

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

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Abstract:

The aim of this *in-vitro* study was to determine the effect of Violet-Blue light on the metabolic activity of early *Streptococcus mutans* biofilm, reincubated at 0, 2 and 6 h after 5 min of Violet-

Blue light treatment. *S. mutans* UA159 biofilm cells grew 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%CO₂. Colorimetric tetrazolium salt (XTT) reduction assay was used 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 differences between metabolic activity in 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 in the light-treated group. This may provide a unique treatment method for caries active patients.

Keywords: *Streptococcus mutans*; Violet-Blue light; biofilm; XTT assay

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 are currently used, which 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 currently being studied in various disciplines such as wound healing, tissue regeneration (1, 2), cancer therapy (3) and skin disorders (3, 4) as well as in the prevention of caries and oral infections (5). This light therapy is widely applied in the control of biofilms with and without the presence of an exogenous photosensitizer (5, 6, 7). 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 (8, 9, 10).

Visible light in the spectral range from 380 to 700 nm is commonly used to inhibit or kill bacteria (5, 11). The synergistic effect of blue light treatment for 20 sec with hydrogen peroxide demonstrated 96% reduction in bacterial growth (12). However, with 10 min of blue light exposure there was a statistically significant reduction only with blue light within the wavelength range of 400 to 500 nm (12). A delayed antibacterial effect (13, 14) was reported by Feuerstein et al. (12) in a 6 h grown *S. mutans* biofilm, whereas Chebath et al. (15) observed increased numbers of dead bacteria after treatment with 3, 5, 7 and 10 min (14). Photoinactivation of *S. mutans* biofilm has been demonstrated earlier with Violet-Blue light of peak wavelength at 405 nm for 5 min (16). This study reported the effect of violet to blue light on biofilm formation, colony forming units and growth kinetics of *S. mutans*. However, 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* (17), including respiration, and fermentation*, 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 form during fermentation of sugars by oxidizing NADH to ATP through the conversion of pyruvate. Enzymes such as various dehydrogenases are involved in *S. mutans* metabolism (Fig. 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.

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Materials and Methods

* Jurtshuk P Jr. Bacterial Metabolism. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 4. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK7919/>

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 strain was 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% CO₂ incubator. *S. mutans* was grown in TSB without sucrose, and in TSB supplemented with 1% sucrose (TSBS) as a biofilm in 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% CO₂ incubator for the cells to reach the logarithmic phase of growth.

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.

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 K₃) 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.

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 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 the wells and the plates were reincubated for either 0 h, or TSB and TSBS were added and the plates were reincubated for either 2 or 6 h, respectively. The metabolic activity of the treated biofilm cells was determined using an adaptation (18) of the method developed originally by Pierce et al (19). 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 37°C. The supernatant fluid was removed and placed into another 96-well microtiter plate followed by measuring the absorbance at 490 nm.

Statistical Analysis:

The metabolic activity determined by the XTT assay and the effect of Violet-Blue light on *S. mutans* and the non-treated control group based on various recovery time periods of 0, 2 and 6 h were analyzed separately based on the growth media without sucrose (TSB) and with 1% sucrose (TSBS) using a mixed-model ANOVA which included fixed effects for the treated and the non-treated groups of Violet-Blue light, time periods of reincubation and the effect of light by

time period interaction and a random effect for each of the experiments. 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.

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 - Fig. 2, 2 h - Fig.3, 6 h - Fig. 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 ($p = 0.0021$; Fig. 3) and at 6 h of recovery time ($p = 0.0012$; Fig. 4). Metabolic activity after recovery times of 2 and 6 h were not significantly different from each other ($p = 0.90$).

The metabolic activity of *S. mutans* 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 (Fig. 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 ($p < 0.0001$; Fig. 3) of recovery time, but there was no significant difference in XTT activity with and without blue light at 6 h of recovery time ($p = 0.30$; Fig. 4). For TSBS, regardless of light treatment, there was no significant effect due to the recovery time ($p = 0.66$).

