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| Original Article |

Effect of Light-Emitting-Diode Irradiation on the Proliferation and Migration in Human Gingival Fibroblasts

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Abstract : *Purpose:* The aim of this study is to evaluate proliferation, migration, and various gene expressions including insulin-like growth factor 1 (IGF-1), SMAD3, and collagen type I (COL1A1) during light-emitting diode (LED) irradiation of human gingival fibroblasts (HGF). *Materials and methods:* HGF was seeded on the culture dish and stimulated using white (1.26 J/cm^2) and red (0.3 J/cm^2) wavelength LED for 9 min during 3 days. The distance between LED light and HGF was about 30 mm. To evaluate cell proliferation, MTT assay was carried out for 3 days after LED irradiation. After culturing for 1 and 3 days, cells were harvested and total RNA was isolated. To assess the expression of IGF-1, IGF-2, IGF-1R, SMAD3, COL1A1, and GAPDH, cDNA was synthesized and RT-PCR was performed. Whether mixed LED irradiation affect to intracellular adhesion molecule-1 (ICAM-1) mRNA expression of HGF, real time PCR was performed. To measure wound healing after LED irradiation, straight scratch was made and observed migrated cells to the scratched region using microscope. Transwell migration assay was carried out to measure migrated cells after LED irradiation. Migrated cells were stained using hematoxilin. *Results*: Cell proliferation was significantly increased both 1 and 3 days after LED irradiation. IGF-1 and COL1A1 mRNA expression was increased in LED irradiated cells compared to control cells. However, IGF-1R, IGF-2, and SMAD3 expression was decreased in LED irradiated cells compare to control cells. In LED irradiated cells, ICAM-1 expression was decreased than control. Interestingly, LED irradiation dramatically promoted wound healing and cell migration compared to control cells. Especially after 20 h, most of the substrate was covered by migrated HGF after LED irradiation. *Conclusion:* These results suggest that simultaneous use of red and white wavelength LED promotes proliferation and migration of human gingival fibroblasts *in vitro*.

Key words: *LED(light-emitting diode), Cell proliferation , human gingival fibroblasts, cell migration, wound healing*

1. Introduction

The periodontium is composed of cementum, periodontal ligament, gingiva, and alveolar bone. Local bacteria-induced inflammation is a major cause of destruction of tooth-supporting structures and periodontium. During periodontitis, lipopolysaccharide (LPS) binds to toll-like receptor 4 (TLR4) and induces expression of interleukin-1 β (IL-1β) and tumor necrosis factor-α (TNF-α). Subsequently, expression of these cytokines induces matrix metalloproteinases (MMPs) expression, resulting in destruction of connective tissues, such as periodontal ligament and alveolar bone, and bone resorption.[1-3]

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Over the past few decades, application of low-level laser therapy (LLLT) in dentistry has increased. Especially, biostimulation using LLLT has been used for treatment of periodontitis.[4] LLLT has been reported to reduce pain, induce cell proliferation, accelerate wound healing, and to have a positive effect on inflammatory processes.[4-7] Recently, Chang *et al*. reported that Light-Emitting-Diode (LED) irradiation could control the periodontal bone levels in the Sprague-Dawley rat model.[8]

Several recent reports have suggested that visible light irradiation is an effective alternative treatment that reduces inflammation and promotes wound healing in periodontal disease. LLLT has been suggested as an adjuvant to traditional periodontal treatment because of its biostimulatory effect on periodontal cells, reduction in the number of periodontopathogens, relief of inflammation, and increase in the periodontal attachment

level.[5, 9-11]

Phototherapy using LED has been suggested as an effective alternative to LLLT. LEDs have an energy density of 0-100 J/cm^2 and negligible temperature change.[6] It was reported that LEDs have the same effect as lasers when used in therapeutic applications. The main advantage of LED is that LED systems allow a larger area to be treated in a short time, with a large wavelengths; while typical laser systems irradiate only small spots.[12-14]

There are many studies of LEDs with different wavelengths[15-21], but no attempt was made to use mixed wavelength simultaneously. In this study, we used a mixed wavelength LED (white and red) for estimating the effect of LED irradiation on human gingival fibroblasts.

The purpose of this study is to evaluate the effect of red and white LED irradiation on gingival fibroblast proliferation and migration.

2. Materials and methods

2.1 Isolation of Human Gingival Fibroblasts (HGF) and Cell Culture

This study was performed at the Department of Periodontology, School of Dental Medicine, Dankook University, Korea. Healthy human gingival tissue was obtained from patients in the Dental and Periodontology Clinic of the School of Dentistry in Dankook University, and the patients provided informed consent.

