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Original Article

630 nm Light Emitting Diode Irradiation Improves Dermal Wound Healing in Rats

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Purpose: To determine the effects of 630 nm light emitting diode (LED) on full-thickness wound healing.
Methods: Twelve male Sprague-Dawley rats were randomly divided into LED (n = 6) and control group (n = 6). Two 19.63 mm^2 wounds were created on the mid dorsum. LED group received a 630 nm LED irradiation with 3.67 mW/cm^2 for 30 minutes (6.60 J/cm^2) for 7 days, while control group received sham LED irradiation. Epithelial gap, collagen density, α-SMA fibroblast and PCNA keratinocyte were measured on histochemical and immunohistochemical staining using image analysis system. An independent t-test was conducted to compare the difference between groups.
Results: The wound closure rate, collagen density, α-SMA fibroblast number, epithelial gap and PCNA keratinocyte number have shown no significant difference between LED and control group at day 3 after the treatment. At day 7 after the treatment, the wound closure rate in LED group was increased when compared with control group ($p < 0.05$). The collagen density ($p < 0.05$) and α-SMA immunoreactive fibroblast number ($p < 0.001$) were increased when compared with control group at day 7. The epithelial gap in LED group was significantly shorten than control group at day 7 ($p < 0.01$). The PCNA positive cell number in LED group was higher than control group at day 7 ($p < 0.01$).
Conclusion: 630 nm LED with 3.67 mW/cm^2, 6.60 J/cm^2 accelerate collagen deposition by stimulating fibroblasts, and enhance wound contraction by differentiating myofibroblasts in the dermis, and accelerate keratinocyte proliferation by facilitating DNA synthesis in the epidermis. It may promote the healing process in proliferation stage of wound healing.

Keywords: Light emitting diode irradiation, Wound healing, Fibroblasts, α-Smooth muscle actin, Keratinocytes, Proliferating cell nuclear antigen

INTRODUCTION

In physical therapy, various electrophysical agents such as electrical stimulation, electromagnetic stimulation, low-level laser therapy (LLLT), ultrasound, ultraviolet light and negative pressure therapy have been used to enhance wound healing.[1] Among these electrophysical agents, electrical stimulation established the evidence for beneficial effect on wound healing. Many clinical practice guidelines recommended electrical stimulation for recalcitrant wound that do not respond to standard wound care.[2,3]

The second commonly used modalities for wound management are LLLT and ultrasound. LLLT were used as a phototherapy for wound care more than 30 years ago. There are contradictory reports on therapeutic benefit of LLLT on wound healing in clinical trials in human subjects. In a Cochrane review, it has found that there is no evidence of any therapeutic benefit associated with LLLT on wound healing.[4] They concluded that well designed clinical trials are required to determine the evidence of LLLT in wound care. The major reason for the failure of establishing the evidence of LLLT on wound healing is the insufficient clinical trials in human models.

The three major therapeutic parameters of LLLT are wavelength, irradiance, fluence. The therapeutic effects of LLLT depend on

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power density and irradiation duration. As evidence based medicine is disseminated, Consolidated Standards of Reporting Trials (CONSORT) statement extension is recommended to improve the reporting of nonpharmacologic randomized controlled trials (RCT). However many reports of laser therapy did not describe key irradiation parameters adequately such as miscalculating the energy density of laser.[5] Also many reports are found to have poor reporting of eligibility criteria for treatment providers and setting.

In animal models, the effectiveness of LLLT on promoting wound healing is well established. In a systematic review from Peplow and his colleagues[5], as they analysed 47 animal wound studies including acute-wound, impaired-healing, and chronic-wound, it is concluded that the studies consistently demonstrated the positive effect of laser therapy to wound healing process.

