Optimal Fluence and Duration of Low-Level Laser Therapy for Efficient Wound Healing in Mice

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Background: Low-level laser (light) therapy is a promising technology that stimulates healing, relieves pain and inflammation, and restores function in injured body parts. However, few studies have compared the effects of light-emitting diodes of different fluence levels or different treatment durations. Objective: Here, we investigated the effects of various fluence levels and treatment durations on wound closure in mice. Methods: Full-thickness wounds were created on the dorsal skin using an 8-mm diameter punch, and the wounds were irradiated at 1, 4, or 40 J/cm² for 5 consecutive days starting on day 1. To determine the optimal irradiation duration, wounds were irradiated at the most potent fluence of previous study for 5, 10, or 15 days. Photographic documentation, skin biopsies, and wound measurements were performed to compare the effects of different treatment parameters. Results: The most effective fluence level was 40 J/cm² at day 5, as determined by monitoring wound closure. There were no statistically significant differences in wound healing with different durations. Conclusion: We have shown that repeated exposure to low levels of light significantly stimulates wound healing in mice and demonstrated more efficient wound closure with certain fluences of 830 nm irradiation. (Ann Dermatol 33(4) 318 ∼ 323, 2021)

INTRODUCTION

Low-level laser (light) therapy (LLLT) is a promising technology used in various fields to stimulate healing, relieve pain and inflammation, and restore function to injured body parts. Since the initial experiments in 1983 studying the effects of low-level HeNe laser irradiation on wounds in rats, many studies have investigated wound healing by LLLT. Low-level lasers can affect lymphocytes, increasing their proliferation and activation; macrophages, increasing their phagocytosis; and fibroblasts, increasing their growth factor secretion and enhancing the uptake of both fibrin and collagen. In addition, LLLT increases the motility of epithelial cells and the amount of granulation tissue produced during healing, and may reduce the synthesis of inflammatory mediators, resulting in reductions in skin wound area in both humans and animals. However, the optimal physical variables for LLLT still lack consensus. A few studies have directly compared the effects of different fluences of LLLT. Da Silva et al. investigated the effects of a 670 nm-wavelength laser on rats, by irradiating skin lesions with 0, 2, or 4 J/cm² for 10 consecutive days. At 4 J/cm², the re-epithelialization process was significantly faster than that in the other groups. A study using a 632.8 nm-wavelength laser reported that 3 ∼ 6 J/cm² photostimulation facilitates the tissue repair process in diabetic wound healing by accelerating the rates of contraction and collagen production. With irradiation at 830 nm, a preliminary investigation demonstrated that 5 J/cm² LLLT improved wound healing, as measured by increased...
wound tensile strength. A study in mice comparing the influences of 632.8, 785, and 830 nm lasers on burn wound healing found that treatment with 830 nm light at a fluence of 3 J/cm² had profound effects on healing compared to untreated controls and mice treated with lasers of other wavelengths. However, no studies have compared the effects of different fluences and irradiation durations on wound size reduction.

In this study, we describe the effects of LLLT on wound size reduction in a standardized model of full-thickness excisional wound healing in mice, using an 830-nm diode laser with various fluence levels and durations of irradiation.

### MATERIALS AND METHODS

#### Animal selection and care

Eight-week-old female albino hairless mice (Skh:hr-1) weighing 25~30 g were maintained in individual ventilated cage systems. The animals were group-housed, ten mice per cage. Constant temperature, humidity, and a 12-hour light/dark cycle were maintained, and the mice were fed a standard diet. All experimental protocols were approved by the Committee for Animal Care and Use of Ajou University (approval no. 2017-0016).

