The Effect of 630 nm Photobiomodulation on the Anti-Inflammatory Effect of Human Gingival Fibroblasts

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1. Introduction

Periodontal disease is the major cause of adult tooth loss and is commonly characterized by a chronic inflammation caused by infection of oral bacteria. Porphyromonas gingivalis (Pg.) is one of the suspected periodontopathic bacteria and is frequently isolated from the periodontal pockets of patients with chronic periodontal disease. The lipopolysaccharide of Porphyromonas gingivalis (Pg. LPS) is a key factor in the development of periodontitis. Human gingival fibroblasts (HGFs) are the major constituents of gingival connective tissue, and it may directly interact with bacteria and bacterial products (including LPS) in periodontitis lesions. It is suggested that HGFs play an important role in the host responses to LPS in periodontal disease [1]. LPS can enhance the production of inflammatory cytokines such as PGE2, interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF-alpha) in HGFs. Inflammatory cytokines are involved in the development of periodontitis, which promotes the release of large amounts of inflammatory cytokines such as PGE2 and IL-8 [2,3]. In addition, several experiments have shown that LPS promotes the release of PGE2 and IL-1β in HGFs [4,5].

Photobiomodulation (PBM) is the use of visible light and near-infrared to treat cells by generating therapeutic effects, such as reducing inflammation and stimulating healing [6].
Amaroli et al. reviewed that PBM is mediated by ATP, ROS, and/or calcium, resulting in cells being manipulated and thus altering their metabolism; they showed that PBM is widely applicable to all forms of life from bacteria to vertebrates [7]. At present, the most common PBM light source is the Light-Emitting Diode (LED) light source. LED has the advantages of energy-saving, high efficiency, small size, safety and stability, long life, wide spectral range, and easy control, etc. PBM has become an important therapy or clinical exploration direction in dermatology, biological rhythm, bone and joint diseases, tumor treatment, stomatology, and other fields [8,9].

The basic parameters of PBM treatment include wavelength, intensity and dose, and different doses of irradiation may have different inhibitory effects on inflammation. Sharma S K et al. irradiated mouse primary cortical neurons with 810 nm PBM (the light intensity was 25 mW/cm²) with various doses at 0.03, 0.3, 3, 10, and 30 J/cm². We found that laser irradiation gradually increased the ROS level, mitochondrial membrane potential (MMP), and Adenosine-triphosphate (ATP) production in mouse neurons with the increase in light energy, and energy enhancement began to have negative effects on the production of intracellular ROS and MMP when the dose exceeded 3 J/cm² [10]. The main target of PBM is mitochondria [11]. Cytochromes of mitochondria can participate in biological regulation as photoreceptors and also indirectly participate in improving mitochondrial and energy metabolism and nerve damage through vibration and energy changes of bound water [12]. Amaroli et al. found that photobiological regulation with 808 nm laser selectively stimulated complexes III and IV of the mitochondrial respiratory chain, while complexes I and II were unaffected. At the wavelength of 1064 nm, complex I, III, and IV are excited, while Complex II and some mitochondrial stromal enzymes do not seem to accept photons at this wavelength [13].

Due to the biphasic dose–response of PBM (too high dose can cause the opposite therapeutic effect) and there being few studies on different PBM doses on the inflammation in HGFs [14], it is very necessary to find out the irradiation dose suitable for the treatment of periodontitis, which will also provide a useful reference for clinical applications. Studies about molecular biological mechanisms have proved that 630 nm PBM can promote cell proliferation, reduce inflammation, and promote wound healing [15–17]. Periodontitis experiments in rats have also demonstrated that 660 nm PBM could promote wound healing and accelerate wound healing [18]. Experiments on HGFs in vitro also showed that 635 nm PBM could reduce the release of PGE2 and achieve an anti-inflammatory effect [19]. However, these studies did not explore different doses of PBM on periodontitis.

Therefore, we used LPS treated gingival fibroblasts to create the periodontitis cell model in this study and used different doses of red light PBM to treat LPS treated HGFs in order to study the therapeutic effect of different doses of red light PBM on periodontitis. Our results provide a theoretical reference for PBM in the treatment of periodontitis.