Up until the end of the 1990's, phototherapy was dominated by these laser sources, because light emitting diode (LED) was highly divergent with low and unstable output powers, and a wide wavelength band. Since National Aeronautics and Space Administration (NASA) has introduced a new generation of near-infrared LED in 1998, 600s nm monochromatic red LED has been developed.[6]

Recently, LED array devices are commercially introduced as an alternative for LLLT. LED phototherapy is used in a large variety of clinical indications such as pain relief, wound healing, musculoskeletal injuries, skin rejuvenation, inflammatory conditions and so on.[6] Laser light source is cumbersome and expensive for routine use. LED light source, however, is more cost efficient than laser source, because it is cheap, can irradiate more surface area, have low risk to tissue damage.[6] The major difference between LED and laser light is coherency. The radiation coherence does not play any significant role in the cellular activity during wound healing process,[7] and coherence does not seem essential to the effects of laser phototherapy.[5] While many studies for LLLT in wound healing have been reported, relatively not much research has been reported for LED in wound healing. Peplow and his colleague's systematic review[5] included 47 animal studies, while 40 studies used LLLT phototherapy, only 7 studies used 600s nm LED phototherapy.

In the proliferative phase, granulation tissue formation, re-epithelialization, angiogenesis, collagen deposition, and wound contraction occur. Promoting cell proliferation, accelerating collagen synthesis and promoting the granulation tissue formation are suggested as the mechanisms of LLLT enhancing wound healing. Fibroblast, myofibroblast and keratinocyte are important cells of the wound healing process. Fibroblasts secrete collagen into the granulation tissue, it increases the strength of the wound. Myofibroblasts differentiated from the fibroblasts are responsible for contraction. Keratinocytes contribute to re-epithelialization of epidermis.[8,9]

Chronic wound care including biological and biosynthetic dressings, skin substiture and growth factors requires much time, effort and money.[9] There is a growing need for study about the effect of LED on wound healing.

The purpose of this study was determine the effects of 630 nm LED array irradiation can influence fibroblast proliferation and collagen deposition, myofibroblast expression and keratinocyte proliferation on full-thickness wound in proliferative phase of wound healing.

METHODS

1. Animals

The study was conducted by Department of Physical Therapy, Wonkwang Health Science University. All animal experimental protocol complied with national guidelines for the humane treatment of laboratory animals. Twelve male Sprague-Dawley rats, weighing 260 ± 10 g (Koatech, Pyeongtaek, Korea), were used for this study. Animals were adapted for a period of 3 days. Rats were housed in standard bio-clean cages (20 × 38 × 56 cm), and bred in the animal room, where environmental conditions were kept constant condition (temperature, 22 ± 1°C; humidity, 60%; 12 hours light-dark cycle). The animals were fed a standard laboratory diet and had water ad libitum.

2. Excisional skin wounds

The hair on the back of the rat was shaved and cleaned with povidine-iodine and alcohol. Two 19.63 mm^2 circular full-thickness wounds including the panniculus carnosus were created on the mid-dorsum using a 5 mm diameter of sterile biopsy punch (Stiefel Laboratories, Inc., Wächtersbach, Germany). The excisions were placed 4 cm apart. The wound was cleaned with a sterile gauze pad, and the rat was carefully observed until it had recovered fully from the anesthesia. Wounds were kept open throughout the entire experiment, without dressing.

3. LED irradiation

Rats were randomly allocated into either LED group (n = 6) or control group (n = 15) without treatment. In LED group, the rats received 30 minutes of LED irradiation over the wound area for seven consecutive days post-operatively. The energy density was 6.60J/cm^2 each session. Control animals were treated the same way but not switched on. LED (IWL-R5R30F-TNB, Itswell Co., Ltd., Incheon, Korea) had the following characteristics: aluminium gallium indium phosphide (AlGaInP), wavelength 630 (618-635) nm (red), diode diameter 5 mm (surface area 0.20 cm^2), irradiance 3.67 mW/cm^2. The calibration of LED was performed with a laser power meter (FieldMax-TOTM, Coherent Inc., Wilsonville, USA).

4. Wound surface area measurement

The wound surface area (WSA) was measured by tracing and planimetry. A transparency film placed over the wound, and traced the perimeter of the wound on the film with a fine-tipped pen, The wound tracings were traced with digital planimetry (Vistrak Digital, Smith & Nephew Medical Limited., Hull, England), then the WSA was determined. Planimetric measurement of WSA has high inter-rater reliability ($r = 0.91$, $p < 0.01$). The wound closure rate (WC) was calculated using the following formula: WC (%) = [(WSA at day 1-WSA at day 7)/WSA at day 1] × 100.

