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*Research Article*

# Effect of 660 nm Light-Emitting Diode on the Wound Healing in Fibroblast-Like Cell Lines

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Light in the red to near-infrared (NIR) range (630–1000 nm), which is generated using low energy laser or light-emitting diode (LED) arrays, was reported to have a range of beneficial biological effects in many injury models. NIR via a LED is a well-accepted therapeutic tool for the treatment of infected, ischemic, and hypoxic wounds as well as other soft tissue injuries in humans and animals. This study examined the effects of exposure to 660 nm red LED light at intensities of 2.5, 5.5, and 8.5 mW/cm$^2$ for 5, 10, and 20 min on wound healing and proliferation in fibroblast-like cells, such as L929 mouse fibroblasts and human gingival fibroblasts (HGF-1). A photo illumination-cell culture system was designed to evaluate the cell proliferation and wound healing of fibroblast-like cells exposed to 600 nm LED light. The cell proliferation was evaluated by MTT assay, and a scratched wound assay was performed to assess the rate of migrating cells and the healing effect. Exposure to the 660 nm red LED resulted in an increase in cell proliferation and migration compared to the control, indicating its potential use as a phototherapeutic agent.

## 1. Introduction

Light in the red to near-infrared (NIR) range (630–1000 nm) generated by low energy laser or light-emitting diodes (LEDs) was reported to have beneficial biological effects in a range of injury models [1, 2]. Such photobiomodulation has been observed to increase the mitochondrial metabolism [3–6], facilitate wound healing [7–9], and promote angiogenesis in the skin [7], bone [10], nerve [11], and skeletal muscle [12–15]. Red and NIR have beneficial effects on cells by "kick-starting" them into immediately creating more adenosine triphosphate (ATP) and increasing the DNA and RNA activity. This effect has been examined extensively since 1987. The positive effects were observed only in injured cells; no benefit was observed in healthy cells. Tissue repair and healing of injured skin are complex processes that involve a dynamic series of events including coagulation, inflammation, granulation tissue formation, wound contraction, and tissue remodeling [16]. The ideal wavelengths were reported to be between 600 and 900 nm with the best results obtained at specific ranges: 610–625, 660–690, 750–770, and 815–860 nm. NIR via LED is a well-accepted therapeutic tool in the treatment of infected, ischemic, and hypoxic wounds, as well as other soft tissue injuries in humans and animals [17, 18]. The mechanism of NIR-LED action is the upregulation of the cytochrome C oxidase activity and the production of ATP, as observed in primary cultures of rat visual cortical neurons inactivated functionally by tetrodotoxin, potassium cyanide (KCN), or sodium azide ($N_3Na$) [19].

Recently, NASA reported that the LED lamp used as a source of 670 nm light has significant effects on injury repair [17]. The efficacy of this high-flux 670 nm LED light source

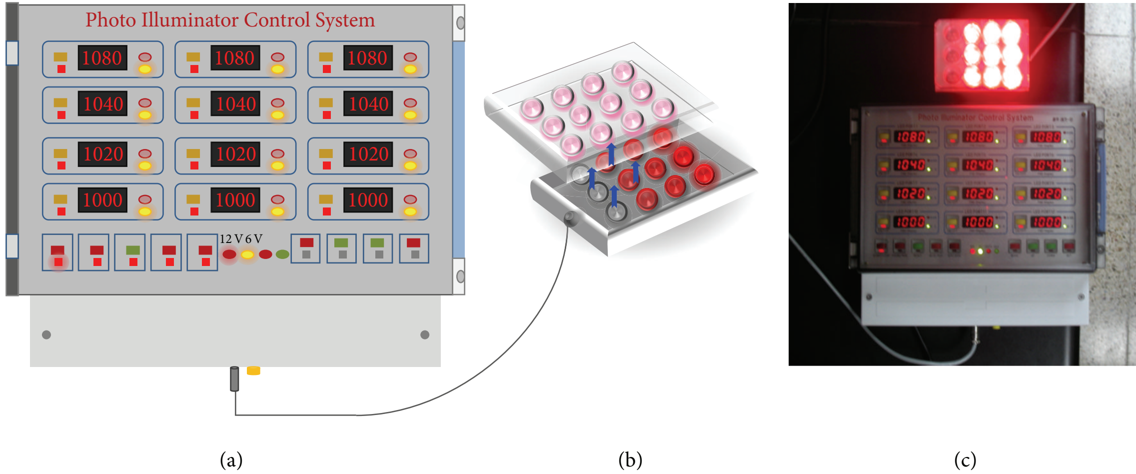



Figure 1: Photo illumination-cell culture system for evaluating the effects of 660 nm red LED exposure on the cell proliferation and wound healing: (a) LED array controller, (b) LED module and cell culture plate, and (c) photograph of red LED illumination system.

in attenuating some types of injury, such as inhibiting the toxic effects of methanol on the retina, was recently reported [20]. Although the effects of LED exposure were reported, there was no direct evidence for cell migration including the influence of the duration and intensity of red LED exposure. These studies suggest that 670 nm LED might be helpful in repairing acute cutaneous wounds. Recently, another study reported the effect of red LED in wound healing [21]. On the other hand, that study did not reveal the direct effects of the red LED on cell migration. Therefore, the present study evaluated cell viability and migration in L929 and human gingival fibroblasts (HGF-1) using MTT and *in vitro* wound healing assay after exposure to 660 nm red LED for several illumination times and intensities.

