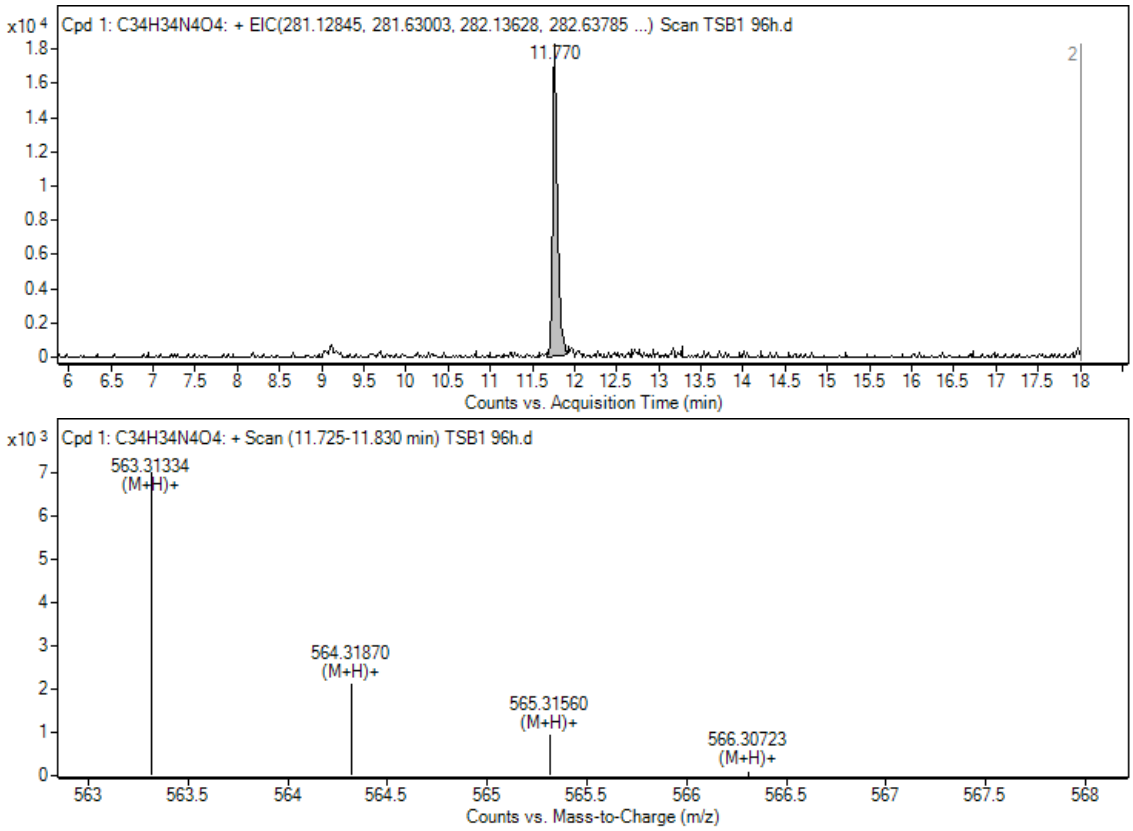

Figure 9.11 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 96 hrs with TSB (0% sucrose) in a microplate.