#### Wound creation and LED irradiation

After anesthesia by intraperitoneal injection of tiletamine/zolazepam (Virbac, Seoul, Korea) and xylazine (Bayer, Seoul, Korea) which was necessary to immobilize the mice, four full-thickness wounds were created on the dorsal skin of each mouse using a 8-mm-diameter punch. The wounds were left exposed without sutures or dressings. A total of 40 mice were used to compare the effects of different irradiation fluences on wound healing. The mice were divided randomly into untreated control (group A) and treated groups (n=10 per group). The wounds of the treated groups were irradiated at fluences of 1 J/cm² (group B), 4 J/cm² (group C), or 40 J/cm² (group D) for five consecutive days starting on day 1 when the wounds were made. To determine the optimal duration of treatment, 30 mice (n=10 per group) were treated with 40 J/cm² infrared light for 5 (IR5), 10 (IR10), or 15 (IR15) consecutive days. A low-intensity LED irradiation device named SHINeY (WON TECH Co., Seoul, Korea) was used as the light source. The intensity was 100 mW/cm² and the spot size was 4.77 mm x 13.15 mm. The distance between the light source and the dorsal skin was approximately 3 cm. Nonirradiated (control) mice were maintained under similar conditions.

#### Photo documentation and wound closure analysis

On days 1, 5, 10, and 15, images of the wounds were acquired, and the wound areas were measured as the primary outcome using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA). The wound size immediately after wound creation was designated the original wound area. The percentage of wound closure at each time point was calculated using the following formula and defined as the secondary outcome.

\[
\frac{\text{original wound area} - \text{area on day } x}{\text{original wound area}} \times 100
\]

#### Statistical analysis

ANOVA was used to compare wound size reduction between treatment groups and the statistical analyses were performed using R software, version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria). \( p \)-values < 0.05 were considered statistically significant. Summary data are expressed as the mean ± standard deviation.

### RESULTS

#### Effect of LED irradiation fluence on wound closure

Our first goal was to identify the optimal fluence of LED irradiation to reduce the time required for wound closure. There were some variations in the sizes of the initial wounds, due to the difficulty in creating wounds in the flexible skin of the mice. Therefore, the relative wound area was analyzed along with the absolute values. Compared to the baseline values for each group, the wound areas in all groups steadily decreased over time (Fig. 1, 2). The wounds of all groups almost closed on day 15. Therefore, to assess when the effects of irradiation on wound...
healing appear, the analysis of wound closure at day 3, 5, 10 and 15 was done. When comparing the relative wound area in group A (control) with irradiated groups (group B, C, and D) respectively, more efficient wound closure was observed in group D (40 J/cm²) on day 5 (Table 1). At the same time, there was no significant difference in wound healing between groups A and B (1 J/cm²), C (4 J/cm²) respectively. Additionally, post hoc analysis was done and compared groups to each other. The most efficient fluence on day 5 was 40 J/cm² (group D), which demonstrated significantly improved wound healing compared to all the groups. After day 5, no significant difference was observed among groups.

**Effect of the duration of LED irradiation on wound closure**

The other goal of our study was to identify the best duration of LED irradiation for wound healing. The effects of 5, 10, and 15 days of radiation (groups IR5, IR10, and IR15, respectively) were compared. Compared to their baseline values, wound areas steadily decreased in all groups (p < 0.001; Fig. 3, 4, and Supplementary Fig. 1). The wound healing was significant at day 5. The wounds of all groups almost closed on day 15. There was no significant difference in wound area reduction between the IR5 and IR10 groups nor between the IR5 and IR15 groups (Table 2). Post hoc analysis revealed no significant difference among the groups.

**DISCUSSION**

Wound closure involves the migration of the boundaries of an injury towards its center and can be assessed through related parameters, such as the percentage of wound contraction. In this study, we investigated the effect of LED irradiation fluence on wound closure and the effect of the duration of LED irradiation on wound closure. Regarding the results of day 5, the most potent fluence was 40 J/cm². Demidova-Rice et al. evaluated the effects of laser therapy on excisional wounds and found that the dose effects are not linear for various fluences of 635-nm light, with a maximum positive effect at 2 J/cm². They reported that intensities of 1 and 10 J/cm² improved healing to a lesser extent, while 50 J/cm² had a negative effect on wound healing. Using 670-nm laser therapy, treatment at 4 J/cm² displayed superior wound healing than treatment at 8 J/cm². Inadequate doses can result in weak and insignificant effects; while excessive doses can cause negative or minimal effects. With even higher doses, a biosuppres-
Fig. 3. Wound area after treatment with different durations of irradiation.