To isolate gingival fibroblasts, we used explant culture methods. Briefly, tissues were minced and placed in culture dishes. Minced tissues were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin (Gibco, Gaithersburg, MD, USA), and 10% fetal bovine serum (FBS, Invitrogen) at 37°C in a humidified atmosphere containing 5% CO_2.

2.2 LED Irradiation

The LED device used in this study was a light-emitting diodes (LEDs) type which provided uniform irradiation of each well (35 mm) in which the cultured cells were seeded. The distance between LED light and HGF was about 30 mm. During experiments, mixed irradiation of red (600~650 nm, 0.3 J/cm^2) and white (400~750 nm, 1.26 J/cm^2) LED wavelength was applied for 9 min until 3 days.

2.3 MTT Assay

Proliferation of LED irradiated HGF was evaluated using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. HGF (3×10^3 cells/well) was seeded on a 96-well plate. Then, 50 μl of MTT (5 mg/mL) was added to each well and incubated for 2 h at 37°C. After removing the MTT solution, purple formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and absorbance was measured at a wavelength of 540 nm with a microplate reader (Multiskan EX; Thermo Electron Corporation, USA). Triplicate samples were analyzed from three independent experiments.

2.4 RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-time PCR

HGF (1×10^5/well) was seeded in 35 mm culture dishes. After irradiation, cells were harvested and total RNA was isolated using Trizol reagent. Then, 2 μg of total RNA was used to synthesize cDNA. cDNA synthesis was performed according to the manufacturer's instructions. The synthesized cDNA was used to amplify insulin-like growth factor 1 (IGF-1), insulin-like growth factor 1 receptor (IGF-1R), Insulin-like growth factor 2 (IGF-2), SMAD3, collagen type I (COL1A1), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by PCR. PCR conditions were 94°C for 1 min, followed by 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s for 30 cycles. Amplified products were electrophoresed on 1% agarose gels and visualized using UV light. Real-time PCR was performed on an ABI PRISM 7300 sequence detection system using the SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. The PCR conditions were 94°C for 1 min, followed by 95°C for 15 s, and 60°C for 34 s for 40 cycles. All reactions were run in triplicate and were normalized to the housekeeping gene, GAPDH. Relative differences in PCR results were calculated using the comparative cycle threshold (CT) method. The primers used in PCR are listed in Table 1.

2.5 Wound Healing Assay

HGF (1×10^5/well) was seeded in 35 mm culture dishes. When the cells reached 100% confluence, a straight scratch was made in the culture dish using a sterilized yellow tip before LED irradiation. After irradiation, wound healing was observed for 6 and 20 h. At each time point, migrated HGF was observed using a microscope (isolution Lite –NFR, Olympus, Japan).

2.6 Transwell Migration Assay

HGF (5×10^3/well) was seeded in transwell inserts (8.0 μm pore size, Falcon 35-3097, Becton-Dickinson, NJ, USA). After 24h, LED irradiated and incubated for 24h to induce migration. After 24h, HGF was fixed using 4% paraformaldehyde (PFA) and stained with hematoxilin. Stained cells were mounted and

Table 1. Primer sequence for RT-PCR and realtime PCR.

Gene	Sequence(5'-3')	Product size (bp)
IGF-1	FW AAGATGCACACCATGTCCTC	389
	RV CCTGTAGTTCTTGTTTCCTGC	
IGF-2	FW CTGGAGACGTACTGTGCTAC	547
	RV GGTGTTTAAAGCCAATCG	
IGF1R	FW CAACCACGAGGCTGAGAAGC	447
	RV CAGCATAATCACCAACCCTC	
Smad3	FW GAGTAGAGACGCCAGTTCTACC	203
	RV GGTTTGGAGAACCTGCGTCCAT	
Type 1 collagen	FW TAAGTTGCCAAGAACGTGCC	103
	RV AATTGAAAGCCAGGAGGCAT	
GAPDH	FW ACCACAGTCCATGCCATCAC	452
	RV TCCACCACCCTGTTGCTGT	
ICAM-1	FW GCCTGGGAACAACCGGAAGGTG	148
	RV GGGTGCCAGTTCCACCCGTTC	
GAPDH	FW AGGTCGGTGTGAACGGATTTG	123
	RV TGTAGACCATGTAGTTGAGGTCA	

counted migrated cells.

2.7 Statistical Analysis

Experiments were carried out in triplicate and the data were presented as mean ± S.D. The statistically significant difference was analyzed using the Student's t test.