## 2. Materials and Methods

*2.1. Red LED Irradiation.* A photo illumination-cell culture system was designed and developed to evaluate the effects of 660 nm red LED light on the cell proliferation and wound healing of L929 and HGF-1 cells (Figures 1(a) and 1(b)). This system consists of three main parts, such as cell culture well plate, LED module, and a LED exposure power and time controller. The red LED module, which was composed of a 12-LED array, was designed to fit the well diameters of a 12-well plate for a fibroblast-like cells culture (Figure 1(b)). The photo illumination controller was designed to allow adjustments of the light intensity and illumination time of each LED array in the red LED module (Figure 1(a)).

The L929 and HGF-1 cells were placed on 12-well plates and exposed to 660 nm red LED light with an intensity of 2.5, 5.5, and 8.5 mW/cm$^2$ for exposure times of 5, 10, and 20 min. The 12-well plates were exposed from a distance of 2 cm. During 660 nm red light exposure, the temperature of the culture medium was maintained at 37 ± 0.1°C using an incubator. Tables 1 and 2 list the detailed experiment conditions.

Table 1: Experimental conditions of photo illumination used to examine cell viability.

| Entry | Exposure intensity (mW/cm$^2$) | Exposure duration (min) | Cell |
|---|---|---|---|
| 1 | | 5 | |
| 2 | 2.5 | 10 | |
| 3 | | 20 | |
| 4 | | 5 | |
| 5 | 5.5 | 10 | L929 |
| 6 | | 20 | |
| 7 | | 5 | |
| 8 | 8.5 | 10 | |
| 9 | | 20 | |
| 10 | | 5 | |
| 11 | 2.5 | 10 | |
| 12 | | 20 | |
| 13 | | 5 | |
| 14 | 5.5 | 10 | HGF |
| 15 | | 20 | |
| 16 | | 5 | |
| 17 | 8.5 | 10 | |
| 18 | | 20 | |

*2.2. Cell Culture.* L929 (mouse fibroblasts) and HGF-1 (human gingival fibroblasts) cells were purchased from the American Type Culture Collection. The L929 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% amphotericin B as antibiotics. The HGF-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS and 1% amphotericin B as antibiotics. Approximately 70%-confluent primary L929 and HGF-1 were plated at 1 × 10$^5$ cells per 100 mm culture dish and cultured until the cells reached 70% confluence.

Table 2: Experimental conditions of photo illumination used to examine wound healing.

| Entry | Exposure duration (min) | Healing time (hr) | Cell |
|---|---|---|---|
| 1 | | 0 | |
| 2 | 5 | 12 | |
| 3 | | 24 | |
| 4 | | 0 | |
| 5 | 10 | 12 | L929 |
| 6 | | 24 | |
| 7 | | 0 | |
| 8 | 20 | 12 | |
| 9 | | 24 | |
| 10 | | 0 | |
| 11 | 5 | 12 | |
| 12 | | 24 | |
| 13 | | 0 | |
| 14 | 10 | 12 | HGF |
| 15 | | 24 | |
| 16 | | 0 | |
| 17 | 20 | 12 | |
| 18 | | 24 | |

A trypsin-EDTA solution (2 mL) was added to the culture dishes to detach the adhered cells. RPMI 1640 and DMEM (8 mL each) were added to the detached cells, which were then aspirated by gentle pipetting. The cell suspensions were each transferred to 15 mL centrifuge tubes and spun down at 1,000 rpm (Sorval RT-6000) for 3 min at 4°C. The supernatant was removed carefully. The cell pellets were resuspended with RPMI 1640 and DMEM of 5 mL. Appropriate aliquots of suspended cells were plated in a 100-mm culture dish, subcultured serially, and passaged until they reached 70% confluence. The third and fourth passage L929 cells and HGF-1 cells, respectively, were used. Each of the subcultured cells was seeded at $1 \times 10^5$ cells per well in a 12-well plate and incubated at 37°C in air containing 5% $CO_2$.

