FULL PAPER *Laboratory Animal Science*

The Effect of Low Reactive-Level Laser Therapy (LLLT) with Helium-Neon Laser on Operative Wound Healing in a Rat Model

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ABSTRACT. The effect of low reactive-level laser therapy (LLLT) with a He-Ne laser on operative wound healing was investigated in a rat model. 10-millimeter surgical wounds were created on the backs of Sprague Dawley rats, and animals were assigned to one of eleven groups (n=5). Ten groups received either 8.5 mW or 17.0 mW irradiation of 15 seconds LLLT a day with one of five different irradiation frequencies, i.e. daily (from the 1st to 6th day following surgery), every other day (the 1st, 3rd, and 5th day), on only the 1st day, on only the 3rd day, and on only the 5th day; the 1st day was the day following the surgery. The control group received no irradiation. A skin specimen was harvested from the dorsal thoracic region on the 7th day to measure the rupture strength. The control group had the lowest rupture strength (5.01 N), and the 17.0 mW every other day irradiation group had the highest rupture strength (13.01 N). Statistical differences were demonstrated in the 8.5 mW irradiation setting between the every other day irradiation group and the control group ($p<0.05$); and in 17.0 mW irradiation setting between the every day irradiation, the every other day, and the 1st day only groups vs. the control group ($p<0.01$). Histological examination demonstrated that wound healing in the 17.0 mW every other day irradiation group was promoted most significantly such as the prevention of excessive inflammation, increased formation of collagen fibers, and recovery in continuity of tissues. The control group showed poor wound healing and the other experimental groups showed intermediate healing. Thus LLLT with a He-Ne laser was found to promote the healing of operative wounds in the present rat model, in which the most favorable application of LLLT was the 17.0 mW setting of 15 seconds a day with a frequency of every other day.

KEY WORDS: He-Ne laser, LLLT, low reactive-level laser therapy, wound healing.

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Low reactive level laser therapy (LLLT) [26] has been utilized clinically since the first successful cases reported by Mester [21–23]. Many studies have been published and various effects of LLLT have been reported such as (1) promotion of wound healing [3, 5, 14–19, 28, 29, 31–33], (2) alleviation of pain [4, 7, 32], (3) improvement in local circulation [20], and (4) alleviation of inflammation [2, 7, 8], and (5) bactericidal effects [12].

In spite of this various fundamental questions have not yet been answered. Regarding wound healing, for example, it is not certain when LLLT should be performed following injury, or what level and frequency of irradiation is effective [35, 36]. There are also reports that deny any effect of LLLT on wound healing [9, 28], and doubts about its effect on neurological functions have also been voiced [7]. One of the reasons why such controversy arose may be the lack of an evaluating method to enable us to examine the effects of LLLT with sufficient accuracy.

Focusing attention on the promotion of wound healing by LLLT, and on the fact that the functional evaluation of the wound healing could be realized by utilizing mechanical tests, we developed a precise method for measurement of the rupture strength of the wound. Hence in this study, in order to find an irradiation setting that promotes wound healing effectively, we performed an experimental animal study. We made operative wounds on the backs of rats, irradiated the wounds with an He-Ne laser with specific irradiation settings and frequency, and harvested the tissue surrounding and including each wound on the 7th day following surgery. Then we measured the rupture strength of the operative wounds by means of mechanical tests, and examined the specimens histologically.

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MATERIALS AND METHODS

Test animals: 55 male SD rats aged 8 weeks (body weight: 238–330 g, mean: 264.1 g) were used. The experiment was conducted in compliance with the protocol approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University, and in compliance with the Committee's guidelines.

Preparation of the operative wounds: The rats were anesthetized with isoflurane (administered by means of inhalation), and 10 mm operative wounds were made using a No. 11 scalpel on the dorsal thoracic region (from the superior angle of the scapula and in the inferior direction), dorsal lumbar region (from the 13th lumber vertebra and in the inferior direction) and dorsal buttock region (from the iliac crest and in the inferior direction) as shown in Fig. 1. The incisions were deep enough to reach just above the fascias. Each wound was then closed with simple interrupted 4–0 nylon sutures; specifically, three sutures were used per wound, one at the middle of the wound (5 mm from each end), and one near each end of the wound (1 mm from each

