J Osaka Dent Univ 2023 (April); **57 (1)**: 131−136.

Effect of blue LED irradiation on the cariogenic bacteria *Streptococcus mutans* and gingival epithelial cells

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We investigated the effect of blue light-emitting diode (LED) irradiation on the cariogenic bacteria *Streptococcus mutans* (*S. mutans*) and the gingival epithelial cell line GE-1 cells. Light-emitting diodes with a wavelength of 465 ± 10 nm were used to irradiate the *S. mutans* and GE-1 cells, and the cell viability was measured. The induction of the antioxidant enzyme heme oxygenase-1 was evaluated using quantitative reverse transcription polymerase chain reaction and western blotting. The results showed that blue LED irradiation with vitamin B partly suppressed the growth of *S. mutans,* and induced reactive oxygen species in GE-1 cells, thereby reducing cell viability. (J Osaka Dent Univ 2023; 57: 131-136)

Key words: Blue LED light; *Streptococcus mutans*; Gingival epithelial cells; Oxidative stress

INTRODUCTION

Owing to the increasing number of antibiotic-resistant bacteria, researchers have proposed antimicrobial therapies based on photodynamic therapy (PDT), which has a low risk of causing the emergence of these resistant bacteria.[1] Different from most clinical studies that chose red light (approximately 630 nm) as the excitation source to obtain a deep penetration depth, we selected blue light which has been applied in the treatment of tooth bleaching and the restoration of teeth with resin-based composite fillings.[2] This was done because of its shallow penetration depth that avoids damage to deep healthy tissues.

Our previous study reported that violet light-emitting diode (LED) irradiation suppresses the growth of the periodontal disease bacterium *Fusobacterium nucleatum.*[3] However, it has been reported that reactive oxygen species (ROS) are generated when a photosensitizer called riboflavin (vitamin B) is irradiated with blue LED light, and that the ROS have bactericidal effects.[4, 5] Therefore, in this study we examined the effect of blue LED irradiation and induced ROS on the cariogenic bacterium *Streptococcus mutans* and the gingival epithelial cell line GE-1.

MATERIALS AND METHODS

LED light source

Blue light was applied using an LED irradiator G-Light Prima-Ⅱ Plus (GC, Tokyo, Japan) with a wavelength of 465 ± 10 nm at mode 20. The distance from the LED module to the cells was set to 2.5 cm prior to each experiment to ensure that the irradiated area was the same as that of the 12-well plate (Iwaki, Ibaragi, Japan). The LED output power was measured using an Astral AI310 handheld analog meter (Scientech, Boulder, CO, USA), and the corresponding irradiation time was calculated using the following equation:

Power Density = Power ÷ Area

Irradiation Dose = Power Density × Time

S. mutans growth conditions

S. mutans ATCC 25175 was cultured in brain heart

infusion (BHI) broth (BD BBL, Sparks, MD, USA) with added agar (1.5%) (Wako Pure Chemical Industries, Osaka, Japan) at 37°C under aerobic conditions.

Bactericidal viability assay

S. mutans single colonies were taken from the culture plate and precultured in sterile culture tubes containing 3 mL of BHI broth at 37°C and put in a bio-shaker (BR-3000 LF; Taitec, Saitama, Japan) at 120 oscillations/min for 17 h. Bacterial suspension was mixed using a vortex mixer, and aliquoted (1 mL) into two 1.5 mL micro-tubes. The tubes was centrifuged at 10,000 × g for 1 min at room temperature, and the supernatant was discarded. One sample was resuspended in phosphate-buffered saline (PBS), and the other in 0.01% vitamin B (Nacalai Tesque, Kyoto, Japan) solution. All samples were adjusted to an optical density of 0.1 at 600 nm (OD_{600}). The diluted resuspension was aliquoted (500 μL) into 12-well plates. For irradiation with blue LED, samples were divided into the control group (C), the vitamin B group (V), the LED group (L), and the vitamin B + LED group (VL). The L and VL groups were irradiated with blue LED at 0, 10, 25 and 50 J/cm^2, with irradiation times of 0, 1 min 58 s, 4 min 55 s, and 9 min 50 s, respectively. The C and V groups were left to stand still for the same amount of time. After a series of operations, 500 μL BHI broth was added to each well and mixed thoroughly by pipetting. Then the suspension was transferred into new sterile culture tubes containing 2.5 mL BHI broth, and incubated at 37°C in a bio-shaker with 120 oscillations/min for 19 h. Next, the suspension was mixed using a vortex mixer, and the optical density at 600 nm was measured.