*2.3. Cell Proliferation.* The level of cell proliferation after irradiation with red LED light was assessed at an intensity of 2.5, 5.5, and 8.5 mW/cm$^2$ for an exposure time of 5, 10, and 20 min using MTT assay. MTT is a direct mitochondrial activity assay that can indicate the cell viability indirectly. The filtered tetrazolium salt solution, 3-[4,5-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) (Sigma, Saint Louis, Missouri, USA), was added to the cell cultures at 5, 10, and 20 min after red light exposure. The wells were incubated for 4 h at 37°C. MTT was reduced to a dark-blue insoluble formazan precipitate by the mitochondrial succinic dehydrogenase of viable cells. The medium was drawn out and the dark-blue crystals remaining in the wells were dissolved in 500 $\mu$L of a solution of ethanol-dimethyl sulfoxide (DMSO) (Amresco, Solon, USA). A 200 $\mu$L aliquot of the solution from each well was aspirated and poured onto a 96-well plate to measure the absorbance at 540 nm using an ELISA reader (Thermo Fisher Scientific).

*2.4. Wound Healing Assay.* The *in vitro* wound healing determined by irradiation with the red LED light was measured at 5, 10, and 20 min after exposure. Each well was seeded with the cells to a final density of $1 \times 10^5$ cells per well and maintained at 37°C and 5% $CO_2$ for 24 h. After the cells reached 90% confluence, they were incubated in the culture media without serum for 18 h. In each plate, artificial wounds, approximately 0.4–0.5 mm in width, were scratched into the monolayer using a sterile plastic 10 $\mu$L micropipette tip. After washing 3 times with 1x PBS, the scratched cells were exposed to 660 nm LED light with an intensity of 8.5 mW/cm$^2$ for 5, 10, and 20 min. All scratch assays were performed in quadruplicate. Wound healing was monitored using an inverted microscope (Olympus TS100, Japan) and a digital camera (Canon, C4742-95, Japan) at 12 and 24 h after wounding. The micrographs were analyzed using an image analysis program (Zeiss, Germany). The rate of cell migration was determined using the following equation: (initial wound − wound at 12 h)/12 or (initial wound − wound at 24 h)/24. Table 2 lists the experimental conditions of photo illumination on *in vitro* wound healing.

*2.5. Data Analysis.* All experiments were performed at least in triplicate. The data is reported as the mean and standard deviation determined using Excel 2007 statistical software (Microsoft, USA). The significant difference ($^* p < 0.05$) was determined using Student's $t$-test.

## 3. Results and Discussion

*3.1. Cell Proliferation.* The HGF-1 cells were spindle-shaped fibroblasts and grew as a monolayer. To confirm the change in HGF-1 cell morphology, the confluent cultured cells were irradiated with 660 nm light under an intensity of 2.5, 5.5, and 8.5 mW/cm$^2$ and exposure time of 5, 10, and 20 min. The cell morphology was examined by phase-contrast microscopy (Olympus CKX41, Japan) equipped with a digital camera (Canon, CKI-PowerShot G10, Japan). As shown in Figure 2, no significant changes in the morphology of the HGF-1 cells were observed, regardless of the intensity or exposure time.

MTT assay was performed to evaluate the cell proliferation depending on the light intensity and time at a 660 nm wavelength (Figure 3). The cell proliferation was measured at 24 h after irradiation and expressed as a percentage of the control value. Under an exposure time of 5 min at 5.5 and 8.5 mW/cm$^2$, the cell proliferation in the light exposure group increased by 10 to 18% with increasing light intensity compared to that of the control group ($p < 0.01$). Under other light exposure conditions, at exposure times of 10 and 20 min, the cell proliferation of HGF-1 cells was similar to that of the control group ($p < 0.01$). In this experiment, the optimal dose of 660 nm light was determined to be 8.5 mW/cm$^2$ for 5 min, which corresponded to an energy density of 2.55 J/cm$^2$. Khadra et al. reported that low-level laser (LLL) irradiated HGF cells on the titanium specimen showed slightly higher