3. Results

3.1 LED Irradiation Promoted Proliferation of Gingival Fibroblasts

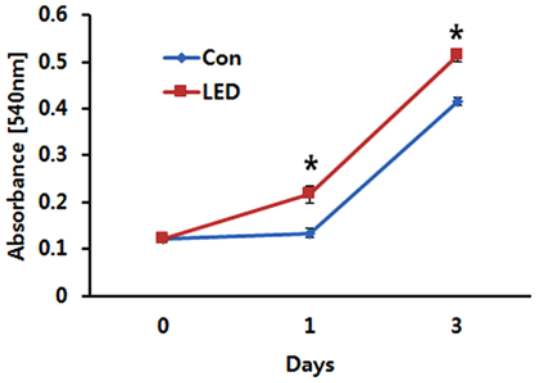

Figure 1. LED irradiation promoted proliferation of HGF. Cultured HGF was seeded in a 96-well plate and stimulated using red and white LED. Proliferation was measured by the MTT assay for 3 days. In the LED irradiated group, proliferation was significantly increased than that in the control group. Data are presented as the mean ± S.D. n = 3, *, $P < .01$.

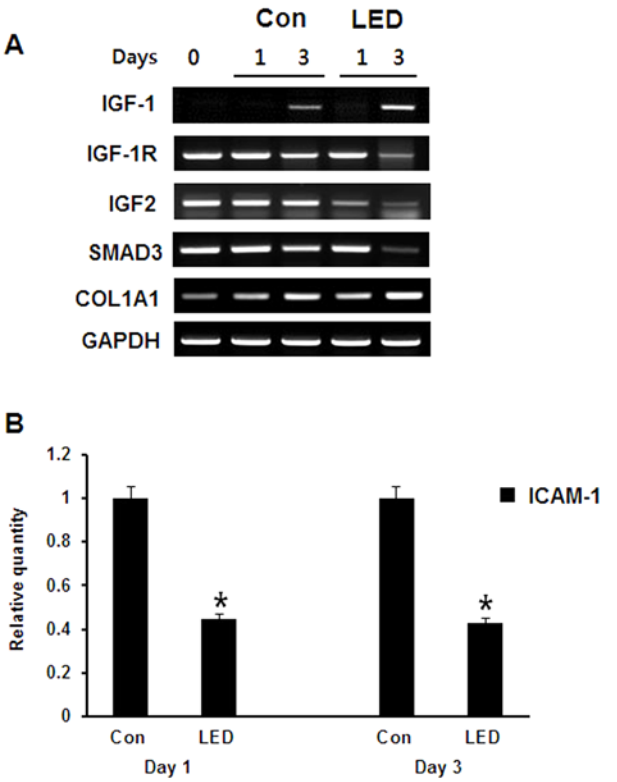

Figure 2. LED irradiation increased IGF-1 and COL1A1 mRNA expression, but decreased IGF-1R, IGF-2, SMAD3 and ICAM-1 mRNA expression in HGF. A. Cultured HGF was seeded in a 35 mm culture dish and stimulated using red and white LED. After 3 days, cells were harvested and total RNA was isolated. RT-PCR was carried out to assess the expression of IGF-1, IGF-2, IGF-1R, SMAD3, and COL1A1. GAPDH was used as a control. B. Real-time – PCR was carried out to check the expression of ICAM-1. Abbreviations: IGF-1; Insulin-like growth factor 1, IGF-2; Insulin-like growth factor 2, IGF-1R; Insulin-like growth factor 1 receptor, COL1A1; Collagen type I, GAPDH; glyceraldehyde 3-phosphate dehydrogenase, Intracellular adhesion molecule-1; ICAM-1. Data are presented as the mean ± S.D. n = 3, *, $P < .01$.

To measure cell proliferation, MTT assay was carried out after LED irradiation. Cell proliferation was significantly increased at both 1 and 3 days after LED irradiation. This result shows that LED irradiation accelerated proliferation of HGF (Fig 1).

3.2 LED Irradiation Increased IGF-1 Expression and Decreased IGF-1R and SMAD3 Expression

To assess the effect of LED irradiation on HGF, expression of various genes was amplified using RT-PCR. As a result of LED irradiation, IGF-1 expression was increased in LED irradiated cells than in control cells. Conversely, the expression of IGF-1R and IGF-2 was decreased in the LED irradiated cells.