Cell culture conditions

The mouse-derived gingival epithelial cell line, GE1, was obtained from the Riken Cell Bank, Ibaragi, Osaka, Japan. The cells were cultured in SFM-101 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 1% fetal bovine serum (FBS; Biowest, Nuaille, France), a 1% penicillin-streptomycin mixed solution (Nacalai Tesque), and 10 ng/mL murine epidermal growth factor (EGF; Wako Pure Chemical Industries) in an atmosphere of 5% CO_2 at 37°C.

Application of blue LED on cells and cell viability assay

Adherent GE1 cells were removed by adding 3 mL 0. 25 w/v% trypsin-1 mmol/L EDTA·4 Na solution (Wako Pure Chemical Industries) and incubated in an atmosphere of 5% CO_2 at 37°C for approximately 5 - 10 minutes. Then, the suspension was transferred into a 15 mL centrifuge tube, and 7 mL SFM-101 medium was added before centrifuging at 4°C and 10,000 × g for 5 min. After the supernatant was aspirated, the pelleted cells were resuspended and adjusted to 1.5×10^5 cells/mL using SFM-101 medium. The suspension was aliquoted (1 mL) into four corner wells of five 12-well plates (Iwaki) and incubated in an atmosphere of 5% CO_2 at 37°C overnight. The suspension was aliquoted (500 μL) into cell chambers and incubated under the same conditions as the cell viability assay, which was performed using a live-dead cell staining kit (Bio Vision, Milpitas, CA, USA), following the manufacturer's instructions. The spent medium in the 12-well plates was aspirated, and the wells were washed once with PBS.

The plates were divided into a control group (C) and four vitamin B + LED groups (VL). PBS (500 μL) was aliquoted into group C, and 0.01% Vitamin B PBS solution was aliquoted (500 μL) into the VL group. The VL groups were irradiated at 25 J/cm^2, corresponding to an irradiation time of 4 min 55 s. Group C was allowed to stand for the same time. Spent PBS and Vitamin B solution were aspirated, and 1 mL of SFM-101 medium was added to the wells. VL groups were incubated in an atmosphere of 5% CO_2 at 37°C for 3, 6, 9 and 12 h. Cells were scraped using a scraper and collected with spent medium into 1.5 mL micro tubes. The tubes were centrifuged at 4°C, 10,000 × g for 5 min. The supernatant was aspirated and pelleted cells were stored at -80°C for later quantitative polymerase chain reaction (qPCR) and western blot analyses.

RNA isolation and quantitative reverse transcription polymerase chain reaction analysis

Total RNA from pelleted GE1 cells was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. cDNA was obtained from 1,000 ng of total RNA using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). qPCRs were carried out as follows: 30 s at 95°C, 40 cycles of 5 s at 95°C, and 10 s at 60°C. The following primers for heme oxygenase-1 (HO-1) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were used. HO-1: 5'-GCTAGCCTGGTGCAAGATACT-3' and 5'-GCCAACAGGAAGCTGAGAGT-3'; GAPDH: 5'-GGGTCCCAGCTTAGGTTCATC-3' and 5'-CCAATACGGCCAAATCCGTTC-3'.

Western blot analysis

Pelleted GE1 cells were lysed in a cell lysis buffer (50 mM Tris-HCl containing 2% SDS, pH 6.8). The protein concentration was measured using a BCA Protein Assay Kit (Takara, Shiga, Japan). The samples were placed on 12.5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Merck Millipore, Carrigtwohill, Ireland). After incubation with Blocking One (Nacalai Tesque) for 1 hour at room temperature, the membranes were incubated with the following primary antibodies: anti-HO-1 (GTX 101147, 1:1000; GeneTex, Irvine, CA, USA) or β-actin (GTX 110564, 1:5000; GeneTex) overnight at 4°C. Subsequently, the membranes were incubated with the following secondary antibodies: goat anti-mouse IgG (1:10000; Proteintech Group, Rosemont, IL, USA) or mouse anti-rabbit IgG (1:10000; Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for 1 h. After washing the membranes with Tris-buffered saline containing Tween 20 (Santa Cruz Biotechnology), chemiluminescence was visualized using Amersham ECL Select Western Blotting Detection Reagent (Cytiva, Florence, Italy), following the manufacturer's instructions. The concentration of the proteins in all lanes was previously confirmed to be similar.