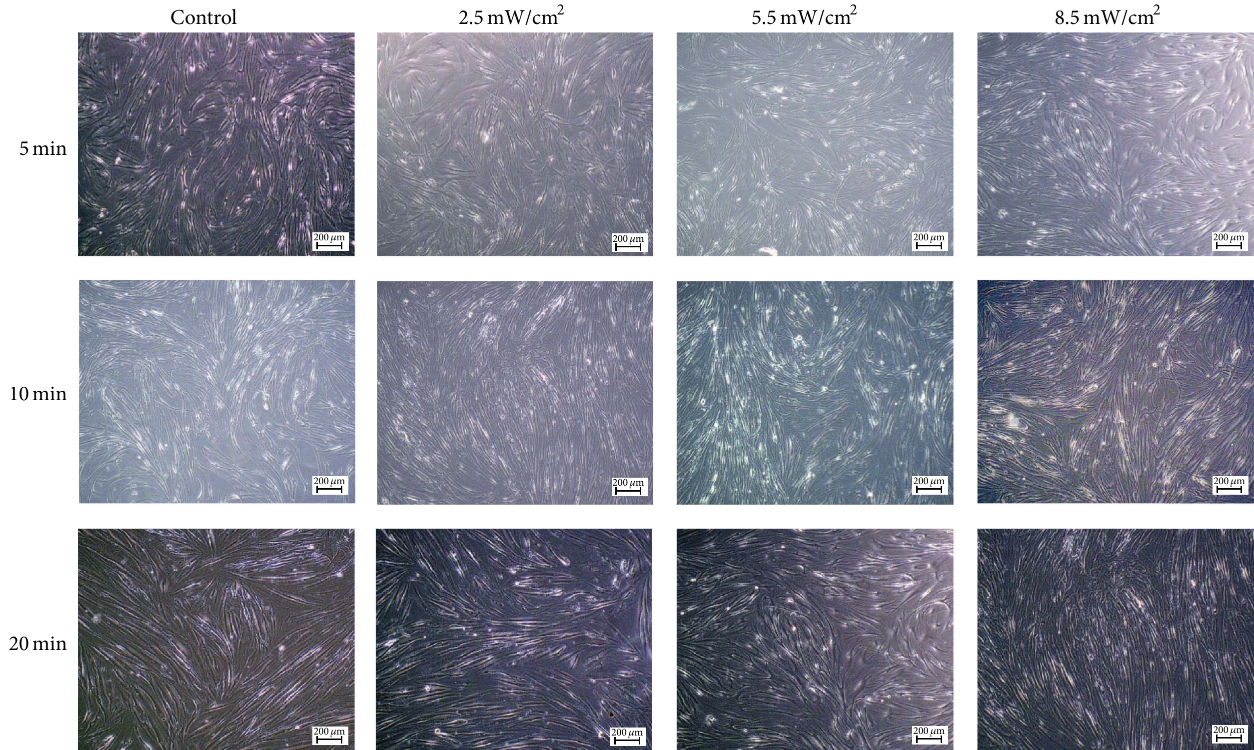



Figure 2: The changes in the HGF cell morphology as a function of the red LED illumination time and intensity. The cell morphology was observed by phase-contrast microscopy. Control is in a dark environment without LED light exposure.

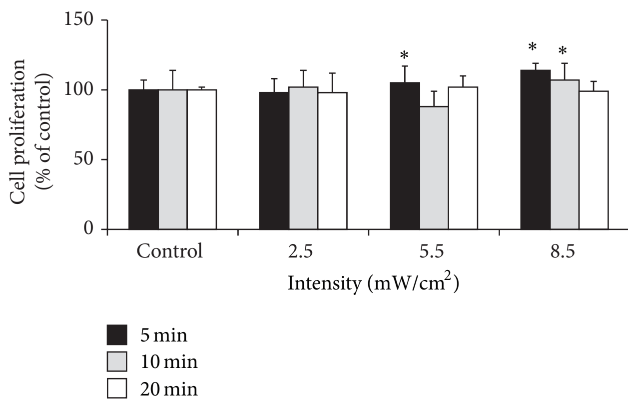



Figure 3: HGF cell proliferation as a function of the red LED illumination time and intensity. The cell proliferation was examined at 24 h after LED light exposure on HGF cell and expressed as a percentage of the control value. The optimal dose was 8.5 mW/cm$^2$ for 5 min.

proliferation than the nonirradiated HGF cells, but the difference was not significant [22]. Interestingly, cell proliferation was stimulated significantly by multiple doses of 1.5 and 3 J/cm$^2$ as the experiment was continued to 72 h and 7 days [22]. These findings are in agreement with those reported by Almeida-Lopes et al. [23]. Several studies reported satisfactory tissue healing rates at helium-neon laser exposure levels between 1 and 4 J/cm$^2$ [23–25]. Laser-enhanced biostimulation was reported to induce intracellular metabolic changes, resulting in faster cell division, rapid matrix production, and cell movement. On the other hand, higher laser energy doses were reported to inhibit cell proliferation [26, 27]. Yu et al. reported that fibroblasts irradiated with laser energy at 2.16 J/cm$^2$ showed increased cell proliferation, whereas laser energy of 3.24 J/cm$^2$ suppressed cell proliferation compared to the control group [28].

Figure 4 shows the changes in morphology of the L929 fibroblast after exposure to 660 nm light with an illumination time of 5, 10, and 20 min and an intensity of 2.5, 5.5, and 8.5 mW/cm$^2$. Under these exposure conditions, 660 nm LED irradiation did not alter the cell morphology in L929 cells.