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 (16). In this study *S. mutans* metabolic activity was decreased after 0 and 2 h of recovery time in the Violet-Blue light treated groups providing an indirect estimation of *S. mutans* cell viability. Metabolic activity remained unaffected after 6 h of recovery time 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 (16). However, bacterial cells which are encompassed within extracellular glucans may be improperly reduced by tetrazolium salts (20) in the assay used in this study. This suggests the significant reduction of bacterial cells in TSB compared to TSBS as the microcolonies protect *S. mutans* from Violet-Blue light. The respiring bacterial cells produce NADH, and the hydrogen ions are accepted by tetrazolium salt (XTT), which is further converted to colored formazan compound (21). Compared to 0 h, 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 and the biofilm formed correlated with the time of reincubation. However, the Violet-Blue light-treated groups had reduced metabolic activity compared to the non-treated group.

There was some impairment 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 observed in this study. The pathogenic nature of cariogenic biofilm is determined by metabolic activity (22). 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 through intact cell walls and membranes 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 through 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 a longer time than 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 in minutes. 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 repeated treatments may be necessary to prevent the regrowth of biofilm. Within the limitations of the current study more innovative approaches are needed.

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 after 6 h of treatment in the presence of sucrose, suggesting that, repeated Violet-Blue light treatment is highly recommended for the prevention and control of oral biofilms to prevent dental caries. Violet-Blue light at home and in clinical settings, could serve as an adjunct prophylactic treatment in controlling the bacterial numbers based on its significant inhibitory effect for a longer period of time in the absence of sucrose.

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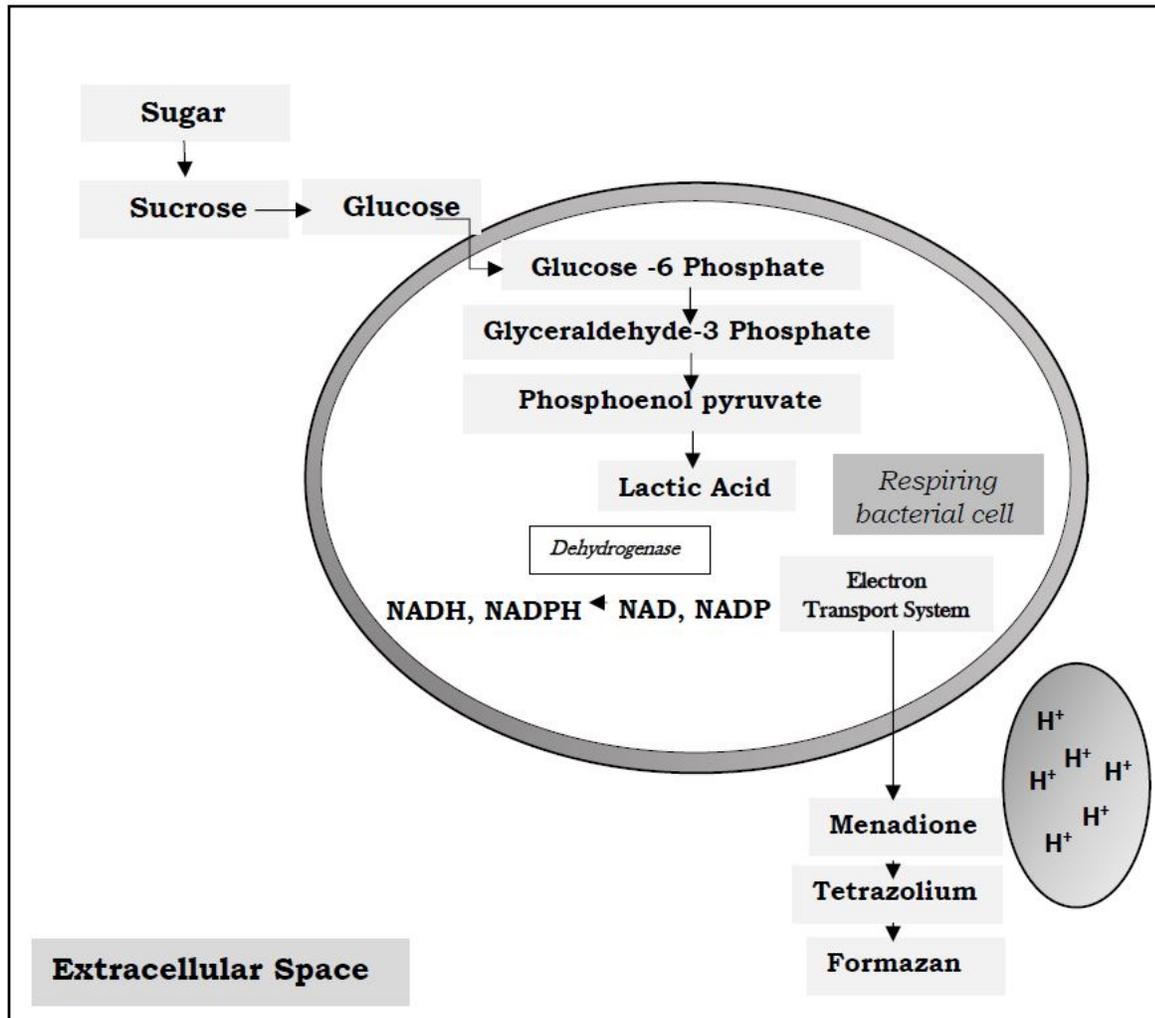