Statistical analysis

Statistical differences in the bactericidal and cell viability assays are expressed as the mean and standard deviation. Statistical differences in qPCR results were analyzed using StepOne Software v2.3 (Applied Biosystems, Foster City, CA, USA)

RESULTS

Vitamin B coupled with blue LED irradiation partly suppressed the growth of *S. mutans*

As shown in Figure 1, the optical density at 600 nm of the samples in the vitamin B + LED groups significantly decreased with irradiation doses equal to or greater than 25 J/cm^2, whereas the differences in the control group, vitamin B group, and LED group were not significant.

Vitamin B coupled with blue LED irradiation reduced the viability of gingival epithelial cells

As shown in Figure 2 A, the merged images of live-dead staining of gingival epithelial cells in the control group (C), vitamin B group (V), and LED group (VL) were green, whereas the merged images of staining of cells in the vitamin B + LED group are yellow. Figure 2 B shows that the viability of gingival epithelial cells in the vitamin B + LED group was significantly decreased compared to that in the control, vitamin B, and LED groups.

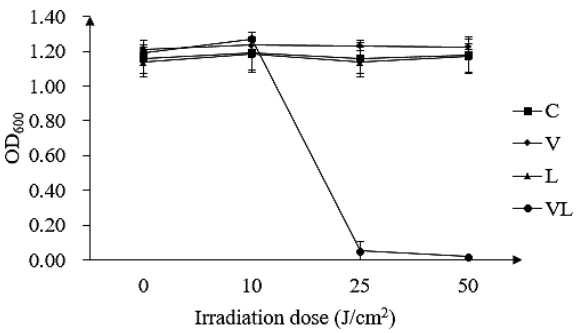

Fig. 1 Changes in bacterial suspension OD_{600} with increasing blue LED irradiation dose in different groups after 19 h of incubation. No treatment was done for the control group (C), the vitamin B group (V) was treated with vitamin B solution, the LED group (L) was irradiated with blue LED, and the vitamin B + LED group (VL) was irradiated with blue LED and treated with vitamin B solution.