Under light exposure and an intensity of 1200 s and 5.5 mW/cm$^2$, respectively, the proliferation of L929 cells was slightly higher than under the other conditions ($p < 0.01$) (Figure 5). For L929 cell proliferation, the optimal dose was determined by irradiation with 5.5 mW/cm$^2$ for 20 min and corresponded to an energy density of 6.6 J/cm$^2$. In that study, the exposure time had a significant effect on cellular proliferation. De Moura et al. examined the effects of the polarized light source (400–2000 nm) on the Hep.2 and L929 cell line in a range of *in vitro* models [29]. A comparison of the cellular viability of irradiated and nonirradiated L929 cultures showed that the treatment had a significant effect on the cellular viability and that time was significant in both groups [29]. Pires-Oliveira et al. reported that the

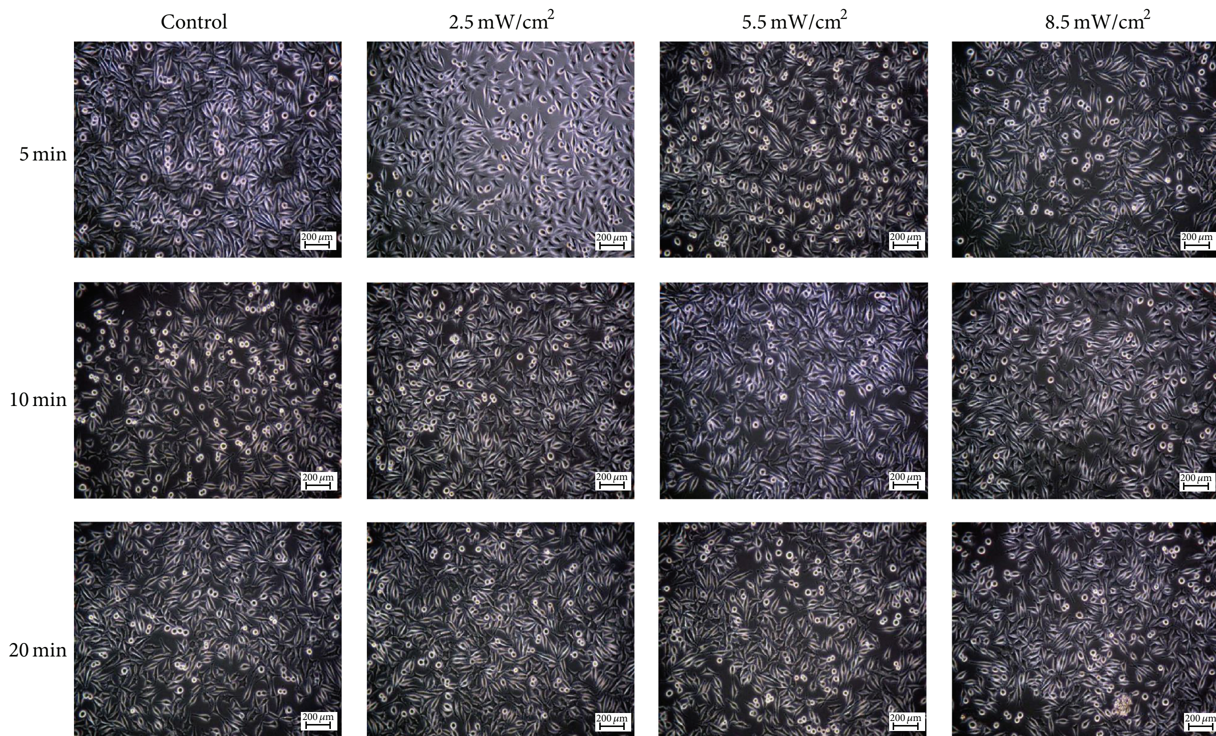



FIGURE 4: Changes in the L929 cell morphology as a function of the red LED illumination time and intensity. The cell morphology was observed by phase-contrast microscopy. Control is in a dark environment without LED light exposure.

