Title

Photo Inactivation of *Streptococcus mutans* Biofilm by Violet-Blue light

Grace F. Gomez, Ruijie Huang, Meoghan MacPherson, Andrea G. Ferreira Zandona, Richard L. Gregory

Abstract

Among various preventive approaches, 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 96-well flat-bottom microtiter plates using Tryptic Soy broth (TSB) or TSB with 1% sucrose (TSBS). Biofilm was irradiated with violet-blue light for 5 min. After exposure, plates were re-incubated at 37°C for either 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 compared to the non-treated groups after the 2 or 6h recovery period. Growth rates of planktonic and biofilm cells indicated a significant reduction in the growth rate of the violet-blue light-treated groups 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. Visible Violet-blue light of the electromagnetic spectrum has the ability to inhibit *S. mutans* growth and reduce the formation of *S. mutans* biofilm. This in-vitro study demonstrated that Violet-blue light has the capacity to inhibit *S. mutans* biofilm formation. Potential clinical applications of light therapy in the future remain bright in preventing the development and progression of dental caries.

Keywords *Streptococcus mutans,* phototherapy, Violet-blue light, biofilm.

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Introduction

The human oral cavity is a cornucopia of microbes with a symbiotic relationship to the human host [4, 32]. Commensal oral microbes share space in the oral cavity in a state of quiescence, protecting the human host from pathogenic bacteria [19]. These non-pathogenic bacteria have the potential to become pathogenic, when factors related to changes in the oral environment disrupt their homeostasis [43]. 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 [4, 27, 28, 29]. Oral microbial biofilm has been established to be an etiological factor for dental caries and other oral diseases [34]. 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” [19, 35]. *S. mutans*, a facultative anaerobic, acidogenic and aciduric bacterium, is a major pathogen of dental caries [5, 15, 20].

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 effectively [28]. New technologies and approaches have been suggested to control the formation of biofilms [41]. Biofilms, are more resistant to antimicrobial treatment than planktonic free-floating bacteria [16, 39]. 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, by visualizing carious lesions at their incipient stage. Quantitative light induced fluorescence (QLF), one of the earliest caries detection devices, which

uses a violet–blue light having a peak wavelength at 405 nm, was employed in this study.

Previous studies demonstrated that blue light within a specific wavelength or range of wavelengths has a potent antibacterial effect [2,8,11,13,21,22,29]. 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) [37]. Recent studies by Chebath-Taub et al. [6] and Steinberg et al. [38] 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 specifically from a QLF device with an exposure time of 5 min has the ability to 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.

Materials and Methods

Bacterial Strain and Growth Media

S. mutans strain UA159 (ATCC 700610) was cultivated in Tryptic Soy broth (TSB, Acumedia, Baltimore, MA) overnight in a 5% CO_2 incubator. Biofilm was grown in 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 to reduce light scatter between treated and untreated wells. The plates were incubated for 12 to 16 h at 37°C in a 5% CO_2 incubator.

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 which 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 (Fig. 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.

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, 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 to Violet-blue light and without Violet-blue light [17]. The biofilm assay experiments were repeated independently

more than three times with similar results and one representative experiment is reported.

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

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 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.

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 to be statistically significant.

Results

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 (Fig. 2).

Effect of Violet-blue light on *S. mutans* biofilm viability

The bacterial cell viability of *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 (Fig. 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 (Fig. 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 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 compared to the treated group (Fig.4C).

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 has an effective capacity to inactivate and kill *S. mutans* biofilm without any photosensitizer. This study indicated that 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 also the type of a particular organism or a group of microorganisms, plays a vital role in the application of photodynamic therapy [14, 18, 37]. The mechanism behind photoinactivation of S. *mutans* is not known, and to our knowledge only one study has used Violet-blue light with no photosensitizer on S. *mutans* biofilms [6]. 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 [10], determined the effect of light from a Xenon lamp with a wavelength ranging from 450 to 490 nm and with an average power of 440 mW on *S. mutans* biofilm treated with hydrogen peroxide. They demonstrated a 3% reduction of bacterial viability at an exposure time of 10 min in the absence of hydrogen peroxide, and a 30% reduction of 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 such that the integrity of the bacterial cell membrane is affected, causing the contents to leak 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 causing cytotoxicity to the bacterial cells [1, 11, 13, 30]. Studies also indicate that the presence of endogenous bacterial porphyrins act as photosensitizers causing bacterial cell death due to similar photochemical reactions [3, 23, 42, 44]. Presence of a fluorophore or a photosensitizing compound within the absorption spectrum of Violet-blue light 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, dental curing lights with LED, halogen and tungsten filament lamps. None of the 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, which 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 [27]. However, several studies indicated that *S. mutans* does not exhibit red fluorescence, but appears green [7, 9]. 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 correlating orange to red fluorescence seen on carious lesions in QLF images with lesion progression [12].

