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Effects of photosensitizers in a high-power, red light-emitting diode irradiation on human gingival epithelial cells

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Antimicrobial photodynamic therapy has been used in clinics to minimize the susceptibility to bacterial resistance in patients with periodontal disease. It has been proven to be effective in treating bacterial, fungal, and viral infections. However, it is hard to ignore the effects of photosensitizers on the body. We investigated the effects of two typical photosensitizers, and assessed the proliferation of human gingival epithelial cells (HGECs) and the production of inflammatory cytokines after treatment with a high-power, red light-emitting diode (LED) along with photosensitizers. We found that methylene blue promotes proliferation of HGECs, whereas toluidine blue does not. The production of the inflammatory cytokines from HGECs decreases after LED irradiation. Hence, this study suggests that LED irradiation in the presence of either of the photosensitizers reduces inflammation in dental tissues such as the epithelium of periodontal pockets. (J Osaka Dent Univ 2019 ; 53 : 109-113)

Key words : Antimicrobial ; Photodynamic therapy ; Photosensitizer ; Epithelial cells

INTRODUCTION

Light has a variety of effects[1-8] on the human body, and phototherapy, which is frequently used to complement medical treatment, has attracted attention in recent years. Phototherapy was first reported over a century ago after its first application by Niels Finsen[1] for the treatment of dermatological disorders. It induces several effects, including cell proliferation,[2] wound healing,[3] pain relief,[4] and anti-inflammatory responses.[5] Previous studies in the field of dental research have also shown the potential beneficial effects of phototherapy in the treatment of oral mucositis,[6] candidosis,[7] and dentin hypersensitivity.[8] In periodontal disease, antimicrobial photodynamic therapy (a-PDT)[9] has been used in clinics to minimize the patient's susceptibility to bacterial resistance. a-PDT has been proven effective in treating bacterial, fungal and viral infections.[10] One of the major benefits of a-PDT is that it is not influenced by drug resistance to antimicrobial agents by the non-peculiar bactericidal mechanism. It is effective against bacillus resistant to antimicrobial agents, and can eliminate resistant microbes efficiently. Therefore, a-PDT is considered safe for long term use.

a-PDT combines a photosensitizer with a light source to induce production of reactive oxygen species in order to kill periodontal bacterium. Previous studies have reported the beneficial effects of a-PDT light at various wavelengths such as 630 nm,[11] and 670 nm.[12] Its antimicrobial effect has been shown *in vitro*[9] and in clinical[13] studies using a 650 nm high-power, red light-emitting diode (LED). The effect of photosensitizers on the cell wall of the bacillus depends on the charge of the sensitizer. Gram-negative bacteria such as periodontopathic bacteria have strong affinity towards positively charged photosensitizers. Hass *et al.*[14] reported that the bacillus in the biofilm formed on the surfaces of implants is completely eradicated when the diode light is irradiated after spreading positively charged phenothiazium dyes on the affected area. In contrast, there are a few studies reporting the effects of photosensitizers on the human body. In particular, the effect of photosensitizers used for

LED irradiation on human gingival epithelial cells (HGECs) is not yet clear. The aim of this study was to investigate the effect of two typical photosensitizers, methylene blue and toluidine blue, which are used for high-power, red LED irradiation. We investigated their effect on cell proliferation of HGECs as well as the production of inflammatory cytokines from these cells.

MATERIALS AND METHODS

Cell culture

The SV40-antigen-immortalized gingival epithelial cell line, Epi4 was kindly provided by Prof. Shinya Murakami of the Osaka University Graduate School of Dentistry, Osaka, Japan. Epi4 were seeded in T-75 flasks (Falcon ; Becton Dickinson Labware, Franklin Lakes, NJ, USA) and maintained in Humedia-KG2 medium (Kurabo, Osaka, Japan) containing 10 g/mL insulin, 0.1 ng/mL hEGF, 0.67 μg/mL hydrocortisone hemisuccinate, 50 μg/mL gentamycin, 0.4% BPE, and 50 ng/mL amphotericin B under 5% CO_2/95% air at 37℃. When the cells reached sub-confluence, they were harvested and subcultured.