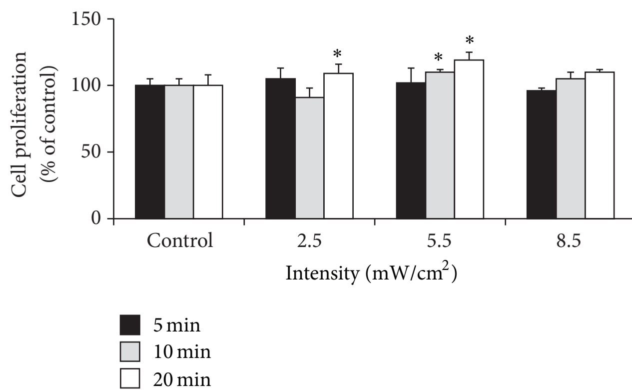
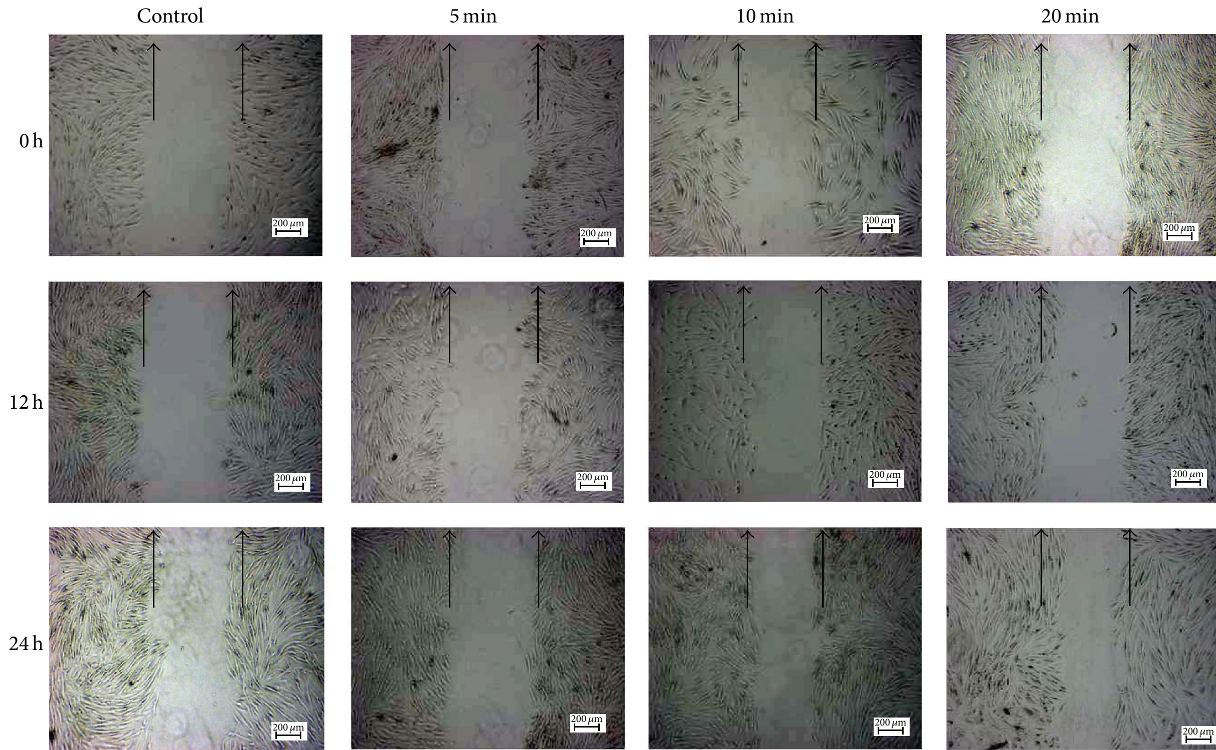



FIGURE 5: L929 cell proliferation as a function of the red LED illumination time and intensity. The cell proliferation was examined at 24 h after LED light exposure on L929 cell and expressed as a percentage of the control value. The optimal dose was 5.5 mW/cm$^2$ for 20 min.

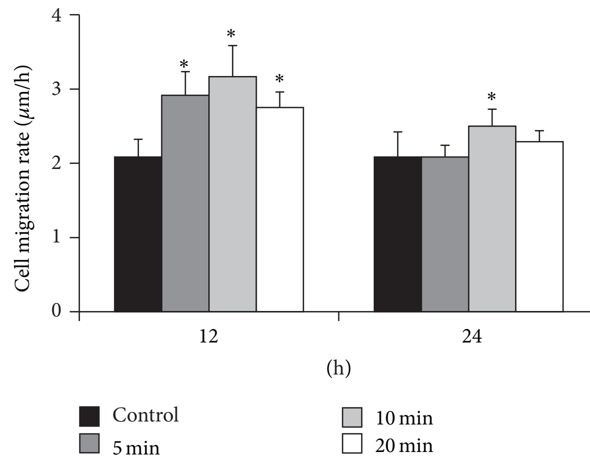
irradiation of L929 cells with a pulsed laser in the NIR region (904 nm) at two distinct energy densities increased cell proliferation in both cases. This result was slightly higher for an energy density of 50 mJ/cm$^2$ than for 6 J/cm$^2$, as revealed by fluorescence microscopy. This growth occurred at 24, 48, and 72 h after irradiation with more growth between 24 and 48 h [30]. When these cells were irradiated with a 670 nm LED at 5 J/cm$^2$ per treatment/24 h (50 J/cm$^2$ total energy dose), the treatment frequency that produced the highest rate of cellular proliferation was reported to be two treatments per day at 144 h after treatment [31]. The different cellular effects induced by laser irradiation were attributed to the redox potential of the target cells, which is associated with stimulation of the cellular functions if it shifts toward oxidation and with inhibition if it moves toward reduction [32].