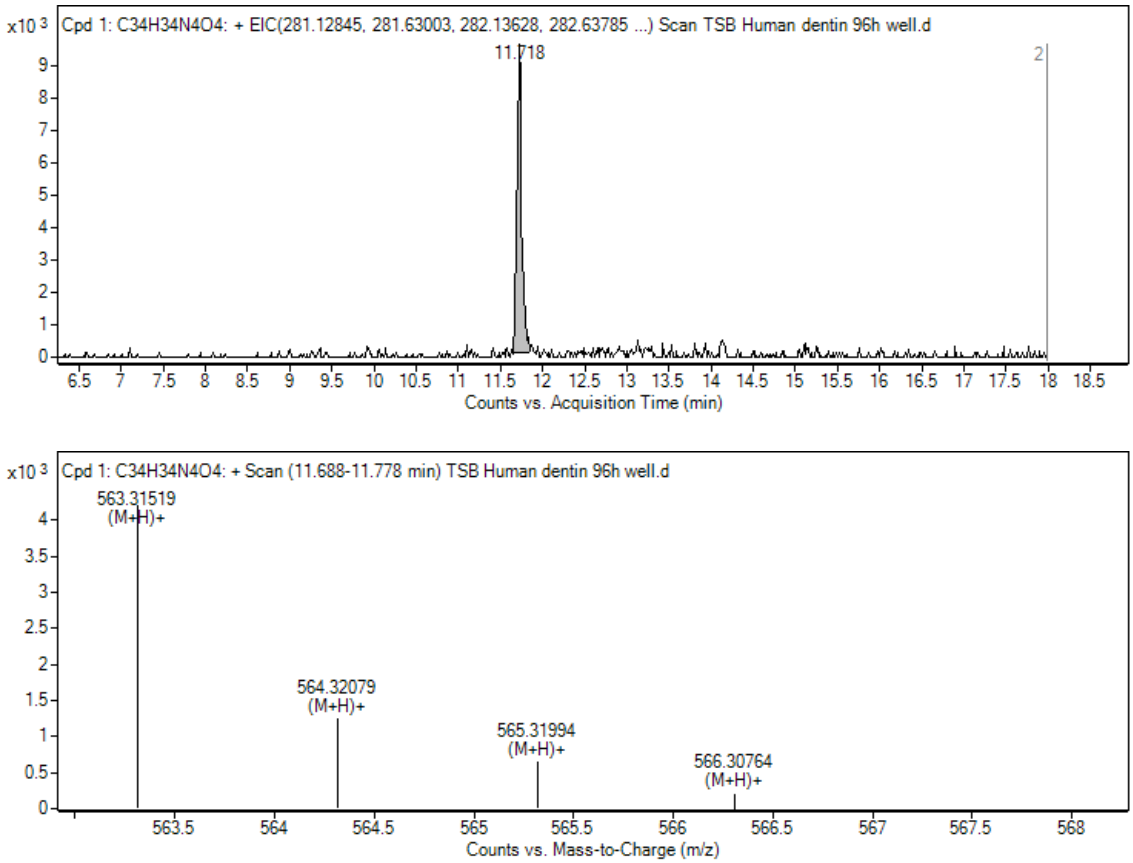

Figure 9.12 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 96 hrs with TSB (0% sucrose) co-cultured with human dentin specimens in a microplate.

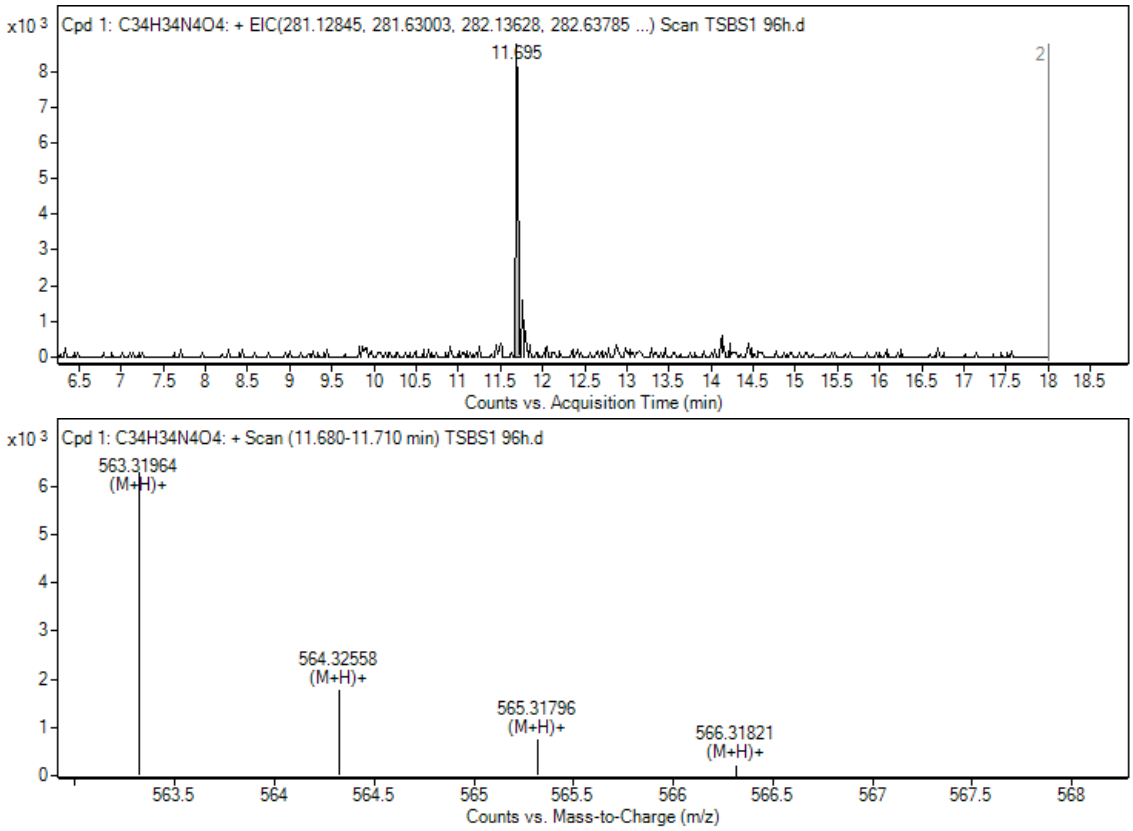

Figure 9.13 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* grown for 96 hrs with in TSBS (1% sucrose) in wells of a microplate.