2. Materials and Methods

2.1. Cell Culture and Chemicals

Human gingival fibroblasts (HGFs) were obtained from a healthy 18 year old man visiting Shanghai Oral Disease Prevention and Treatment Institute and cultured in Dulbecco’s modification of Eagle’s medium (DMEM) (Gibco, MD, USA) supplemented with 1% streptomycin and penicillin (HyClone, Logan, UT, USA) and 10% fetal bovine serum (Gibco, Logan, UT, USA). The cells were maintained in 5% CO₂ in a 37 °C humidified incubator (Qiqian, Shanghai, China). The medium was replaced with fresh medium every two days. One day before light treatment, the cells were detached using the trypsin (GibcoBRL) solution and plated in 96-well plates at a density of 5 × 10³ cells/well [6].

2.2. Light Source

The source of light used for irradiation was a continuous-wave light-emitting diode (LED) with a wavelength of 630 nm. The irradiation was performed by the LED source in a light humidified incubator (Figure 1) (LightEngin technology, Shanghai, China). In this experiment, the irradiance was 5 mW/cm² and kept constant. The light dose was changed
with the irradiation time of different groups. The cells were treated with PBM in a DMEM medium. Our previous study found that the DMEM medium did not affect cell viability after PBM treatment; thus, we chose to treat HGFS with PBM in DMEM medium [6].

Figure 1. Light humidified incubator and spectrum of the LED light source.

2.3. Experimental Conditions and Groups

According to the irradiation dose and HGFS incubated in the presence or absence of LPS, the experiment was divided into seven groups. The light doses were 1, 3, 9, 18, and 36 J/cm². The concentration of LPS was 16 µg/mL; the light intensity was 5 mW/cm². The specific experimental conditions and groups are shown in Tables 1 and 2. Irradiance technical parameters were measured by an optical power meter (Thorlabs, Newtown, CT, USA).

Table 1. PBM parameters.

| Wavelength (nm) | Average Irradiance (mW/cm²) | 630 ± 30 | 5 |
|----------------|----------------------------|---------|---|
| Time (s)       | 200 | 600 | 1800 | 3600 | 7200 |
| Dose (J/cm²)   | 1   | 3   | 9    | 18   | 36   |

Dose (J/cm²) = Irradiance (mW/cm²) × Time of irradiation (s)/1000.

Table 2. Experimental conditions and groups.

| Conditions/Groups | with or without LPS |
|-------------------|---------------------|
| Control group     | (+)                 |
| LPS group         | (+)                 |
| 5 mW/cm², 1 J/cm² | (+)                 |
| 5 mW/cm², 3 J/cm² | (+)                 |
| 5 mW/cm², 9 J/cm² | (+)                 |
| 5 mW/cm², 18 J/cm²| (+)                 |
| 5 mW/cm², 36 J/cm²| (+)                 |

2.4. Enzyme-Linked Immunoassay for PGE2 and IL-8

After HGFS were incubated in the presence or absence of LPS and PBM for 24 h, PGE2 in the supernatant was measured using an enzyme-linked immunosorbent kit (R&D System, MN, USA). IL-8 in the supernatant was measured by using an enzyme-linked immunosorbent kit (Cloud-clone corp, Houston, TX, USA). Absorbance for PGE2 and IL-8 was measured at 450 nm using a microplate reader (Thermo Scientific, Waltham, MA, USA).
2.5. Cell Viability

Cell viability was tested by CCK-8 assay (Dojindo, Shanghai, China). The human gingival fibroblasts (HGFs) were seeded in 96-well plates (Corning Incorporated, Corning City, NY, USA). After 24 h, the 96-well plates were treated with LPS and PBM treatment (cells were first treated with LPS and then treated with PBM), and the cells were subsequently incubated at 37 °C for 24 h. Cell viability was tested with 10 µL/well CCK-8 solutions and 100 µL/well medium; then, the cells were incubated for 1.5 h in a humidified incubator. Ultimately, the absorbance at 450 nm was measured by a microplate reader (Thermo Scientific, Waltham, MA, USA).

2.6. Detection of Intracellular ROS

The level of intracellular ROS was detected by using the fluorescence probe (2,7-Dichlorodihydrofluorescein diacetate, DCFH-DA) (Beyotime, Shanghai, China). Cells were first laid in 96-well plates and treated with PBM 24 h later. A DCFH-DA probe was added immediately after PBM treatment and then incubated with 20 µM/mL DCFH-DA in a serum-free medium at 37 °C for 30 min. The cells were washed with serum-free medium and observed with a fluorescence microscope at an emission wavelength of 500 nm (Olympus BX53, Tokyo, Japan).