Based on this result, the light intensity of the 660 nm red LED in this experiment was insufficient to evaluate the effect on the cell proliferation of HGF-1 cells and L929 cells. More studies will be needed to confirm the effects of the intensity of 660 nm light.

*3.2. Wound Healing Effect.* Scratch wounds were made to determine the effects of red LED light irradiation on cell migration. As shown in Figure 6, the wound area was reduced more in the 10 and 20 min group at 12 and 24 h after wounding than in the 5 min group. The rate of HGF-1 cell migration was increased significantly after 5, 10, and 20 min irradiation compared to that of the control at 12 h after wounding ($p < 0.05$). On the other hand, the rate of migration at 5 min was similar to that at 20 min irradiation. At 24 h after wounding, 10 min irradiation increased the rate of HGF-1 cell migration. The 10 min irradiation stimulated the migration of HGF-1 cells between 12 and 24 h after wounding. These results suggest that 10 min irradiation has a good effect in stimulating the migration of HGF-1 cells, which is consistent with the MTT assay result. L929 cell migration was more active than HGF-1 cells according to the microscopic images.




(a)

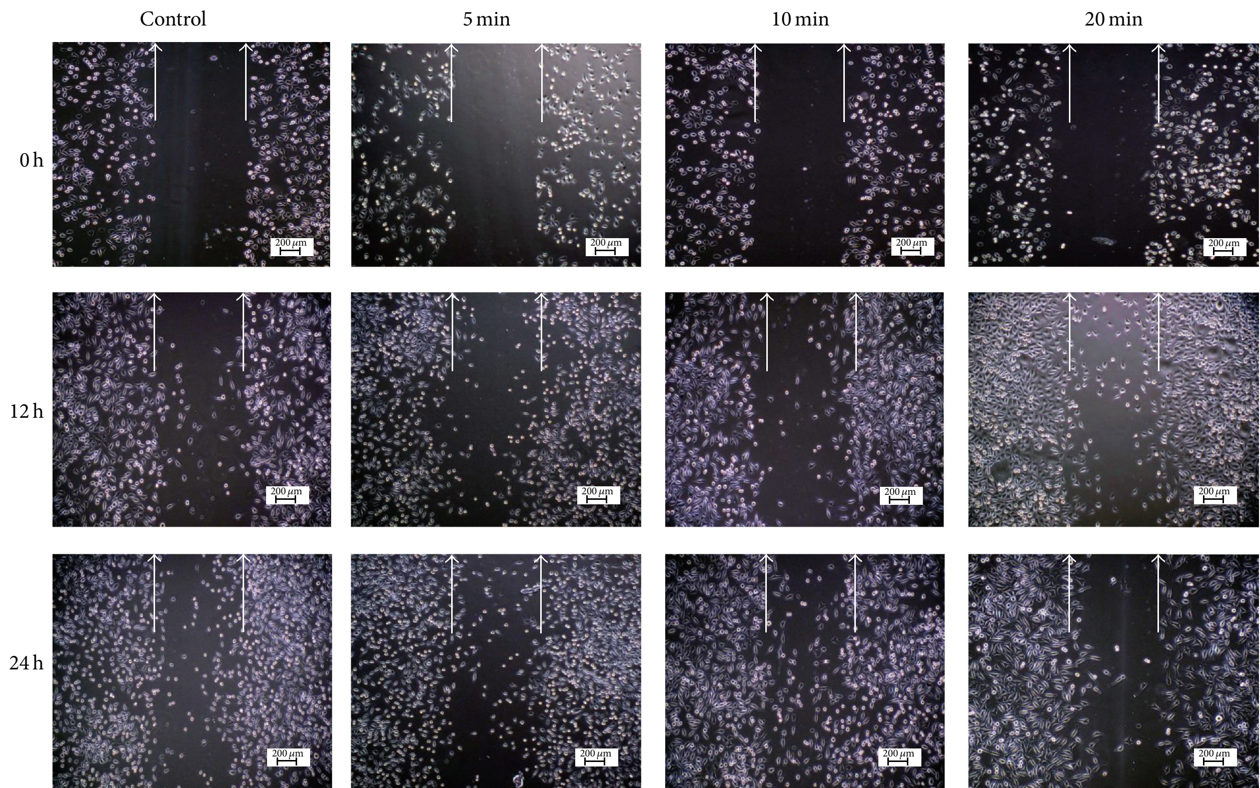



(b)