5. Histochemical and immunohistochemical analysis

For histochemical analysis, a 6 mm diameter of full-thickness wound biopsy samples were obtained at 7 days of post-wound. Samples were fixed in 10% phosphate buffered formalin, they were paraffin embedded, sectioned 5 μm thick, and stained with hematoxylin and eosin (H & E) and Masson trichrome (MT).

For immunohistochemical analysis, the sections were incubated with mouse anti-human alpha smooth muscle actin (α-SMA) antibody (1:400, Dako, Glostrup, Denmark) and mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (Clone PC10, 1:200, Dako, Glostrup, Denmark) after microwave pre-treatment with citrate buffer for 30 minutes at room temperature, respectively. After slides were rinsed in TBS, the horse radish peroxidase (HRP)-polymer (Thermo Scientific, Walthman, USA) was applied for 30 minutes at room temperature. Visualization of the reaction was performed using diaminobenzidine (DAB) as a chromogen. After washing, slides were counterstained with Gill's hematoxylin and mounted with DPX. For negative control, sections were stained without primary antibody.

The deposited collagen density in regenerating granulation tissue, the epithelial gap distance, α-SMA immunoreactive fibroblast and PCNA immunoreactive keratinocyte were assessed by image analysis system (Image-Pro® Plus, Media Cybernetics, Inc., Silver Spring, USA). The analysis was performed by an experienced pathologist in a double-blind manner.

6. Statistical analysis

All data are presented as means ± SD. An independent t-test was performed to compare the difference between LED and control groups. To assess inter-rater reliability, the Pearson product-moment correlation coefficient was used. The statistical interpretation was based on a 0.05 significance test level. SPSS WIN (ver 12.0) software was used for the analyses .

RESULTS

The wound closure rate, collagen density, α-SMA immunoreactive fibroblast number, epithelial gap and PCNA positive cell number has shown no significant difference between control and LED group at day 3 after the treatment (data not presented). At day 7 post excision wound, wound closure rate, collagen density, α-SMA immunoreactive fibroblast number, epithelial gap and PCNA positive cell number of control and LED group were shown in Table 1.

1. Wound closure rate

In the course of ongoing wound healing, wound surface areas de-

Table 1. The wound healing rate, collagen density, α-SMA immunoreactive fibroblast number, epithelial gap and PCNA immunoreactive keratinocyte number in control and LED group

	Control Group (n = 6)	LED Group (n = 6)	t	p
Wound closure rate (%)	72.42 ± 10.54	85.87 ± 3.11	-2.998	0.025
Collagen density (%/mm^2)	28.72 ± 7.38	37.54 ± 5.69	-2.320	0.044
α-SMA(+) fibroblast (cell/mm^2)	52.18 ± 9.61	133.15 ± 21.50	-8.422	0.000
Epithelial gap (mm)	2.34 ± 0.95	0.74 ± 0.37	3.403	0.007
PCNA(+) keratinocyte (cell/mm^2)	21.82 ± 9.74	78.72 ± 35.78	-3.758	0.010

Values are number or mean ± standard deviation.
α-SMA, alpha smooth muscle actin; PCNA, proliferating cell nuclear antigen.

creased in control and LED groups were compared to the initial wound surface area. However, at day 7 after the treatment, the wound closure rate was 13.45% greater ($p < 0.05$) in LED group compared to control group.

2. Collagen deposition in granulation tissue

In MT staining, collagen fibers were observed across the entire area of granulation tissue in LED group (Figure 1B) in contrast to sparse collagen fibers distributed in a dispersed manner in control group (Figure 1A). The collagen density in LED group was significantly greater than that in control group at day 7 ($p < 0.05$) (Figure 1C).

3. α-SMA immunoreactive myofibroblasts

In immunohistochemical staining, α-SMA immunoreactive myofibroblasts were detected in the granulation tissue in both control and LED groups. α-SMA positive myofibroblasts appeared to be more populous in LED group (Figure 2B) than that in control group (Figure 2A). Myofibroblast number in LED group was significantly greater than that in control group ($p < 0.001$) at day 7 (Figure 2C).