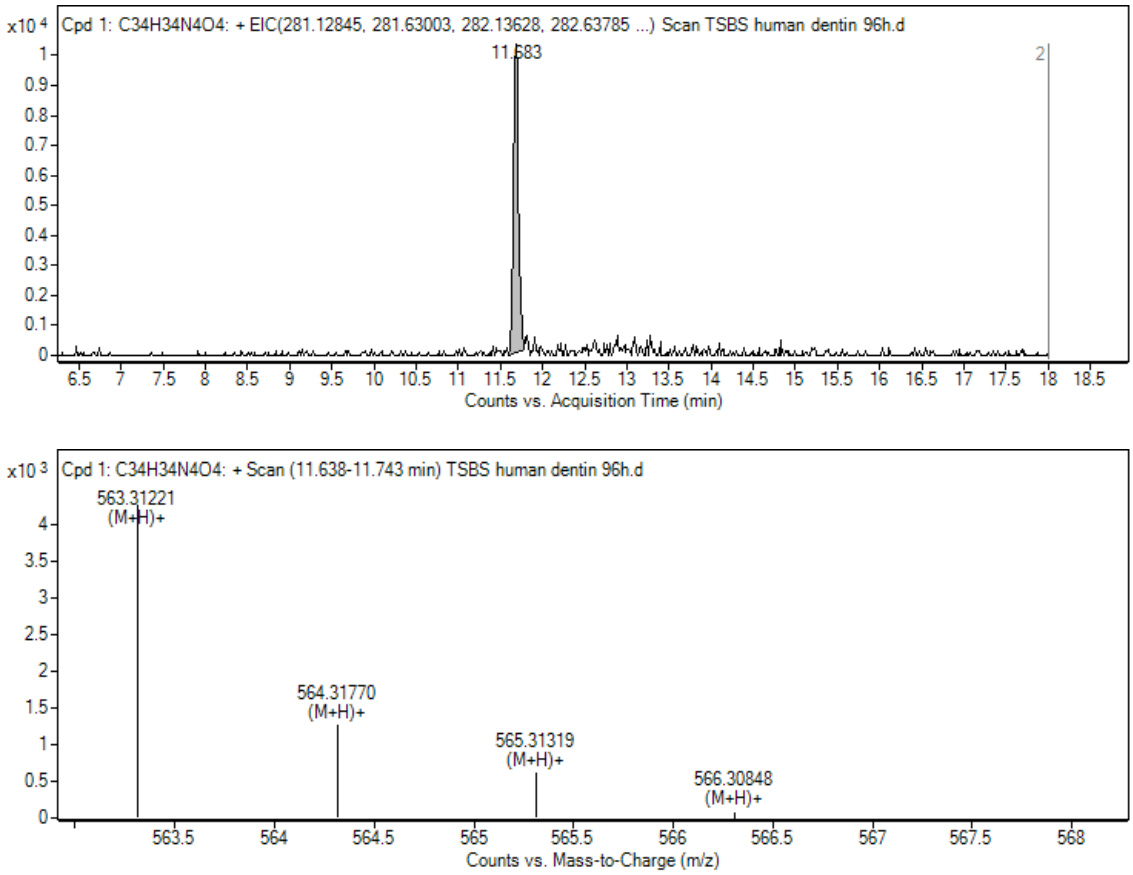

Figure 9.14 Mass spectrum and chromatographic peaks of PPIX of *S. mutans* co-cultured with human dentin specimens for 96 hrs with in TSBS (1% sucrose) in wells of a microplate.