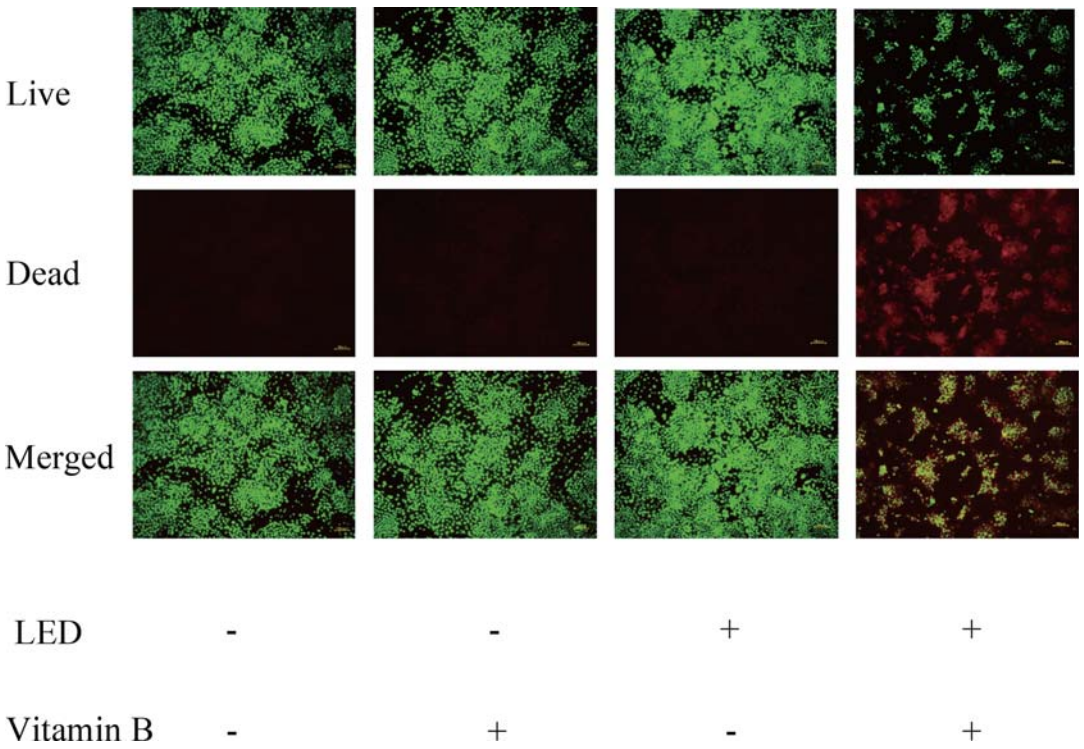

Fig. 2 A Live-dead assay of gingival epithelial cells under immunofluorescence microscopy after a series of treatments and incubation at 37°C for 24 h. These cells were treated with LED doses of 50 J/cm^2 and 0.01% vitamin B solution. Green represents live cells and red represents dead cells. The merged images are the superposition of live and dead cells.

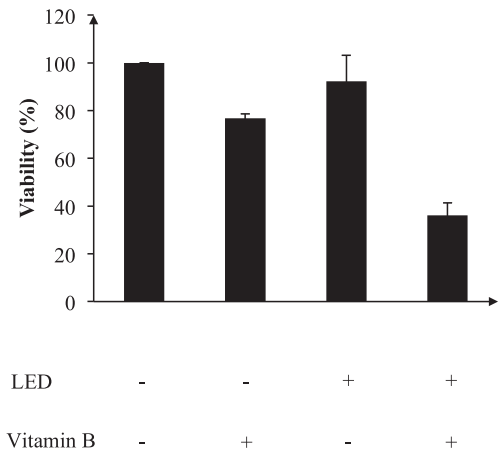

Fig. 2 B Viability of the cells for various treatments.

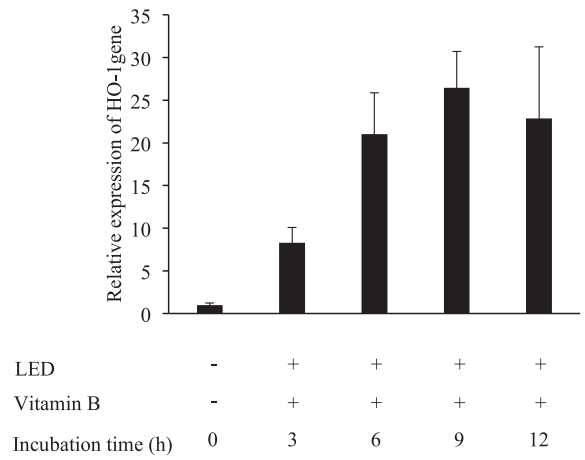

Fig. 3 The relative expression of the HO-1 gene in mouse-derived epithelial gingival cells for various treatments.

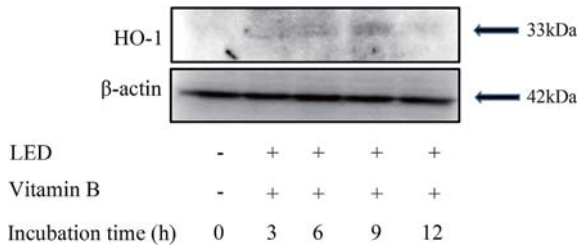

Fig. 4 Western blotting analysis of the HO-1 protein in mouse-derived gingival epithelial cells for various treatments. No treatment was done on the control (C).

Oxidative stress was induced in gingival epithelial cells using blue LED irradiation coupled with vitamin B solution.