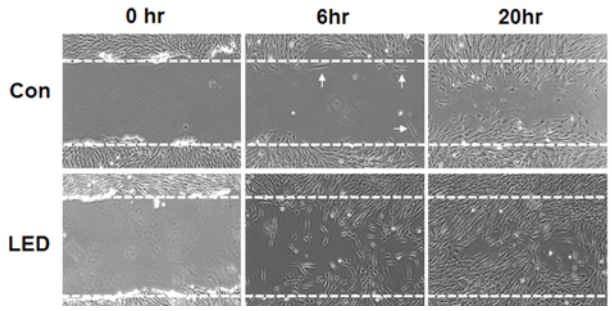

Figure 3. LED irradiation promoted wound healing in HGF. HGF was cultured until they reached 100% confluence and a straight scratch (white dot line) was made before LED irradiation. After irradiation, wound healing was observed for 6 and 20 h. The white arrows represent migrating cells in the control group. Magnification: × 40.

Interestingly, SMAD3 expression was decreased after LED irradiation. COL1A1 expression was increased after LED irradiation (Fig 2A). To measure the ICAM-1 mRNA expression, real-time PCR was carried out. After LED irradiation, both 1 and 3 day ICAM-1 expression was significantly decreased about 60% than control (Fig 2B).

3.3 LED Irradiation Promoted wound Healing and Cell Migration

We assessed cell migration after LED irradiation using the wound healing assay and transwell assay. Compared with that

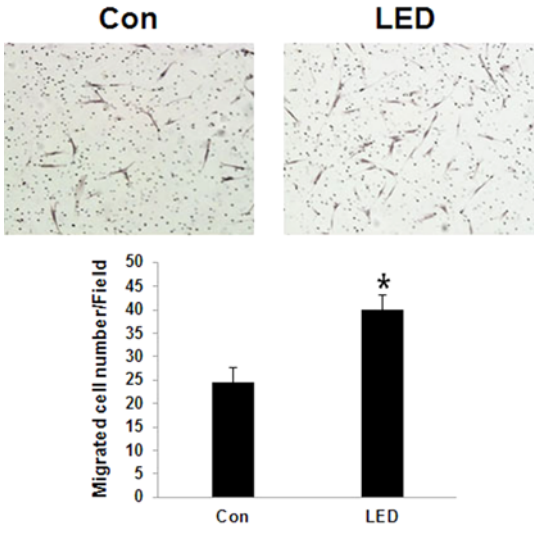

Figure 4. LED irradiation promoted cell migration in HGF. HGF was LED irradiated and cultured for 24 h. After 24 h, cells were fixed using 4 % PFA and stained with hematoxilin. Stained cells were mounted and counted migrated cells. Magnification: × 40. Data are presented as the mean ± S.D. n = 3, *, $P < .01$.

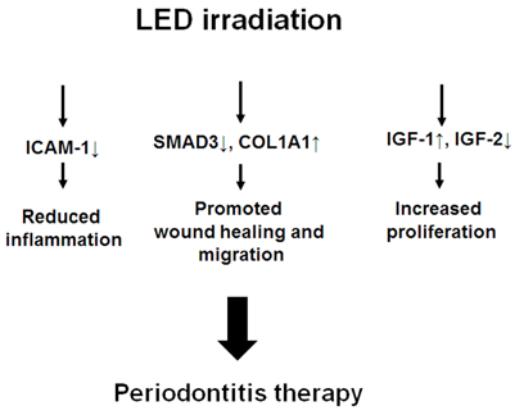

Figure 5. Model of LED irradiation on periodontitis therapy. Combination of red and white wavelength LED could stimulate IGF-1 expression and inhibit IGF-2 and SMAD3 expression. Increased IGF-1 expression promoted proliferation and decreased SMAD3 expression stimulated wound healing in HGF. Decreased ICAM-1 expression by LED irradiation might be reduced inflammation caused by periodontitis.

in control cells, LED irradiation increased migration of HGF. In the control group, after 6 h, few cells start to migrate to the wounded substrate (white arrows). However, in the LED irradiated group, about 50% of the wounded substrate was filled with migrated cells. Especially after 20 h of irradiation, most of the substrate was covered by migrated HGF in the LED irradiated group (Fig 3). Using transwell assay, we stained migrated cells and counted migrated HGF. As a result, LED irradiated HGF migration was significantly increased than control. Transwell assay result indicate that LED irradiation accelerate the migration of HGF about 1.5 fold (Fig 4). This result shows that LED irradiation promoted cell migration

4. Discussion

During treatment of periodontitis, wound healing is essential to restore the barrier function rapidly.[22-24] In the oral cavity, gingival fibroblasts play a key role in the late wound healing phase. Fibroblasts secrete multiple growth factors during wound healing and participate actively in the formation of granulation tissue and synthesis of a complex extracellular matrix, such as collagen. Therefore the stimulation of fibroblasts might be beneficial in accelerating the wound healing process.[3, 25, 26]