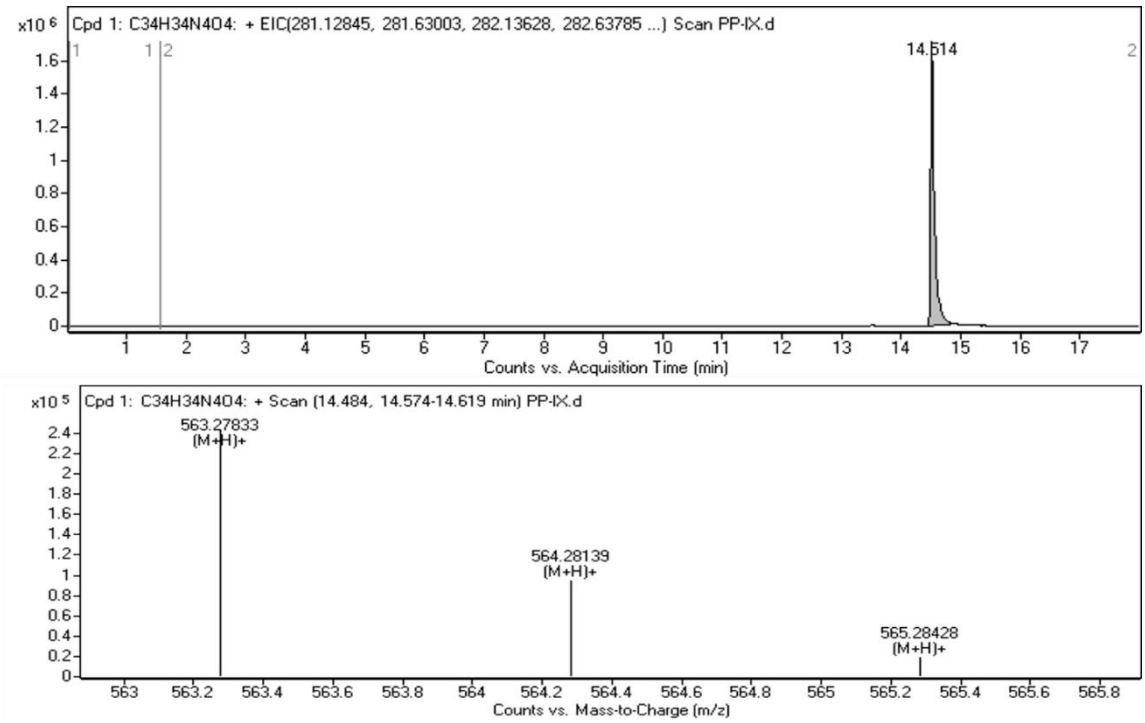

Figure 9.15 Mass spectrum and chromatographic peaks of standard PPIX (10 ug/mL).

Chapter 10

Discussion

Dental caries is a reversible disease process on the surface of the tooth with multiple etiological factors including bacteria, diet, time, host, and civic factors. The risk factors are interrelated; however, oral bacteria in their biofilm mode is the primary etiological factor for the initiation of a carious surface (Selwitz et al, 2007). Though it is an infectious, chronic, and a progressive disease, it is preventable if it is detected at an earlier stage. The father of modern dentistry, Dr. Greene Vardiman Black introduced the concept of extension for prevention in his principles of cavity preparation as a part of restoration. He also stated that “The day is surely coming and perhaps within the lifetime of you young men before me when we will be engaged in practicing preventive rather than reparative dentistry” (Ruby et al, 2010). This statement has come true with introduction of fluorides in the 1900’s, which helps in the remineralization of tooth surfaces. Fluoridation of drinking water and tooth paste brought a worldwide revolution in the prevention of dental caries (Centers for Disease Control and Prevention [CDC], 2000). Indiana University has the pride in introducing fluoridated tooth paste around the world in partnership with Procter and Gamble (Yael Ksander, 2006). The prevalence of caries has been reduced in developed countries due to these intervention programs; however caries remains epidemic in the world.

In the US, there was a slight decline in the prevalence of dental caries between the 1970's and the 1990's. Later in the 1990's, despite the earlier success of fluoridation, there was an increase in the prevalence of dental caries in school children and in adults (Peterson and Lennon, 2004). According to the National Health and Nutrition Examination Survey (NHANES), 92% of adults aged 20 to 64 years old had caries in their permanent teeth, while 42% of children aged 2 to 11 had caries in their primary dentition, and 21% of school-aged children had dental caries in their permanent dentition (NHANES,1999-2004).