Fig. 1. *S. mutans* metabolizes carbohydrates including sucrose, glucose, lactose fructose and other intermediates of sugars to generate energy. The glycolytic metabolic pathway includes various intermediates such as glucose-6-phosphate, glyceraldehyde 3-phosphate, phosphoenol pyruvate and finally pyruvate. Pyruvate is converted to final 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 in addition to ATP with a conversion of pyruvate to acetate, and the hydrogen ions are accepted by tetrazolium salt (XTT), which is further converted to a colored formazan compound.

Metabolic Activity of *S. mutans* Biofilm Treated with Violet-Blue light (0 h reincubation)

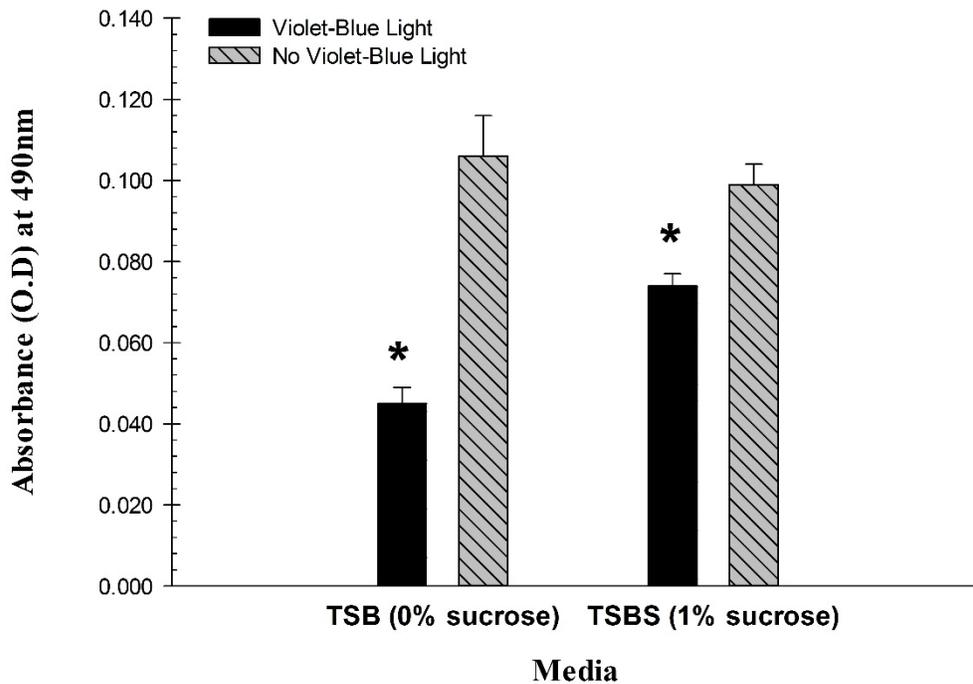


Fig. 2. The effect of Violet-Blue light for TSB-grown *S. mutans* at 0 h had significantly lower metabolic activity in the light-treated group compared with the non-treated group ($p < 0.0001$). Metabolic activity of *S. mutans* grown in TSBS (1% sucrose) was significantly lower in the treated than the 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.