2.7. Measurement of Mitochondrial Membrane Potential (mt.∆Ψ)

A mitochondrial membrane potential kit with JC-1 (Jiancheng, Nanjing, China) was used to detect the mt.∆Ψ. 24 h after the Human gingival fibroblasts (HGFs) were treated by LPS and irradiation and then incubated with JC-1 fluorescent probe at 37 °C for 20 min. The cells were washed three times with JC-1(1X) buffer and observed with a fluorescence microscope at an emission wavelength of 510 and 580 nm (Olympus BX53, Tokyo, Japan).

2.8. Temperature Measurement

Since the maximum irradiation dose was 36 J/cm², the temperature of the cell medium may change during irradiation. The temperature changes of the cell medium during irradiation were measured by a thermal resistance thermometer (Shimaden, Tokyo, Japan).

2.9. Statistical Analysis

All experiments were performed in at least three biological replicates and three independent experiments. Data were expressed as means ± SD. The results were analyzed by using ANOVA analysis of variance by Graphpad Prism, and p < 0.05 was designated to be significant.

3. Results

3.1. Determination of LPS Concentration

In this experiment, Pg.LPS was used to stimulate the inflammatory response of HGFs. A high concentration of LPS may have a significant effect on the cell viability of HGFs, however, a low concentration of LPS could not significantly stimulate the inflammatory response of HGFs. HGFs were cultured in medium containing various concentrations of LPS for 24 h. The concentrations of HGFs were 0, 4, 8, 16, 32, 64, and 128 µg/mL. Then, the HGFs' cell viability was tested by a CCK-8 assay. The experimental results are shown in Figure 2. When the concentration of LPS was over 32 µg/mL, the cell viability of HGFs was decreased significantly (p < 0.05). The amount of 16 µg/mL LPS did not have a significant effect on the cell viability of HGFs (p > 0.05). Therefore, 16 µg/mL LPS was chosen to stimulate the inflammatory response of HGFs for further study.
Figure 2. HGFs were cultured in a medium containing LPS for 24 h. Over 32 µg/mL LPS could reduce the cell viability (p < 0.05), 16 µg/mL LPS had no significant effect on the cell viability. * p < 0.05.

3.2. The Effects of PBM on the Production of PGE2 and IL-8

After HGFs were incubated in the presence or absence of LPS and PBM for 24 h, the concentration of PGE2 and IL-8 in cell supernatant was measured. Figure 3A shows the results compared with the control group. The cells in the LPS group had a significant increase in the production of PGE2 under the stimulation of LPS (p < 0.001). The PBM treatment with a dose of 3, 9, 18, and 36 J/cm² could inhibit the production of PGE2 in HGFs stimulated by LPS (p < 0.01). The irradiation with dose of 1, 3, and 9 J/cm² had no significant effect on inhibiting the production of IL-8 in HGFs (p > 0.05).

Figure 3. LPS could promote the production of PGE2 and IL-8 in HGFs, and a certain dose of PBM treatment could inhibit the production of PGE2 and IL-8 in inflammatory HGFs. (A) The effect of various doses of PBM on the production of PGE2 in HGFs. (B) The effect of various doses of PBM on the production of PGE2 in HGFs. ** p < 0.01; *** p < 0.001.

3.3. The Effects of PBM on ROS Production

Many researchers have found that the production of intracellular ROS could be inhibited by some medicinal herbs, such as resveratrol, prunella Vulgaris extract, and rosmarinic acid [20,21]. However, few researchers have studied the effect of PBM with various doses on the production of ROS in inflammatory cells. Therefore, we detected the intracellular ROS after HGFs were incubated in the presence or absence of LPS and PBM for 24 h. Figure 4 shows that LPS increased the intracellular ROS level in HGFs. PBM with doses of 3, 9, 18, and 36 J/cm² could inhibit the production of ROS in HGFs induced by LPS. The dose of 1 J/cm² PBM had no significant effect on the production of intracellular ROS.
Figure 4. The effects of irradiation with doses of 1, 3, 9, 18, and 36 J/cm\(^2\) on ROS production in HGFs induced by LPS. (A) Immunofluorescence of cellular reactive oxygen species. (B) Statistical analysis of the average fluorescence intensity of reactive oxygen species. * \(p < 0.05\); ** \(p < 0.01\).