One of the goals in preventing dental caries was to target microbial agents that cause caries. Researchers have studied various preventive approaches ranging from natural products to nanotechnological approaches. Though immunization studies related to anti-caries vaccines were popular in the 1970s and 1980s, it has waned lately. There are surface antigens which are present on *S. mutans,* and they may be cross reactive with human heart muscle. Anti-caries vaccines have shown to be effective in primates. Natural defense against caries is mediated by innate host factors and mucosal salivary IgA (Zhang et al 2013). Unicellular microorganisms are in excess compared to eukaryotic cells in the human host environment. Some of the microorganism are beneficial and protect the human host from pathogenic microorganisms. Some of the commensals become pathogenic due to changes in environment. Also, aerobic, anaerobic, and facultative anaerobic bacteria in the oral cavity tend to form biofilm which contributes to dental caries. Various preventive treatments are currently used, which have been shown to reduce, inhibit, and even eliminate oral biofilm. Non-

invasive phototherapy/photodynamic therapy is a method currently studied against microbial infections to overcome the emergence of antibiotic-resistant bacterial strains (Ten cate, 2006).

Phototherapy is a new non-invasive avenue for prevention and treatment of dental caries. It is simple, efficient and has the potential of being used at home. It is safe since there are no exogenous photosensitizers such as phthalocyanines, hematoporphyrin HCl, erythrosine, toluidine blue O, methylene blue, etc. Therefore, toxic effects such as photosensitivity and other side effects such as pain and staining of the teeth are avoided. It is a localized treatment on a specific area, which eliminates systemic side effects (Gursoy H et al, 2013, Konopka K and Goslinski T, 2007). The research done as a part of this dissertation project has great potential in the future to be used as an in-office or at-home treatment application. It could be used as an adjunct treatment in addition to traditional prophylactic oral care.

Alternative therapeutic approaches have been studied to overcome the emergence of antibiotic-resistant bacterial strains. No studies have reported bacterial resistance due to light (Steinberg et al, 2008). Researchers are working on new approaches to control oral biofilm, and to prevent antibiotic resistance (Ten cate, 2006). Non-invasive phototherapy is one of the approaches currently being studied in various disciplines such as wound healing, tissue regeneration, cancer therapy, and skin disorders (Chaves, et al 2014, Hamblin MR and Demidova TN, 2006), as well as in caries prevention. This therapy along with photodynamic therapy is widely applied in the control of biofilms (Soukos NS and Goodson JM,

2000). This dissertation work is significant in the natural history of caries formation and prevention. Findings from this study will help to understand the cellular mechanisms involved in light therapy and will promote new conservative approaches in the prevention of caries.

Phototherapy with no exogenous photosensitizers or photodynamic therapies with exogenous photosensitizers on *S. mutans* are innovative approaches to control oral biofilm. The light source used in this study is from a caries detection device called QLF. This study extends the application of an early caries detection device to the field of treating the etiology of caries. The use of QLF as a source of light with a peak wavelength at 405 nm to mediate bacterial destruction was the first developed study in the field of phototherapy studies on oral biofilm.

The origin of this concept was based on an observation of orange to red fluorescence in carious lesions in QLF images. This fluorescence was probably due to bacterial metabolic byproducts called porphyrins (al-Khateeb S et al ,1997, Konig K et al, 1998, Buchalla W, 2005, Heinrich-Weltzien R et al, 2003). The concept of interlinking fluorescence due to a stokes shift, which is absorption of a shorter wavelength of Violet-Blue light and emission at a longer wavelength in synergism with photo-mediated destruction was adopted for this research project.

The workhorse strain UA159 of *S. mutans* was used in the study, since it is a serotype c strain, and is widely prevalent in the population. Complete genome sequencing of UA159 has been done, which helped us to choose this strain of *S. mutans*. A systematic analysis of the literature related to light therapy studies on

S. mutans with visible light was conducted. This helped us to narrow our phototherapy research project without the presence of photosensitizers based on the limited number of studies. Since none of the studies were done with a QLF device, which employs Violet-Blue light and have not been done without the presence of sucrose, we conducted our experiments in the presence and absence of 1% sucrose.

We determined that there was a photoinhibitory effect of Violet-Blue light on biofilm formation of *S. mutans* without the presence of sucrose when they were allowed to recover for 2 and 6 hrs after 5 minutes of continuous irradiation with Violet-Blue light. However, the viability and kinetics of the growth were significantly reduced at both 2 and 6 hrs in the presence and absence of sucrose (Gomez et al 2016). This demonstrates that the Violet-Blue light had a direct effect on the bacteria. Some studies also have shown a reduction in biofilm formation with changes in morphology. This advocates for study in detail in the changes of size, shape and morphology of individual bacterial cell on irradiation with a specific wavelength ranging from Violet-Blue light of the electromagnetic spectrum. *S. mutans* has an interesting history with its acquisition of its name in 1924 by Clarke. It was reported to have forms of coccal as well as bacilli morphologies with changes in the medium used and fluctuations in pH (Clarke, 1924). This characteristic feature could be studied in the future for a single bacterial cell compared to changes in the morphology of a biofilm in the presence of Violet-Blue light.