4. Epithelial gap

In H & E staining, the epithelial gap of LED group showed shorter distance between the wound edges than control group at day 7 (Figure 3A, B). There is a significant decrease in the epithelial gap in LED irradiate rats compared to that of control rats at day 7 ($p < 0.01$) (Figure 3C).

5. PCNA immunoreactive fibroblast

In PCNA immunohistochemical staining, PCNA immunoreactive keratinocytes were observed in the epidermis (Figure 4A, B). PCNA immunoreactive keratinocytes showed more populous in LED group. The PCNA positive cell number in LED group was higher than control group at day 7 ($p < 0.01$) (Figure 4C).

DISCUSSION

Macroscopic, quantitative histochemical and immunohistochemical analyses enable the assessment of the progress of wound healing and treatment effect. This study demonstrated that 630 nm LED irradiation with irradiance of 3.67 mW/cm^2, energy density of 6.60 J/

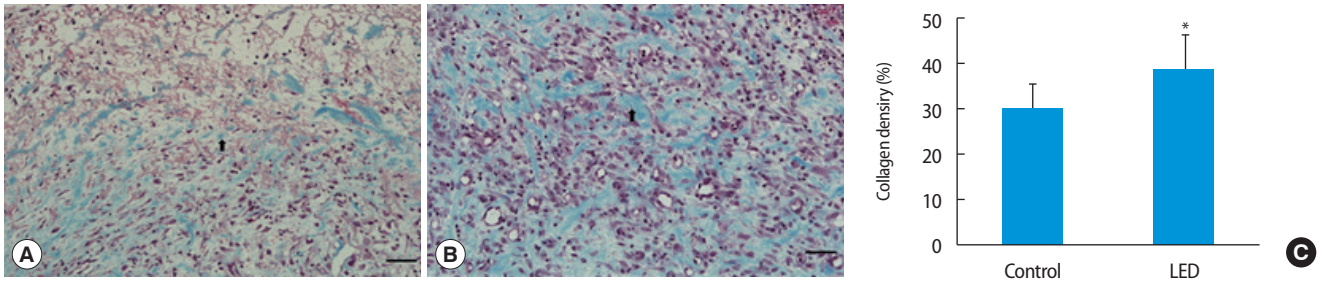

Figure 1. Comparison of the collagen density of wound between control and LED group. The green stained collagen fibers (arrow) in the granulation tissue of control (A) and LED group (B). LED group showed more collagen deposition in dermis. Masson trichrome stained, Magnification: × 200; Scale bar: 20 μm. There is a significant increase in collagen density in LED group compared to that of control group at day 7 ($p < 0.05$). The bars and error bars indicate mean and standard deviation, respectively (C).

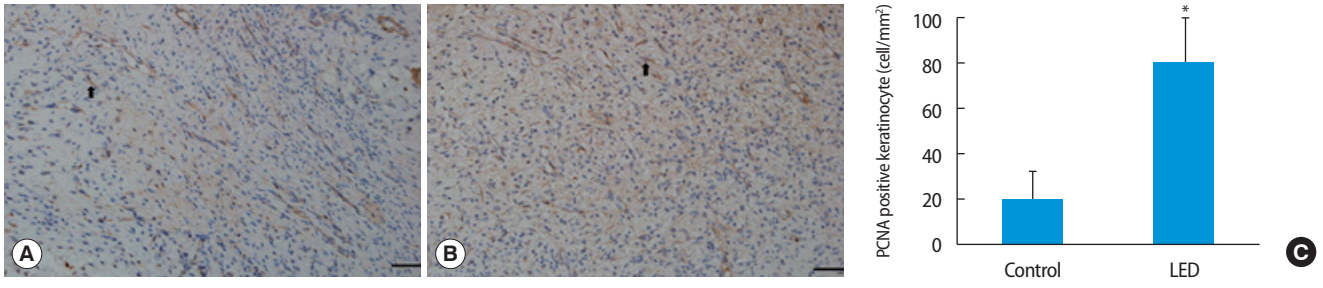

Figure 2. Comparison of the α-SMA positive fibroblast number (cells/mm^2) of wound between control and LED group. The brown stained α-SMA immunoreactive fibroblast (arrow) in the granulation tissue of control (A) and LED group (B). LED group showed greater α-SMA positive fibroblast. α-SMA immunostained, Magnification: ×200; Scale bar: 20 μm. There is a significant increase in α-SMA positive cell number in LED group compared to that of control group at day 7 ($p < 0.001$). The bars and error bars indicate mean and standard deviation, respectively (C).