Metabolic Activity of *S. mutans* Biofilm Treated with Violet-Blue Light (2 h reincubation)

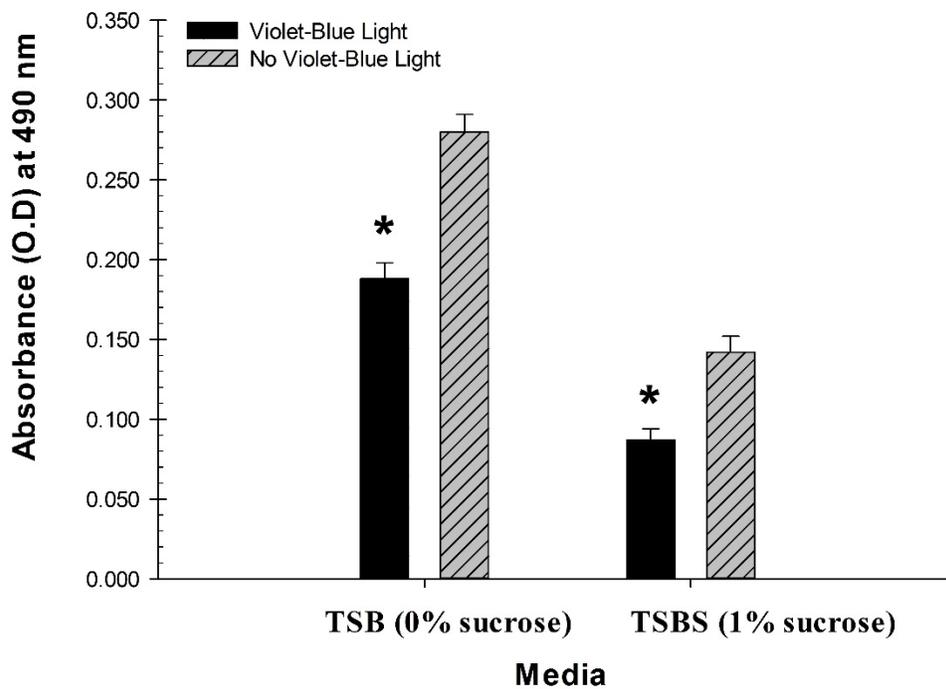


Fig. 3. The effect of Violet-Blue light for TSB-grown *S. mutans* at 2 h had significantly lower metabolic activity in the light-treated group compared with the non-treated group ($p < 0.0001$). Metabolic activity of *S. mutans* grown in TSBS (1% sucrose) was significantly lower in the treated than the 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.

Metabolic Activity of *S. mutans* Biofilm Treated with Violet-Blue Light (6 h reincubation)

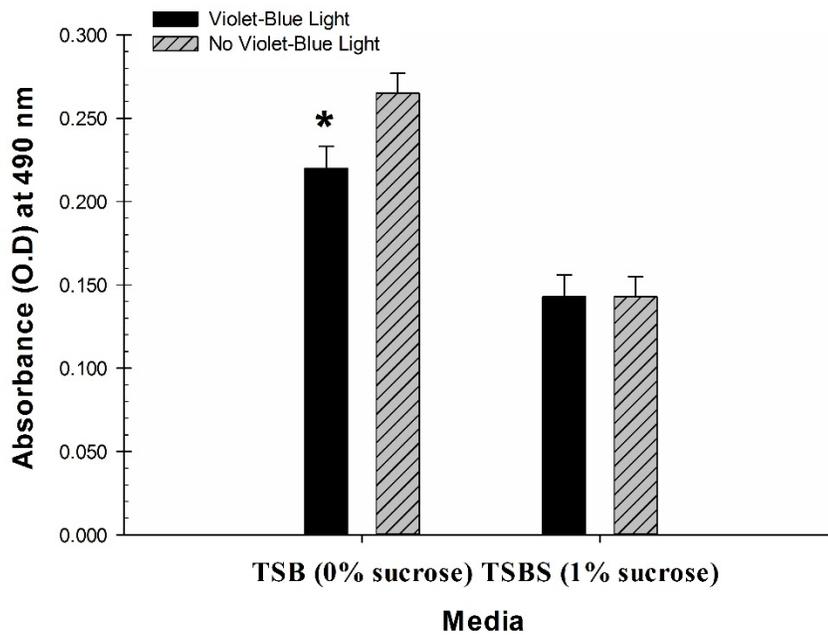


Fig. 4. The effect of Violet-Blue light for TSB-grown *S. mutans* at 6 h had significantly lower metabolic activity in the light-treated group compared with the non-treated group ($p < 0.0001$).

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.

