**Abstract**

Although the anti-inflammatory effects have been reported in clinical fields for specific wavelength irradiation during wound healing, the physiological mechanism has not yet been clarified. The aim of the present study is to investigate the anti-inflammatory effect of Light-Emitting-Diode (LED) irradiation. The present study was designed to determine the effect of LED irradiation on TNF-α, IL-1β and IL-6 detection and NO production in Lipopolysaccharide (LPS) - stimulated RAW264.7 macrophages. Cell toxicity was determined by MTS assay. The amount of NO was measured using the NO Detection Kit and the ELISA was performed by coating 96-well plates of monoclonal antibody with specificity for TNF-α and IL-6 respectively. 440nm LED irradiation reduced TNF-α and IL-6 detection and NO production without cytotoxicity. Our results suggest that 440nm LED irradiation may have an anti-inflammatory property through suppressing inflammatory mediator productions and appears to be useful as an anti-inflammatory tool.

**Keywords:** Inflammation, LED (Light Emitting Diode), Light Therapy, NO

**1. Introduction**

In curing the various diseases, a laser and LED are now applied to many people of a worldwide. LED related research has been continuously progressed and in the specific LED wavelength, it was clarified that there was an effect including wound healing acceleration\(^1\)–\(^4\), collagen proliferation\(^5\), anti-inflammation\(^6\)–\(^8\), pigmentation prevention\(^9\), and etc. In addition, it was clarified that there is an effect of the cellulite removal\(^11\),\(^12\) and depilation\(^13\). And in the recent research\(^14\), the visible ray (400-500nm) was applied for treating the patient who has the atopic eczema.

Nitric Oxide (NO) involves in an anti-cancer and not only the bacteriostasis but also pathogenesis of the various inflammatory diseases as the free radical distributed widely in the organism controlling the various biological functions including a vasodilation, smooth-muscle contraction, nerve signal transmission, inhibition of platelet aggregation, immune-modulating\(^15\),\(^16\). However, if NO is produced in more than necessity, the proceed of an inflammation and septic shock by the excessive vasodilation are induced and it engages in the suppression of the wound healing and pathogenesis of the systemic lupus erythematosus and cell apoptosis and degradation of the immune function are induced. And in some, the disease like cancer is caused\(^17\). NO is the organism generating numerator having the high reactivity and is produced by the Nitric Oxidesynthase (NOS) from L-argnine. NOS has three different isoforms of NOS :iNOS, endothelial nitric oxide synthase (eNOS), and neuronal nitric oxide synthase (nNOS)\(^18\),\(^19\). Particularly, when the macrophage is stimulated with LPS, iNOS is expressed and NO is produced. In this way, produced NO plays the role of mediating an inflammation\(^20\)–\(^22\).

The light treatment using an ultraviolet rays for the several ten year of late was used in managing the general skin disease\(^23\) but there was the side effect relating to the harmfulness of an ultraviolet rays\(^24\),\(^25\). So we decided to use LED light, visible rays which no side effect has been reported, in the study. In this research, by using the LED light as a source of the visible ray wavelengths, we proved

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2. Materials and Methods

2.1 Light Source and Irradiation
The source of light for irradiation was a continuous-wave LED (U-JIN LED, Goyang-City, Korea) emitting at a wavelength of 400nm, 440nm and 495nm and each LED wavelength (50mA current) was irradiated 10cm above the cell surface for 1 hour. The manufactured LED irradiation tool kit was built in a 5% CO₂ humidified chamber at 37°C.

2.2 Cell Culture
The murine macrophage cell line RAW 264.7 was purchased from the Korean Cell Line Bank (KCLB; Seoul, KOREA). RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO Inc., NY, USA) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% fetal bovine serum (FBS; GIBCO Inc., NY, USA). The cells were incubated in an atmosphere of 5% CO₂ at 37°C and were subcultured every 3 days.

2.3 Cell Viability Assay
The effects of 400nm, 440nm and 495nm LED irradiation on cell viability were determined using CellTiter 96® AQueous One Solution Assay for cell proliferation, which employs colorimetry for counting the number of viable cells. This assay was utilized to determine the number of viable cells remaining after the completion of the culture process. RAW264.7 cells were plated at a density of 1×10⁵ cells/well in 96-well flat-bottomed plates, and were incubated with 400nm, 440nm and 495nm LED irradiation for 1 hour. After 24h incubation, the number of viable cells was counted in accordance with the manufacturer’s instructions.

2.4 Measurement of Nitrite
The amount of nitrite produced by the mouse macrophages was measured in RAW264.7 cells culture supernatant. RAW264.7 cells were plated at a density of 1×10⁵ cells/well in 96-well cell culture plate with 200ul of culture medium and incubated for 24h. They were then irradiated with various wavelengths of LED in the presence of 200ng (0.2ug)/ml of LPS for 1h and incubated for another 24h. The amount of nitrite was measured using the NO (Nitric Oxide) Detection Kit (iNtRON Biotechnology), according to the manufacturer’s instructions.

2.5 Enzyme-Linked Immunosorbent Assay for TNF-α, IL-1β and IL-6 Detection
RAW 264.7 cells were seeded in a 96-well plate at a density of 1.0×10⁵ cells/well and cultured for 18 hours and pretreated LED irradiation with for 1 hour before LPS (200ng/mL) stimulation. The ELISA was performed by coating 96-well plates of monoclonal antibody with specificity for TNF-α, IL-1β and IL-6 respectively.