Following the photoinactivation of *S. mutans* with Violet Blue light, we determined the metabolic activity of *S. mutans* biofilm at various time periods such as 0, 2, and 6 hrs. One of the limitations in the previous studies was biofilm formation; viability and kinetics of growth were not determined at zero hours which was addressed in this study. Recovery of biofilm cells after treatment at 2 and 6 hrs were found in both studies. There was a reduction in metabolic activities of *S. mutans* biofilm cells except that formed in TSBS with 1% sucrose after 6 hr of recovery (Gomez et al, 2017, J. Oral Sci., in press). This might be due to bacterial cells trapped in the EPS matrix.

Based on the proof of concept studies, we conducted translational research studies on human dentin and enamel. However, we chose to treat the *S. mutans* biofilm grown on human dentin and enamel specimens twice daily with a reincubation period of 6 hrs. The common consensus on these studies was that the Violet-Blue light inhibited the viability of bacterium. These experiments were monitored for a period of 5 days with 2 treatments per day for the first 4 days and one treatment on the final day. Both dentin and enamel were allowed to progress towards carious lesions by *S. mutans*. Data in loss of mineral and lesion depth were obtained through QLF-D and Transverse Microradiography (TMR). This data helped us to determine the effectiveness of Violet-Blue light in the prevention of carious lesions. Though there was a reduction in the QLF-D parameters, lesion depth and mineral loss in the Violet-Blue light treated group were not significantly different from those of the control group, except for TSBS cultures with dentin and mineral loss in TSB cultures with enamel.

As stated in the introduction of this thesis, an orange to red fluorescence was observed in QLF images. This observation of fluorescence on teeth was proposed to be due to porphyrins. Blue light was used for photoinactivation of bacteria such as *Propionibacterium acnes* (Ashkenazi H et al, 2003). This helped us to undertake the phototherapy studies with QLF. Simultaneously, we also carried out a retrospective study on images of carious lesions to investigate the orange to red fluorescence in progressive carious lesions compared with non-progressive lesions. The results indicated that there were increases in the amount of orange-red fluorescence with progression to cavitation. Also, there were increases in orange fluorescence at baseline on surfaces that progressed to cavitation after 48 months.

This helped us to develop our central hypothesis that bacteria produce endogenous fluorophore(s) to absorb the Violet-Blue light, which becomes excited resulting in photo-mediated inactivation. This fluorophore was hypothesized to be a porphyrin-related compound, since it has been proposed previously that porphyrins in carious lesions fluoresce in the red region of the spectrum when excited with Violet-Blue light.

Autofluorescence of *S. mutans* was then studied and finally the presence of Protoporphyrin IX in *S. mutans* biofilm was confirmed by LC/MS. Photoinactivation was mediated through an excitation of fluorophore. The fluorophore, which absorbs light, gets excited to mediate photo inactivation of bacteria by generation of oxygen radicals and emits light at a longer wavelength causing fluorescence. The use of QLF will help in caries risk assessment for

patients. The project determined the fluorophore, specifically Protoporphyrin IX, for the first time in *S. mutans* biofilm. This work has also brought new insights towards future strategies to detect and prevent dental caries.

Very few studies have examined gene expression of *S. mutans* in response to Violet-Blue light treatment (Sol A et al,2011 Steinberg et al, 2008, Sol A et al, 2013), and the virulence factors related to cariogenicity in *S. mutans* have not been completely identified. Light has been shown to affect the expression of genes and proteins under some environmental conditions. Upregulation of genes related to biofilm formation, glucan production, or protein synthesis in response to light irradiation has been reported. However, most of the genes were downregulated upon laser irradiation. In the future, the virulence factors of *S. mutans* affected by light and are responsible for the formation of dental caries will be studied. The cell surface antigenic receptor gene *spaP* encodes the protein Antigen (Ag) I/II, also called Pac1 or P1. The *spaP* gene is conserved among serotypes c, e, and f of *S. mutans* (Ma et al, 1991). Antigen I/II, is an adhesin, known for its non-sucrose mediated bacterial attachment to tooth surfaces. It also interacts with glycoproteins, other oral bacteria, exopolysaccharides, and cell surface receptors (Ajdic et al, 2002, Brady et al, 2010). It is a major polypeptide protein with a molecular weight of 185,000 and is associated with the cell wall of the bacteria. Electron micrographs show a fuzzy coat layer on the cell wall of *S. mutans* which has the P1 or Ag I/II protein (Ayakawa, GY et al,1987). Investigation of how Violet-Blue light regulates the expression of *spaP*, and the way it affects the production of antigen I/II should be studied in detail. Four glucan binding proteins have been