Several previous studies reported that various LED wavelengths have a clinical and histological influence. Oliveira *et al*. demonstrated that the use of LED (700 nm) irradiation induced increased fibroblastic cell proliferation and wound

healing in anemic animals.[11] Chang PC *et al*. reported that LED (660 nm) irradiation appeared to be a suitable treatment modality for experimental periodontitis in rats by temporarily reducing inflammation, facilitating collagen realignment and bundle bone deposition.[4] De Sousa reported that the use of green and red LED light is effective in increasing fibroblastic proliferation.[27] Choi *et al.* reported that LED irradiation inhibits activation of pro-inflammatory cytokines via MAPK signaling pathway in human gingival fibroblasts.[28] In rat tendinitis model, low-power LED irradiation also showed anti-inflammatory effect.[29] In this study, LED irradiation (combination of red and white wavelengths) promoted proliferation of HGF cells.

IGF-1 (Insulin-like growth factor 1) family has 49% homology to insulin, IGF-1, and IGF-2. IGF-1 and IGF-2 are known to have a similar structure, but different functions. The IGF family contains three ligands (Insulin, IGF-I, and IGF-II) and three cell surface receptors (Insulin, IGF-1 and IGF-l-mannose G- phosphate receptors). IGF-1 is an important factor that regulates the cell cycle, inhibits cell division via apoptosis. Recently, it was reported that IGF-1 promotes proliferation and osteogenic differentiation of human periodontal ligament cells.[25] IGF-2 is an important growth factor which is highly active during fetal development but is less active in the adult body.[30, 31] Insulin-like growth factor-1 receptor (IGF-1R) is a tyrosine kinase class of membrane receptors. IGF-1R forms a tetramer composed of two αβ subunits and can hybrids with αβ subunit pair from the highly homologous insulin receptor. The activated IGF-1R take part in cell growth and survival control.[32] According to our RT-PCR result, IGF-1 expression was increased while IGF-2 and IGF-1R expression was decreased in LED irradiated HGF. These results suggest that increased IGF-1 expression caused by LED irradiation promoted proliferation of HGF. However, biological correlation between IGF-1 and IGF-1R expression by treating LED irradiation in HGF is not cleared.

SMAD3 is a mediator of the TGF-β signaling system and directly activates transcriptional regulators and signal transducers through SMAD2/3. Jinno *et al.* showed that palatal wound healing was promoted in SMAD3 deficient mice than in wild type mice.[33-35] In this study, the wound healing assay showed more concentration of HGF at 20 h after LED irradiation. LED irradiation caused recovery of most of the scratched region compared to that in control cells. In addition, SMAD3 expression was decreased after LED irradiation. Therefore, these results suggest that a decrease in SMAD3 expression caused by LED irradiation promoted wound healing in HGF.

Intracellular adhesion molecule-1 (ICAM-1, CD54) is known to a transmembrane protein and plays important role in inflammation and cell adhesion.[36] It is reported that lipopolysaccharide (LPS) from Porphyromonas gingivalis (P. gingivalis) induces ICAM-1 expression via toll-like receptor (TLR).[37] In our result, LED irradiation decreased ICAM-1 mRNA expression about 60% both 1 and 3 days. Therefore, these results suggest that mixed irradiation thought to take part in regulation of ICAM-1 mRNA expression.

Type I collagen (COL1A1) is an important protein for connective tissue formation.[1] Phases of wound healing include: haemostatic phase (formation of blood clot), inflammatory phase (parallels with haemostatic phase), and tissue formation phase (granulation tissue formation).[24] During the periodontal wound healing process, matrix formation requires the removal of granulation tissue with revascularization. New collagen and elastin fibers substitute the granulation tissue.[22] These processes are regulated by various growth factors and cytokines.[24, 38] A previous study showed that low-intensity laser irradiation (LILI) at 660 nm increased COL1A1 expression in diabetic wounded fibroblasts.[15] A previous study showed that mRNA expression of COL1A1 was present only in the biostimulation setting of diode laser, and that the settings used did not increase the proliferation indexes of the fibroblasts.[19] This study showed that LED irradiation not only induced an increase in COL1A1 gene expression, but also promoted proliferation of HGF.

In conclusion, our data suggest that the combination of red and white wavelength LED could stimulate proliferation and migration of human gingival fibroblasts via regulating IGF-1, COL1A1, and SMAD3 expression. LED irradiation might be reduced inflammation caused by periodontitis through regulation of ICAM-1 expression (Fig 5).

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Conflicts of Interest: Jong-Tae Park and Ki Seok Hong declcare that they have no conflicts of interest.

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