LED irradiation

A portable, red LED prototype emitter with a peak wavelength of 650 nm, supplying a power of 5 W was constructed in a very small package. The red LED was a LZ1-00R205 Deep Red LED (Led Engin, Santa Clara, CA, USA), which emits specific red wavelengths (600-700 nm), with a peak wavelength of 650 nm and a power density of 1100 mW/cm^2. The light intensity of the emitter was confirmed by a power meter (Nova II ; Ophir, North Andover, MA, USA). The LED emitter was used to irradiate samples at a distance of approximately 22 mm for either 10 or 20 sec.

Cell Proliferation

HGECs were plated in normal culture medium (100 μL/well) at a density of 5×10^4 cells/mL into the wells of 24-well cell culture plates (Becton Dickinson Labware) that could be separated. In previous studies,[21] cells were seeded in disassembled wells of cell culture plates, and each well was irradiated separately. After 24 h incubation, the medium was replaced with fresh medium and the wells were irradiated. Cells were allowed to recover for 3, 24 or 72 h. Proliferation was assessed using a mixture of 50 μL of CellTiter-Blue Reagent (Promega, Madison, WI, USA) and 250 μL of PBS (Nacalai Tesque, Kyoto, Japan) added to each well. After incubation at 37℃ for 1 h, the solution was removed from the 24-well tissue culture plates (Becton Dickinson Labware) and 100 μL was added to a new 96-well tissue culture plate. The OD560/590 of the remaining solution was measured using a multi-microtiter reader. The difference between the two optical densities was defined as the proliferation value.

Photosensitizers

Methylene blue (MB ; Nacalai Tesque) powder and toluidine blue O (TBO ; Nacalai Tesque) powder, which has a maximum absorption of 626 nm were dissolved at concentrations of 0.01, 0.1 and 1 μg/mL in sterile saline solution.

Production of inflammatory cytokines using photosensitizer

HGECs were cultured at a density of 5×10^4 cells/mL in 24-well cell culture plates using Humedia-KG 2 medium containing the optimal concentrations of both photosensitizers. We analyzed the supernatants from each culture of HGECs quantitatively using interleukin 8 (IL-8) and interleukin 6 (IL-6) ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions.

Statistical analysis

Data was analyzed using SPSS 19.0 software (SPSS IBM, Armonk, NY, USA). All experiments were performed in triplicate. The data is shown as the mean and standard deviation (SD). In all analyses, statistical significance was determined using one-way analysis of variance (ANOVA) followed by a Fisher's least significant difference test. Values of $p<0.05$ were considered significant.

RESULTS

Optimal LED irradiation

Figure 1 shows that the cell proliferation of HGECs is enhanced in LED irradiated samples compared to the non-irradiated ones. We observed better proliferation of HGECs subjected to 8 J/cm^2 irradiation than 4 J/cm^2 irradiation after 24 h culture. However, the difference in cell proliferation was not significant after 72 h. Therefore, a LED dose of 4 J/cm^2 was chosen for the subsequent assays.

Proliferation of HGECs stimulated with the photosensitizers

Figure 2 shows that the cell proliferation of HGECs was promoted in the MB group in a dose dependent manner. However, the TBO group showed the opposite effect. The proliferation of HGECs treated with MB alone was greater than that with TBO.

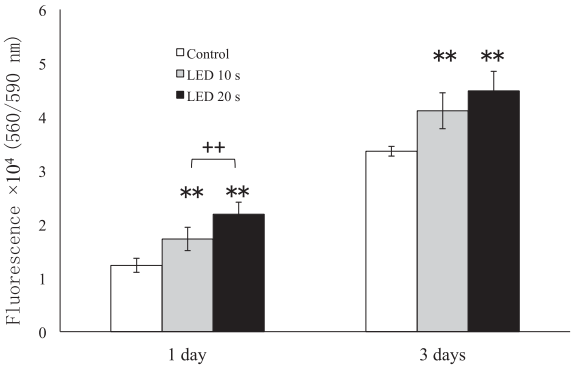

Fig. 1 HGEC proliferation for 1 and 3 days after LED irradiation (**p＜0.01 vs. Control, ＋＋p＜0.01 vs. LED 20s).

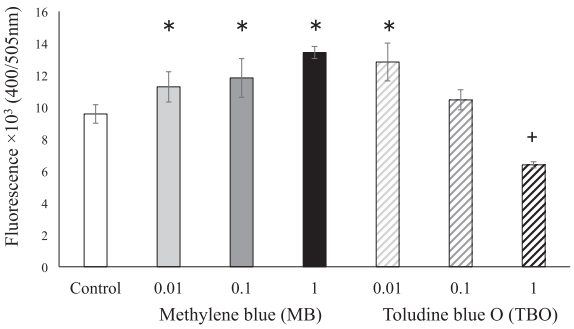

Fig. 2 HGEC proliferation for 24 h after LED irradiation including photosensitizers (*p＜0.05 Upregulate vs. Control, ＋p＜0.05 Downregulate vs. Control).