There was a statistically significant ($p < 0.05$) reduction in the total biofilm mass formed in TSB (Fig. 2A), but not in TSBS (Fig. 2B), a statistically significant reduction in the number of viable bacterial colonies in both TSB and TSBS (Fig. 3) and reduced growth rate in both TSB and TSBS Violet-blue light treated groups (Fig. 4). Before exposure to Violet-blue light, bacterial cultures grown in TSBS were more turbid than cultures 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 thickness of the 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 [10]. that using hydrogen peroxide with Violet-blue light causes increased light penetration into the deepest layers of the *S. mutans* biofilm. We believe that the architecture of biofilm grown in sucrose-supplemented cultures is such that that the microcolonies and cell aggregates e 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* 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 specific wavelength or a wavelength range with a higher intensity might be required for the inactivation of biofilm with sucrose.

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. One specific treatment may be useful in all applications.

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 and 6 h of recovery. The reduction in bacterial viability and the rate of kinetic growth were 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 have a role as an effective prophylactic procedure. However, more studies are necessary to determine the effectiveness and

application of light treatment in the visible light spectrum specifically in the Violet-blue light wavelengths.

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Compliance with Ethical Standards:

Conflict of Interest The authors declare no conflict of interest.

Ethical approval This was an in-vitro study with no human or animal subjects.

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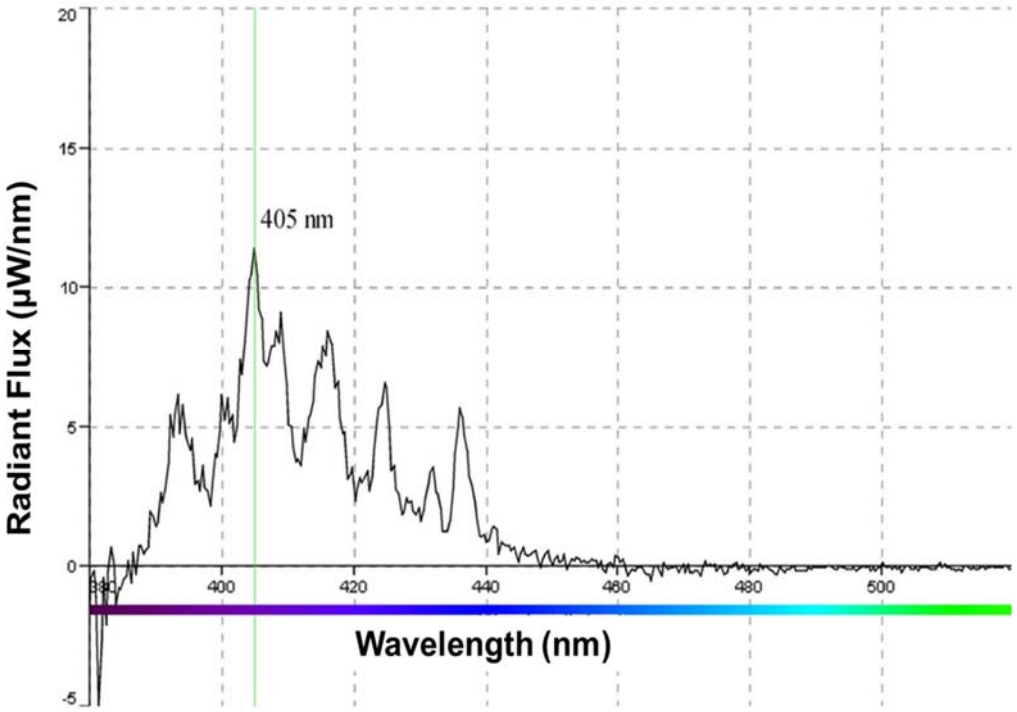
Table 1 Effect of Violet-blue light on *S. mutans* grown in TSB and TSBS measured by maximum absorbance, time to max, lag time and Vmax

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)