3.4. Temperature Changed

Figure 5 shows the results under the condition of light intensity (5 mW/cm\(^2\)). As the light dose increased, the temperature in the medium increased slightly and remained at about 37.4 °C ± 0.4 °C, the temperature changed within 1 °C. Our previous studies have demonstrated that the effect of light on cells is not caused by changing the temperature of the culture medium [19]. This result also demonstrated that the inhibitory effect of PBM treatment on the inflammation of human gingival fibroblasts was independent of changing temperature.

Figure 5. In vitro cell culture temperature.

3.5. Effects of Various Concentrations of LPS on the Production of ROS and PGE2

Figure 6 showed that the higher the concentration of LPS, the stronger the promotion effect on the production of ROS and PGE2 in HGFs. When the concentration of LPS was 16 µg/mL, LPS could significantly increase the level of intracellular ROS and the concentration of PGE2 in the supernatant, which meant that 16 µg/mL LPS could cause inflammation in HGFs.
3.6. Effects of PBM with Various Doses on Cell Viability and Mitochondrial Membrane Potential

After HGFs were incubated in the presence or absence of LPS and PBM for 24 h, cell viability and mitochondria membrane potential were measured. Figure 7 shows that when HGFs was treated with 16 µg/mL, PBM with dose of 1, 3, 9, 18, and 36 J/cm² had no significant effect on cell viability and mitochondrial membrane potential, which meant that LPS and irradiation with various doses would not result in mitochondrial dysfunction.

4. Discussion

Periodontal disease is the most common oral chronic inflammatory disease in humans. Studies have found that the plaque microorganisms that cause periodontal disease are mainly anaerobic bacteria, such as treponema tartaricum and porphyromonas gingivalis [22]. However, high concentrations of LPS inhibit hepatocyte growth factor activity. Fu et al. used different concentrations of lipopolysaccharide to treat hepatocytes and found that high doses of lipopolysaccharide had a significant inhibitory effect on hepatocyte growth factor cell viability [23,24]. Therefore, we used various concentrations of LPS in this experiment (0, 4, 8, 16, 32, 64, and 128 µg/mL) to treat HGFs for 24 h and then detected the cell viability of HGFs. We found that LPS could inhibit the cell viability of HGFs (p < 0.05) when the LPS concentrations were 32, 64, and 128 µg/mL, and LPS had no significant effect on the cell viability of HGFs (p > 0.05) when the concentrations of LPS were 4, 8, and 16 µg/mL.

Then, we studied the effect of various concentrations of LPS (0, 4, 16, and128 µg/mL) on the production of ROS and PGE2 in HGFs. Figure 6 shows that the higher the concentration of LPS, the greater the promoting effect on the production of ROS and PGE2 in HGFs. Scherz-Shouval et al. found that reactive ROS levels of HGFs increased after stim-
ulation by LPS, resulting in a decrease in MMP, and high ROS would activate the NF-κB signaling pathway, which ultimately resulted in autophagy and decreased proliferation and cell viability of HGFs [25]. We found that 16 µg/mL LPS could significantly promote the production of PGE2 in HGFs without significantly inhibiting cell viability. Therefore, 16 µg/mL LPS was selected to induce HGFs to simulate cell inflammation in vitro.

PGE2 is a product of intracellular arachidonic acid metabolism. Excessive PGE2 will cause inflammatory reactions such as pain, fever, vasodilation, etc. PGE2 is closely related to periodontitis and alveolar bone resorption [26]. In this study, we used 16 µg/mL LPS and irradiation with doses of 1, 3, 9, 18, and 36 J/cm² to treat HGFs. It was found that LPS could induce HGFs to produce more PGE2 and IL-8. Furthermore, a certain dose of 630 nm PBM could inhibit the production of PGE2 and IL-8 in HGFs. Interestingly, irradiation with a dose of 3 J/cm² and 9 J/cm² could significantly inhibit the production of PGE2 but had no obvious inhibitory effect on the production of IL-8. However, the PBM treatment with dose of 1 J/cm² had no significant effect on the production of PGE2; the dose of the irradiation might have been too low to induce an inhibitory effect. Figure 3B shows that the PBM treatment with dose of 18 and 36 J/cm² could inhibit the production of IL-8 in HGFs stimulated by LPS. Serrage et al. found that 450 nm blue light PBM could significantly inhibit the TGFβ1 secretion of HGFs [27]. The results indicated that red and blue light PBM can inhibit periodontitis. Therefore, perhaps the production of PGE2 and IL-8 are both regulated by 630 nm PBM in inflammatory HGFs, but the production of PGE2 is more sensitive to the dose.