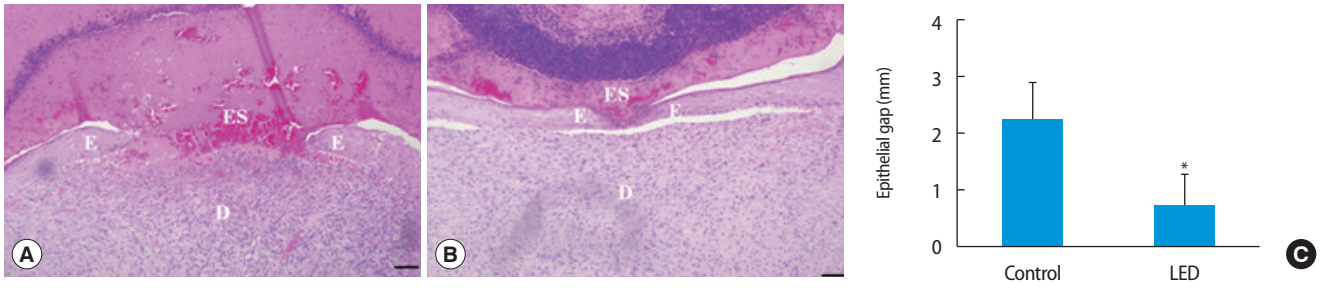

Figure 3. Comparison of the epithelial gap of wound between control and LED group. The epithelial gap of control (A) and LED group (B). The epithelial gap of LED group showed shorter distance between the wound margins. Hematoxylin and eosin stained, ES: eschar, E: epidermis, D: dermis. Magnification: ×100; Scale bar: 50 μm. There is a significant decrease in the epithelial gap in LED irradiate rats compared to that of control rats at day 7 ($p < 0.01$). The bars and error bars indicate mean and standard deviation, respectively (C).

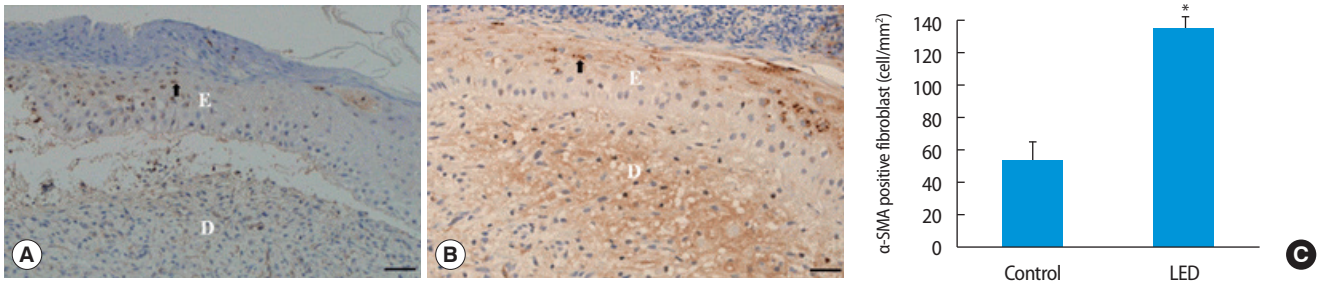

Figure 4. Comparison of the PCNA positive keratinocyte number (cells/mm^2) of wound between control and LED group. The brown stained PCNA-immunoreactive keratinocyte (arrow) in the regenerated epidermis of control (A) and LED group (B). LED group showed more PCNA positive keratinocyte in epidermis. PCNA immunostained, Magnification: ×200; Scale bar: 20 μm. There is a significant increase in PCNA positive cell number in LED group compared to that of control group at day 7 ($p < 0.01$). The bars and error bars indicate mean and standard deviation, respectively (C).