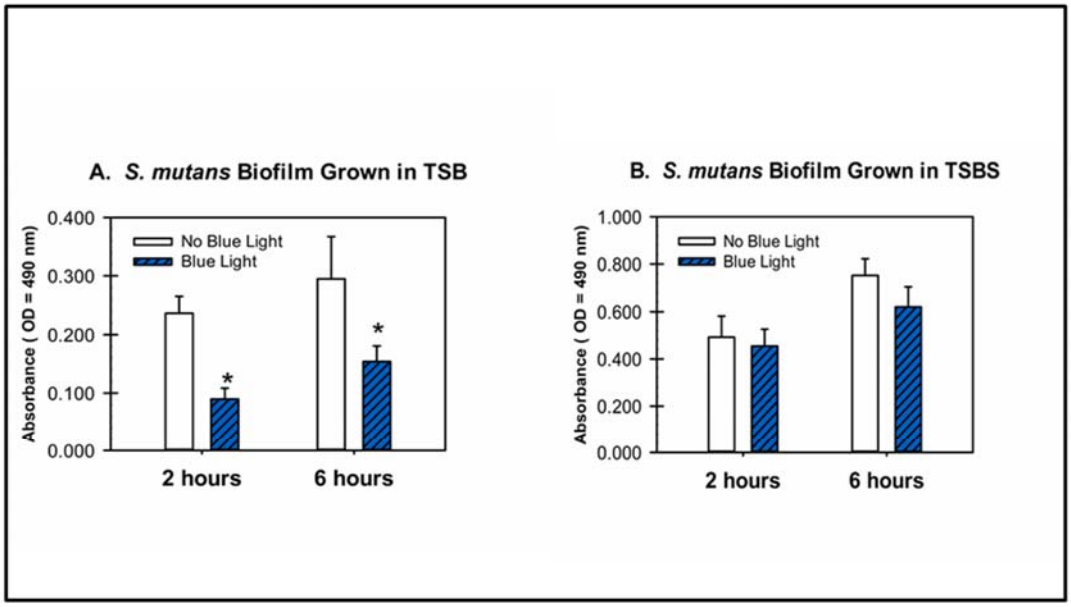
[a]Maximum absorbance: highest absorbance measured during the 6 h period of recovery growth at 37°C. [b]Time to max: Time to maximum absorbance. [c]Lag time: The length of time from incubation until the bacteria begins logarithmic growth. [d]Vmax: slope of exponential growth in logarithmic phase. [*]Statistical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

Fig1 Measurement of the wavelengths emitted by the QLF light.



The wavelengths emitted from the QLF instrument (QLF^{TM}/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.

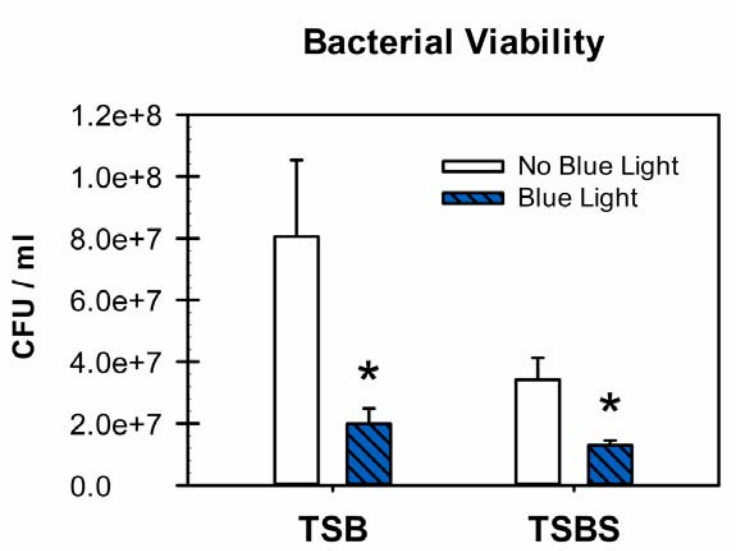
Fig2 Effect of Violet-blue light on *S. mutans* biofilm formation.



(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.

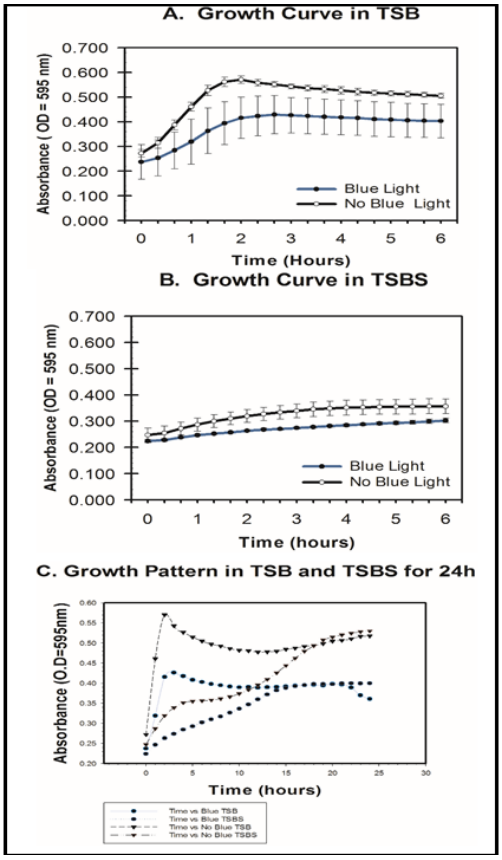
(B) Absorbance values of Violet-blue light-treated *S. mutans* biofilm (n=3) grown in TSBS after staining with crystal violet and allowed to recover for 2 or 6 h. There was no statistical significance between the Violet-blue light and no Violet-blue light groups. Error bars indicate standard deviation.

Fig3 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.

Fig4 Effect of Violet-blue light on the growth rate of biofilm/planktonic *S. mutans.*



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 (4A). Growth curves of S. *mutans* cultures formed in TSBS and treated with Violet-blue light was compared with the no Violet-blue light control group (4B). Growth pattern of *S. mutans* in TSB and TSBS for a period of 24h (4C).