identified, including GbpA, GbpB, GbpC and GbpD. They play a role in the sucrose mediated metabolic pathway of *S. mutans*. They help in the production of extracellular glucans or exopolysaccharides. GbpA is antigenic. Similar to glucosyltransferases, it is involved in attachment of the bacteria to surfaces. GbpB, also known as *sagA* gene, encodes peptidoglycan hydrolase. It functions to maintain cell wall integrity. GbpC is a dextran related cell wall anchored protein and has some similarities with Antigen I/II. GbpB and GbpC have functions related to stress related conditions (Ajdic et al 2002, Mattos-Graner, RO et al, 2006).

Glucosyltransferases (Gtf's): Gtf's are exoenzymes responsible for water insoluble and soluble glucan production. Glucan is mainly produced from sucrose. There are 3 genes gtf B, C and D, which encode glucosyltransferases (Gtf I, SI, and S), fructanase, fructosyltransferase, and dextranase (Ajdic et al 2002, Mattos-Graner, RO et al, 2006). Gtf's are responsible for dental plaque formation. Gtf's will aggregate oral bacteria together and contribute to sucrose mediated attachment. GtfC is hydrophilic, and Gtf B and D are hydrophobic. Below is a proposed model for the effect of phototherapy on *S. mutans* (**Figure 10.1**). Future studies on genes related to cariogenicity of *S. mutans* such as *spaP*, Gbps and Gtfs and the light-affected virulence factors of *S. mutans* responsible for the formation of dental caries should be conducted.