cm^2 promotes healing of excision wound in the rat. The parameters of LED irradiation used in this study are based on previous studies.[7,10,11]

LED irradiation was increased the wound closure rate and collagen density in situ at day 7 post excision wound, This result suggest that LED irradiation stimulate the fibroblast proliferation and collagen deposition in the granulation tissue. In addition, LED irradiation was increased α-SMA immunoreactive fibroblasts. This result could indicate that LED irradiation stimulated the differentiation of fibroblasts, and myofibroblast may have contributed to reduction of the wound area. The presence of α-SMA represents the most reliable marker of the myofibroblastic phenotype.[12] The fibroblasts differentiate into myofibroblasts during wound healing process. Myofibroblasts are characterized by their cytoskeleton, which contains contractile α-smooth muscle actin, an actin isoform also present in smooth muscle cells. This contractile cytoskeleton can develop tensile force, and decrease wound area by contracting the edges of the wound.[13,14]

Our results are similar with the results from other studies. Several studies have been reported that 630 nm and 640 nm LED irradiation with 4-24 J/cm^2 decreased wound surface area.[10,15-17]

Our results agree with the findings from several other studies that used 640 nm and 700 nm LED with 15-16 J/cm^2 to stimulate fibroblast proliferation and collagen production on excision wound in rats.[16,18,19]

In the present study, epithelial gap of LED group showed significantly shorter distance between the wound edges at day 7 post-wound in H & E stained specimens. Moreover, PCNA immunoreactive keratinocytes increased significantly in LED group in PCNA immunostained specimens. This result indicates that LED irradiation promotes the proliferation and migration of keratinocytes in epidermis. This result could indicate the role of keratinocytes in re-epithelialization of epidermis by 630 nm LED irradiation during wound healing process.

The process of re-epithelialization of a wound in the epidermis comprises the following steps: proliferation of basal keratinocytes, migration of epidermal cells to the wound surface, and differentiation.[20] PCNA is an antigen that is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle. PCNA is a marker of cell proliferation.[21] Expression of PCNA-immunoreactive kerati-

nocytes reflects cell proliferation.[22]

Previous studies showed that 670 nm LED irradiation with 40 mW/cm^2, 3.6 J/cm^2 improved the wound gap on incision wound in mice,[6] and 630 nm LED irradiation with 300 mW/cm^2, 24 J/cm^2 improved the process of reepithelialization on surgical wound in rats.[17] Our results agree with the results of these studies, despite the difference of irradiance and fluence of LED.

Previous studies reported the collagen synthetic activity by measurement of hydroxyproline level in the cultured fibroblasts or from the wound tissue. Biochemical assay of the collagen in wound tissue is inaccurate method. Whereas histological assessment can be visualised and quantify the collagen density in situ. In contrast with other previous studies, we confirmed the collagen deposition in the granulation tissue by histochemical examination. Futhermore, in previous studies, there has been few reports of α-SMA and PCNA immunohistochemical assays, our study performed quantitative assessment of the myofibroblasts and proliferating keratinocytes by immunohistochemistry in proliferative phase of wound healing process.

The major difference between LED and laser light is coherency. Laser emits collimated and coherent light, which has high energy and potential risk to tissue damage. Unlike laser, LED emits noncoherent light. LED light is more divergent than laser light, and has less energy per spectral band as the photons spread over a larger area. Although these differences exist, LED can irradiate on the action area with similar energy concentration of laser.[23] The radiation coherence does not play important role during wound healing process.[5,7] Furthermore, LED arrays can be irradiated in large areas and can minimize the risk of tissue damage. Also LED is inexpensive than laser. Therefore, LED phototherapy seems to be an alternative for the traditional LLLT phototherapy.

We concluded that LED with 630 nm, 3.67 mW/cm^2, 6.60 J/cm^2 accelerate collagen deposition by stimulating fibroblasts and enhance wound contraction by differentiating myofibroblasts in the dermis. Also these LED enhance keratinocyte proliferation in the epidermis. It may improve the healing process in a proliferation stage of wound healing. LED light could be used for wound management as an inexpensive phototherapy modality. Further studies are needed to determine the optimal parameters of LED for wound healing and the effect of human wounds for clinical setting.

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