Table 2. Comparisons of wound areas according to group by time

| Variable | Day 1 (baseline) | Day 5 | Day 10 | Day 15 |
|----------|-----------------|-------|--------|--------|
| Group    |                 |       |        |        |
| IR5      | 100 (0)         | 35.26 (7.78) | 9.85 (4.36) | 7.57 (3.32) |
| IR10     | 100 (0)         | 36.02 (11.26) | 8.85 (2.53) | 7.31 (1.71) |
| IR15     | 100 (0)         | 36.27 (12.24) | 8.64 (3.59) | 7.01 (1.98) |
| p-value* | NA              | 0.905 | 0.276  | 0.598  |

Post-hoc analysis†

| IR5 vs. | IR10 | IR5 vs. | IR10 vs. |
|---------|------|---------|----------|
| NA      | >0.999 | 0.644 | >0.999 |
| NA      | >0.999 | 0.401 | 0.936 |
| NA      | >0.999 | >0.999 | >0.999 |
| NA      | >0.999 | >0.999 | >0.999 |

Values are presented as mean (standard deviation). NA: not applicable. IR5: 5 days of irradiation, IR10: 10 days of irradiation, IR15: 15 days of irradiation. *p-values are obtained by using ANOVA. †Data are p-values, which are obtained by using t-test and corrected using the Bonferroni adjustment, which are significant when <0.05.

In contrast to these studies, we used 830-nm light and observed an optimal fluence of 40 J/cm². As light at this wavelength can penetrate the skin more deeply, we hypothesize that a higher fluence of irradiation might be required for wound healing at 830 nm. Further study is needed to investigate whether over 40 J/cm² of fluence of irradiation has harmful effect on wound healing.

However, after day 5, we observed no statistically significant differences between the groups. Wound closure begins with an inflammatory phase and re-epithelialization, followed by the remodeling phase, which generally begins 5 to 7 days after injury. In a previous study, while healing curves generated for control mice demonstrated an initial decrease in wound size during days 1 to 4 after injury, the wounds of LLLT-treated mice started to contract immediately after illumination. Therefore, 5 days of irradiation could be adequate to reduce the wound area.

The basic biological mechanism behind the effects of LLLT is thought to involve the absorption of red and near-infrared light by mitochondrial chromophores, in particular cytochrome c oxidase (CCO), a component of the mitochondrial respiratory chain. CCO activation results in increased production of adenosine triphosphate (ATP), which provides both the energy and phosphate required to regulate a variety of cellular functions. Consistent with this notion, the addition of exogenous ATP stimulated wound healing in an animal model. Although wound contraction did not increase in mice treated with external ATP, in vitro observations suggest that ATP increases wound contraction by serving as an energy source for motility and contractile force generation, and as a phosphate donor for kinases regulating contraction.

Regarding the wound healing of human skin, not only wound closure but also prevention of hypertrophic scars and keloids have great importance. In the context of formation of hypertrophic scar, the remodeling phase has critical role. Fibroblastic proliferation and excess collagen deposits are their two main characteristics, and imbalances in the rates of collagen biosynthesis and degradation, along with individual genetic predisposition, have been implicated in their pathogenesis. It was recently proposed that poor regulation of interleukin (IL)-6 signaling and TGF-β1 expression may play a significant role in this process.
LLLT can decrease IL-6 mRNA levels\textsuperscript{26}, and has been proposed as an alternative therapy for hypertrophic scars. In three case studies, Barolet and Boucher\textsuperscript{27} reported significant improvements to scars after LLLT following scar revision by surgery or CO\textsubscript{2} laser ablation. In fact, the mice in our study did not show any hypertrophic scar or keloid. Therefore, it is difficult to evaluate the effectiveness of LLLT for prevention of hypertrophic scar or keloid. Further studies to evaluate the effectiveness of LLLT for prevention of human hypertrophic scars or keloids are needed.

In conclusion, we have shown that repeated exposure to low levels of light significantly stimulates wound healing in mice and demonstrated more efficient wound closure with certain fluences of 830-nm irradiation. Conversely, the duration of irradiation did not significantly affect wound healing. Further studies regarding human wound healing will be required to examine the applicability of these results to clinical LLLT.

SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-33-318-s001.pdf

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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