2.6 Statistical Analysis
Results are expressed as the means±standard errors (SEM) of triplicate experiments. Statistically significant values were compared using one-way Analysis of Variance (ANOVA) and p-values of less than 0.05 were considered statistically significant.

3. Results

3.1 Effect of LED Irradiation on the Cell Viability of RAW264.7 Cells
To investigate the cytotoxicity of LED irradiation on RAW264.7 macrophage cells, MTS assay was performed. There was not any cytotoxicity in all LED wavelengths used in an experiment (Figure 1).

RAW 264.7 cells were treated with 400nm, 440nm, 495nm LED irradiation for 1 hr. Cell viability was
determined by MTS assay. Results of three independent experiments were averaged mean value of three independent experiments, and are shown as percentage cell viability compared with the viability of untreated control cells.

3.2 Effect of LED Irradiation on the Production of NO in RAW264.7 Cells Stimulated with LPS

To investigate the effects of LED irradiation on LPS-induced NO in RAW 264.7 cells, culture media were harvested and nitrite levels were measured. 440nm LED irradiation significantly inhibited LPS-induced NO production more than 400nm, 495nm LED irradiation (Figure 2).

RAW 264.7 cells were treated with or without LPS (200ng/ml) and then with 400nm, 440nm, 495nm LED irradiation for 1 hr, and incubated for 24h. The nitrite concentrations in medium were determined by NO Detection Kit. Results of three independent experiments were averaged mean value of three independent experiments, and asterisks indicate significantly different from treatment with LPS alone. (# : p<0.05 compared to control; * : p < 0.05 compared to LPS ; ** : p < 0.01 compared to LPS).

3.3 Effect of LED Irradiation on TNF-α Secretion

To examine the effect of LED irradiation on inflammatory cytokine TNF-α, IL-6 secretion, after LPS (200ng/ml) was solely treated in RAW264.7 cells or LPS and LED irradiation was concentration-dependently treated, TNF-α, IL-1β, IL-6 concentration secreted on the medium was measured by the ELISA method. As a result (Figure 3), concentration-dependent inhibition of TNF-α was observed. In particular, there was significant inhibition in 1000 ug/mL. Many media are involved in inflammation. For cytokine secreted from many cells such as activated lymphocyte and macrophage, such cytokine as TNF-α,IL-1β, IL-6, and IL-8 is involved in inflammatory responses. TNF-α is a major medium of LPS response and plays an important part in an innate immune response. TNF-α secreted from macrophage and mast cell represents cytotoxicity in tumor cells and is associated with chronic inflammation.

RAW 264.7 cells were treated with or without LPS (200ng/ml) and then with 400nm, 440nm, 495nm LED irradiation for 1 hr. Results of the experiments were the mean values of three independent experiments and asterisks indicate the significant differences (*: p<0.05 compared to LPS).

3.4 Effect of LED Irradiation on IL-6 Secretion

To examine the effect of LED irradiation on inflammatory cytokine TNF-α, IL-6 secretion, after LPS (200ng/ml) was solely treated in RAW264.7 cells or LPS and LED irradiation was concentration-dependently treated, TNF-α, IL-1β, IL-6 concentration secreted on the medium was measured by the ELISA method. As a result (Figure 4), concentration-dependent inhibition of TNF-α was observed. In particular, there was significant inhibition in 1000 ug/mL. Many media are involved in inflammation. For cytokine secreted from many cells such as activated lymphocyte and macrophage, such cytokine as TNF-α,IL-6, and IL-8 is involved in inflammatory responses. TNF-α is a major medium of LPS response and plays an
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4. Discussion

Recently, many reports have suggested that a specific wavelength of light irradiation could be an alternative anti-inflammatory tool for wound healing in clinical fields\(^{26,27}\). Different wavelengths have different chromophores and can have various effects on tissue\(^{28}\). And the various cell and tissue types in the body have their own unique light absorption characteristics, each absorbing light at specific wavelengths\(^{29,30}\). To have any effect on a living biological system, LED-emitted photons must be absorbed by a molecular chromophore or photoacceptor.

Inflammation can happen in a number of ways and free radicals (NO, HNO\(_2\), ONOO-) are known to cause subclinical inflammation. LED therapy brings new treatment alternative for such lesions possibly by counteracting inflammatory mediators like NO\(^{31}\). To investigate the cytotoxicity of LED irradiation on RAW264.7 macrophage cells, MTS assay was performed. There was not any cytotoxicity in all LED wavelengths used in an experiment (Figure 1). To investigate the effects of LED irradiation on LPS-induced NO in RAW 264.7 cells, culture media were harvested and nitrite levels were measured. 440nm LED irradiation significantly inhibited LPS-induced NO production more than 400nm, 495nm LED irradiation (Figure 2). 440nm LED irradiation significantly reduced TNF-α, IL-6 secretion more than 400nm, 495nm LED irradiation (Figure 3,4). In our study, 440nm LED irradiation significantly inhibited LPS-induced NO production and TNF-α, IL-6 secretion more than 400nm, 495nm LED irradiation without cytotoxicity in RAW264.7 cells. The inhibition of NO production and TNF-α, IL-6 secretion may be one of the mechanisms responsible for the anti-inflammatory action of 440nm LED irradiation. The results indicate that the LED irradiation with the range of 400~495nm in wavelength is not toxic to the cultured skin cells and suggests that the LED irradiation may be employed as a therapeutic agent to ameliorate skin disease.

5. References