Figures 2 A and 2 B show that 50 J/cm^2 LED-irradiation coupled with vitamin B was cytotoxic to *S. mutans* and GE-1 cells. To analyze the oxidative stress in the GE-1 cells, we examined them under the minimum power density of 25 J/cm^2. As shown in Figure 3, the relative expression of the HO-1 gene in gingival epithelial cells irradiated with 25 J/cm^2 blue LED coupled with vitamin B solution increased gradually compared with that in the untreated cells. In Figure 4, the band of HO-1 protein was found in gingival epithelial cells irradiated with 25 J/cm^2 blue LED coupled with vitamin B solution.

DISCUSSION

In this study, we designed a simple *in vitro* model to examine the effects of blue LED irradiation on *S. mutans* and GE-1 cells. A previous study had demonstrated that irradiation with violet LED at a dose of 10 J/cm^2 could significantly decrease the relative ratio of *Fusobacteriaceae* in dental plaque, and a dose greater than 25 J/cm^2 could significantly decrease the total bacterial density. This showed that violet LED irradiation may partly suppress the growth of plaque microorganisms.[3] Our results showed that even when irradiated with blue LED at a dose of 50 J/cm^2, the density of *S. mutans* did not significantly vary, which suggests a relatively strong tolerance of *S. mutans* to this approach (Fig. 1). However, riboflavin (vitamin B) has been reported to be an efficient photosensitizer that can induce oxidative damage to light-exposed tissue,[6] and riboflavin-mediated blue light photodynamic therapy in the management of periodontitis has been extensively discussed.[7] Although a laboratory investigation revealed that riboflavin-mediated PDT significantly reduced the amount of *S. mutans,* the exact irradiation density was not mentioned.[8] Thus, we decided to determine the specific irradiation density. As shown in Figure 1, a dose greater than 25 J/cm^2 led to a significant decrease in bacterial density. These results are consistent with a previous study, which reported that riboflavin-mediated LED irradiation in PBS at 25°C significantly inactivated *Listeria monocytogenes* cells at 19.2 J/cm^2, while irradiation alone gave the same result at 57.6 J/cm^2.[9] Furthermore, our results demonstrated the high efficiency of photodynamic therapy.

For the oxidative stress detection, we chose evaluating the expression level of the HO-1 gene and protein. HO-1 plays a role in defending the organism against oxidative stress-mediated injures, and its gene expression in response to various stresses.[10, 11, 12] Our results showed that oxidative stress was induced by a 25 J/cm^2 dose of blue LED irradiation coupled with vitamin B (Figs. 3 and 4).

Although this study focused on the irradiation dose of 25 J/cm^2, a dose of 50 J/cm^2 was also investigated initially (Figs. 2 A and 2 B). Although the results showed that vitamin B with a dose of 50 J/cm^2 blue LED irradiation reduced the viability of gingival epithelial cells, the results of western blot assays were not satisfactory for the very low concentration of extracted protein. In addition, the gene expression of HO-1 in qPCR also did not show significant variation. Several studies have shown that HO-1 is always found to be associated with the regulator nuclear factor erythroid 2-related factor (Nrf2) and the Nrf2-Keap 1 pathway.[13, 14] It has also been reported that different doses of curcumin may have opposite effects on ROS levels and the translocation of Nrf2 to the nucleus to upregulate the expression of HO-1.[14] Curcumin and riboflavin are both used as photosensitizers, and findings have indicated that more *in-vitro* and clinical trial studies are needed to find the best protocol, including suitable concentrations of riboflavin accompa-

nied by appropriate laser parameters.[7] Taking this into consideration, our qPCR and western blotting results for the irradiation of 50 J/cm^2 suggested that 50 J/cm^2 is not an appropriate parameter to induce HO-1. Although riboflavin served as a pro-oxidant in this study, previous research has shown that it can alleviate oxidative stress.[15] A possible reason for the pro-oxidative effect of riboflavin in this study could be that the different irradiation densities caused different riboflavin degradation rates, and that the different ratios of pro-oxidants to antioxidants affected HO-1 induction. This is consistent with the concept of oxidative stress, which has been shown to result from an imbalance between the production of free radicals and the ability of antioxidant defense mechanisms to deactivate them.[16]

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