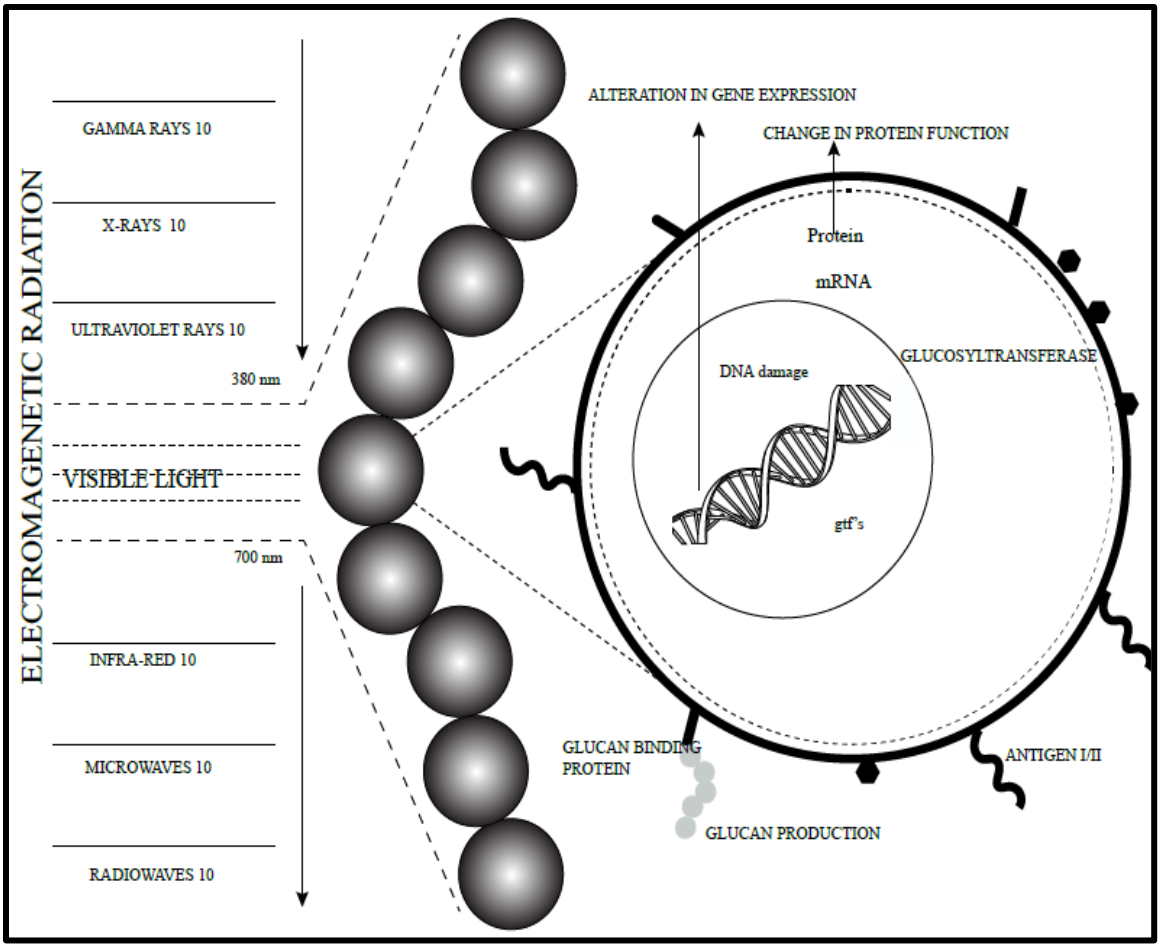

Figure 10.1 Proposed model for the effect of light on the expression of *S. mutans* virulence factors related to cariogenicity.

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Zhang, S. (2013). Dental caries and vaccination strategy against the major cariogenic pathogen, *Streptococcus mutans*. *Current Pharmaceutical Biotechnology, 14*(11), 960-966.

Curriculum Vitae

Grace Gomez Felix Gomez

Education

2012-2018 Ph.D, Indiana University, USA

2008-2010 M.P.H, University of Toledo and Bowling Green State University, USA

1996-2002 B.D.S, Tamilnadu Dr. M.G.R Medical University, Tamilnadu, India

Honors, Awards, Fellowships

2017 Graduate-Professional Educational Grant (GPEG), ($ 500)

2017 President's Dissertation Diversity Fellowship

2016 Graduate-Professional Educational Grant (GPEG), ($ 500)

2016 IUPUI Graduate Travel Fellowship Award ($900)

2015 Travel Fellowship Award from the IUPUI Graduate Office ($630.17)

2014 Educational Enhancement Grant ($500)

2013 Service Learning Assistant Scholarship

2013 Graduate Fellowship Award

Conferences Attended

2017 AADR, San Francisco, CA (poster presentation)

2017 IBASM Turkey Run State Park, IN (poster presentation)

2016 IADR Los Angeles, CA (poster presentation)

2016 IBASM, Fort Wayne, IN (poster presentation)

2015 IADR, Boston, Massachusetts (poster presentation)

2014 AADR, Charlotte, North Carolina (poster presentation)

2013 IADR/AADR, Seattle, WA (oral presentation)

2013 IBASM, McCormick's Creek State Park, IN (poster presentation)

Service and Memberships

2015 - 2017 Indiana Branch of the American Society of Microbiology (IBASM). Student representative of IBASM,2015&2016

2015 - 2017 Member of the American Society of Microbiology

2013 - 2017 American Association for Dental Research (AADR) student membership

2013 - 2017 International Association for Dental Research (IADR) membership

2012 - 2017 Member Advanced Graduate Organization, IUPUI

2015 - 2016 Student representative, IUSD Graduate Student Professional Conduct Committee

Publications

1. Felix Gomez, G. G., Lippert, F., Ando, M., Zandona, A. F., Eckert, G.J., & Gregory, R. L. (2018). Effect of Violet-Blue Light on *Streptococcus mutans*-Induced Enamel Demineralization. Dent J (Basel), 6(2). doi:10.3390/dj6020006. Reproduced with permission from the publisher.

2. Gomez G. F., Huang, R., Eckert, G., Gregory, R. L. (2017). Effect of Phototherapy on the Metabolism of *Streptococcus mutans* Biofilm Based on Colorimetric Tetrazolium Assay. *Journal of Oral Science* (In Press). © Nihon University School of Dentistry. Reproduced with permission from the publisher.

2. Ashkanane, A., Gomez, G. F., Levon, J., Windsor, L. J., Eckert, G. J., & Gregory, R. L. (2017). Nicotine Upregulates Coaggregation of Candida albicans and Streptococcus mutans. *J Prosthodont*.

3. Felix Gomez, G., Eckert, G. J., & Ferreira Zandona, A. (2016). Orange/Red Fluorescence of Active Caries by Retrospective Quantitative Light-Induced Fluorescence Image Analysis. *Caries Research, 50*(3), 295-302. doi:10.1159/000441899 © S. Karger AG, Basel. Reproduced with permission from the publisher.

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5. Tagelsir, A., Yassen, G. H., Gomez, G. F., & Gregory, R. L. (2016). Effect of Antimicrobials Used in Regenerative Endodontic Procedures on 3-week-old *Enterococcus faecalis* Biofilm. *Journal of Endodontics, 42*(2), 258-262.

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