ROS is the normal metabolite of redox reactions in the cell, mainly coming from the mitochondrial respiratory chain, ROS is related to mitochondrial oxidative phosphorylation, apoptosis, immunity, and inflammation [25–30]. In this study, HGFs were stimulated by 16 µg/mL LPS, the production of intracellular ROS increased. In addition, the PBM with doses of 3, 9, 18, and 36 J/cm² could inhibit the production of ROS. We found that the sensitivity of ROS generation to the dose of irradiation is similar to that of PGE2. Many research results show that the level of intracellular ROS increase in HGFs induced by LPS and ROS could promote the expression of the COX-2 enzyme, which in turn promotes the oxidation of arachidonic acid in order to produce PGE2 [31–33]. Therefore, we infer that LED PBM could inhibit inflammation by inhibiting the level of intracellular ROS.

There was a study finding that low-dose LED PBM could significantly stimulate tissues and cells such as: accelerating wound healing, promoting cell proliferation and differentiation, etc. [28]. Wang et al. found that 660 nm red light (3.5 mW/cm²) irradiation with dose of 20 J/cm² could significantly promote the proliferation of rat gingival fibroblasts [26]. However, in this study, we found that 630 nm PBM with doses of 1, 3, 9, 18, and 36 J/cm² had no significant effect on cell viability of HGFs (Figure 7A), which demonstrated that the inhibition of cell inflammation by PBM had nothing to do with changing cell viability. Chang et al. also found that there was no significant change in cellular proliferation in the PBM group (660 nm at energy densities of 5, 10, and 15 J/cm²) [11]. Mitochondria are the main sites of intracellular ROS production. High levels of ROS will damage the mitochondrial membrane, reducing the respiration efficiency of mitochondria and the production of intracellular ATP. It will then produce more ROS through a series of reactions, which ultimately results in a decrease in MMP. When the MMP is low, JC-1 mainly exists as monomer with green-fluorescent. When the MMP is high, JC-1 mainly exists as aggregates with red-fluorescent; thus, the mitochondrial membrane potential can be measured by the relative ratio of red-green fluorescence [6]. Mitochondrial function is impaired, which is an early marker event of apoptosis [11,13,20]. In this study, we had studied the effects of different doses of PBM on the MMP of LPS-treated HGFs. Figure 7B shows that 16 µg/mL LPS and irradiation with doses of 1, 3, 9, 18, and 36 J/cm² had no significant effect on the mitochondrial membrane potential of HGFs, which means 630 nm red light PBM is a harmless method for inhibiting inflammation.
In the future, we can co-culture multiple cells with human fibroblasts in order to study the effect of different cells on inflammatory response under PBM treatment. Our work is only an in vitro study, which needs to be validated by in vivo experiments in the future. We believe that our study provides a reference and theoretical basis for the clinical use of PBM therapy in the treatment of periodontitis.

5. Conclusions

Our findings demonstrated that a certain dose of 630 nm red light PBM could inhibit the inflammatory response in HGFs induced by LPS, which was reflected in the inhibition of the production of PGE2 and IL-8. Moreover, the production of PGE2 regulated by PBM was more sensitive to light dose than that of IL-8. We also discovered that inhibition of inflammatory response in HGFs by PBM is related to the reduction in intracellular ROS, not to changing cell viability. This may be because the high levels of ROS can promote the expression of the COX-2 enzyme, which could promote cells to produce more PGE2 and IL-8. Furthermore, it was found that PBM (630 nm) of various doses could not reduce cell mitochondrial membrane potential and cause mitochondrial dysfunction, which suggested that 630 nm red light PBM is a harmless method for clinical treatment of periodontal inflammation.

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