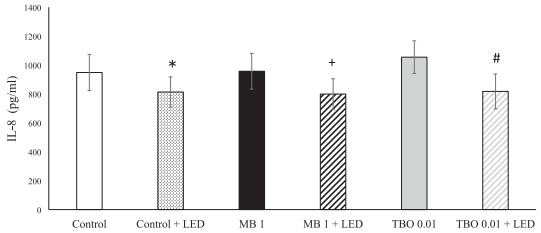

Fig. 3 IL-8 production from HGECs for 24 h after LED irradiation including photosensitizers (*p＜0.05 vs. Control, ＋p＜0.05 vs. MB 1 μg/mL, #p＜0.05 vs. TBO 0.01 μg/mL).

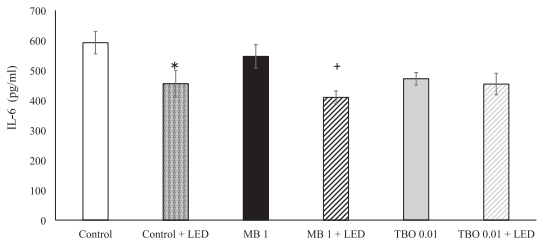

Fig. 4 IL-6 production from HGECs for 24 h after LED irradiation including photosensitizers (*p＜0.05 vs. Control, ＋p＜0.05 vs. MB 1 μg/mL).

Production of inflammatory cytokines stimulated by photosensitizers and LED irradiation

Figure 3 shows that production of IL-8 significantly decreased after LED irradiation with both photosensitizers compared to the control. Figure 4 shows a significant reduction in the production of IL-6 after LED irradiation with MB compared to the control, although the TBO group did not show any major difference.

DISCUSSION

Development of LED has provided a-PDT technology with a new light source. LED devices have the advantage of being significantly less expensive and much safer than lasers. In addition, they can be used to perform much more extensive irradiation compared with current laser devices. However, it is necessary to use some photosensitizers before LED irradiation during a-PDT. This process utilizes three non-toxic components, including a harmless visible light, a non-toxic photosensitizer, and generated singlet oxygen. The inactivation of microbial growth with PDT has been variously termed antimicrobial PDT, photodynamic antimicrobial chemo-

therapy, photodynamic disinfection, and lethal photosensitization.[15] Originally, PDT was developed as a treatment method against cancer cells. It has also been used to eradicate the bacillus causing periodontitis. However, at the same time, questions have arisen regarding the effects of this technology on the body, especially on the epithelial cells of periodontal pockets.

In this study, we examined the influence of irradiating LED after treating HGECs with photosensitizers in the absence of periodontopathic bacteria. Generally, cell proliferation occurs when light is irradiated on the cell. The high-powered red LED used in this study tends to elicit a similar response, as shown in Fig 1. Although there are few reports on the effect of red LED irradiation on HGECs, one previous study reported that red light irradiation activated cytochrome C oxidase, an enzyme within the mitochondria of eukaryotes.[16] This enables the resumption of respiratory chain activity and ATP synthesis,[17] suggesting that an increase in the ATP concentration in HGECs induces intracellular signaling to promote proliferation.

Secondly, this study found that not all photosensitizers can promote cell proliferation of HGECs. Fig. 2 shows that although MB promotes cell proliferation of HGECs, TBO does not. Ichinose-Tsuno *et al.*[13] reported that TBO decreased the cell proliferation of gingival fibroblasts regardless of LED irradiation. Hence, this study as well as previous reports suggest that TBO might be cytotoxic.

Fig. 3 and 4 shows that the productions of the inflammatory cytokines from HGECs decreases after LED irradiation. Fujimura *et al.*[18] reported that irradiation with a low-level diode laser reduces the levels of inflammatory cytokines in epithelial cells. This study suggests that using irradiating LED in the presence of both photosensitizers reduces the inflammation in the epithelium of periodontal pockets. In conclusion, the present study demonstrated that high-powered, red LED irradiation increases the proliferation of HGECs and decreases inflammatory responses when used along with photosensitizers such as MB and TBO.

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