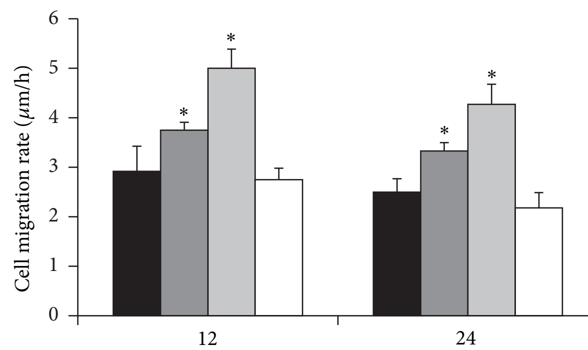
Figure 6: Images of scratch assays of HGF cells (a). Wound healing was observed using an inverted microscope and a digital camera at 12 and 24 h after wounding. Original magnification ×100. Arrows indicate the starting position of the cell migration. Cell migration is toward the center of the wound. Graph of HGF cell migration rate (b). Scratched cells were exposed to 660 nm LED light with an intensity of 8.5 mW/cm$^2$ for 5, 10, and 20 min.

The migration rate increased significantly at 5 and 10 min compared to that of the control L929 cells and was similar to that observed at 20 min after wounding ($p < 0.05$). At 24 h after wounding, 5 and 10 min irradiation increased the rate of L929 cell migration compared to the control, which was similar to the pattern observed at 12 h, but this increase in rate was lower than that observed at 12 h (Figure 7).

Erdle et al. examined the wound healing effect of 670-nm LED light on incisions and burn injuries in hairless mice and suggested that red light exposure might be helpful in postoperative wound repair [21]. Their results showed that although 670-nm LED light was not as effective for burn injuries, it did accelerate healing in the skin of hairless mice with incisions [21]. In addition, DNA synthesis and the growth of osteoblasts were increased by laser light [33]. In blue light emission, the cellular activity was lower in mouse fibroblasts and HGF-1 cells but was enhanced in oral epithelial cells [34, 35]. This suggests that the effect of light emission might depend on




(a)




(b)

FIGURE 7: Images of scratch assays of L929 fibroblasts (a). Graph of L929 cell migration rate (b). Arrows indicate the starting position of the cell migration. Scratched cells were exposed to 660 nm LED light with an intensity of 5.5 mW/cm$^2$ for 5, 10, and 20 min.

the characteristics of the cells. The wavelength range from 670 nm to 880 nm induces the growth of human epithelial cells at different intensities, 50 mW, 40 mW, and 53 mW/cm$^2$ [14]. Moreover, a previous wound healing study showed that the intensity and duration are essential factors during the irradiation of red LEDs to improve the biological activity. This study clearly shows that 660 nm red LEDs promote cell migration, which is consistent with the viability in typical intensity and duration. Nevertheless, the cellular signaling mechanism of 660 nm LEDs on the migration of the cells remains to be determined.

## 4. Conclusion

A red LED illumination-cell culture system was designed and developed to evaluate red LED light's effect on the cellular bioactivity for cell proliferation and wound healing using L929 and HGF-1 cells. The optimal intensity of 660 nm

red LEDs to stimulate and promote cell migration in the 5 and 10 min exposure groups of HGF-1 and L929 cells was 5.5 and 8.5 mW/cm$^2$, respectively. The results were cell dependent. These results suggest that 660 nm red LED might be a good source for phototherapy in wound healing medical treatments.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Myung-Sun Kim and Yong-Ick Cho equally contributed to this study.

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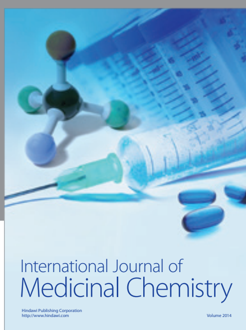
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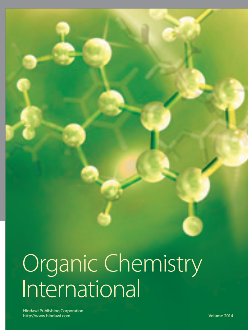
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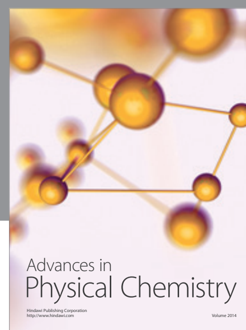
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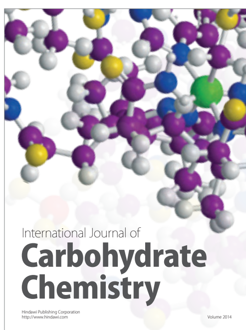
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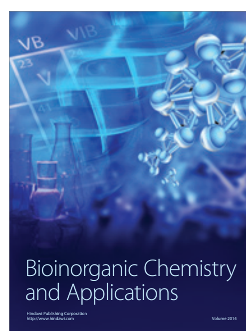
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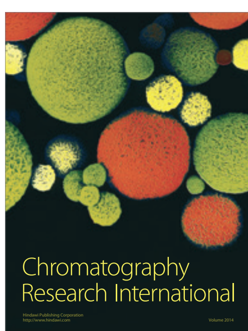
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