Fig. 1

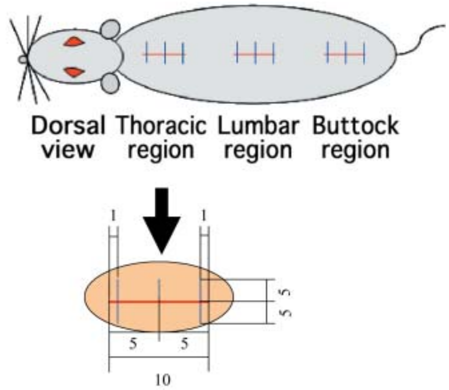

Fig. 2

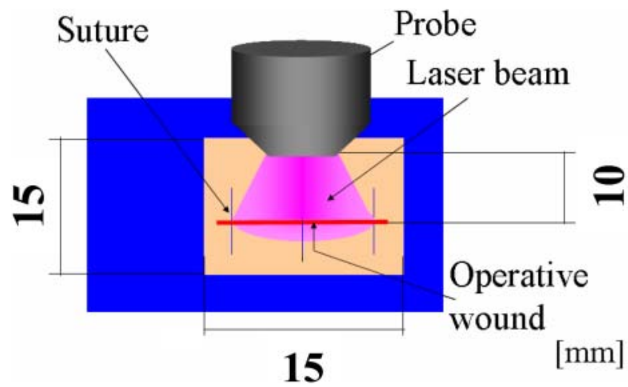

Fig. 3

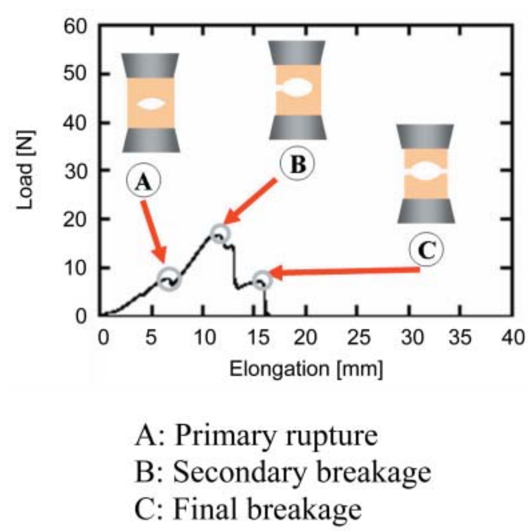

Fig. 1. Positions and size of operative wounds (mm).
Fig. 2. He-Ne laser irradiation method.
Fig. 3. Typical load-elongation curve of a skin specimen containing operative wound. The load at Point A was determined as the rupture strength of the wound.

end). Mosquito forceps were used to arrest bleeding during surgery. Morever, enrofloxacin was used at 5 mg/kg, once a day for 3 days to prevent infection.

Laser system used in the experiment: An He-Ne laser (He-Ne Cold Laser Stimulator Model PDT-A2, Lead Medical Science, Tokyo, Japan) was used. This system has two independent laser source points, each of which emits an 8.5 mW laser beam.

He-Ne laser irradiation settings: In this experiment, we set up two irradiation settings; in the 8.5 mW setting, laser light was emitted from one source point via a straight laser probe, and in the 17.0 mW setting, laser light was emitted from both source points simultaneously via a Y-shaped laser probe. The irradiation period was set at 15 seconds per operative wound. The irradiation of 8.5 mW produced power density (PD) of 139 mW/cm^2 and energy density (ED) of 2.09 J/cm^2, and irradiation of 17.0 mW produced PD of 281 mW/cm^2 and ED of 4.21 J/cm^2 were measured with a light power meter (TQ8212; Advantest, Tokyo, Japan). We assigned 5 rats to each of 11 groups: one group as a non-irradiation control, and ten irradiation groups which received either 8.5 mW or 17.0 mW of 15 seconds LLLT per operative wound a day with one of five different irradiation frequencies, i.e., daily (from the 1st to 6th day following surgery), every other day (the 1st, 3rd, and 5th day), on only the 1st day, on only the 3rd day, and on only the 5th day; the 1st day was the day following surgery.

He-Ne laser irradiation method: Each rat with operative wounds on its back was secured in the prone position in a Ballman cage, and the tip of the laser probe was situated such that the laser beam would irradiate each operative wound perpendicularly as shown in Fig. 2. The distance from the tip of the laser probe to the operative wound was set at 10 mm. In order to prevent the laser beam from reflecting and scattering onto other operative wounds, we prepared drapes made of aluminum foil and paper towel with a window of 15 mm × 15 mm, and placed a double-layer of this drape on the back of the rat such that the target wound would be exposed through the window. The wound was then irradiated for 15 second.

Preparation of skin specimens and tensile testing: On the 7th day, animals were sacrificed with an intraperitoneal overdose injection of sodium pentobarbital. Rectangular specimens of dorsal skin with operative wounds were harvested from all rats in each group. The skin specimens were 40 mm long and 12 mm wide. The specimens from the thoracic region were used in the tensile tests. Each end of the specimen was glued between two stainless steel plates, each 20 mm × 20 mm × 0.5mm, using cyanoacrylate adhesive (Aron Alpha, Toagosei, Tokyo, Japan). The distance between the glued stainless steel plates at each end of the specimen (the gage length of the specimen) was 20 mm. In order to prevent any change in the skin's properties, the specimens were placed in saline until the tensile testing. Tensile tests were conducted with a mechanical testing machine (Instron 1185; Instron Japan. Kanagawa, Japan) within three hours after preparation of the specimens. The sutures used to close the wounds were removed immediately prior to loading. The crosshead speed was 5 mm/min.

Analysis of data obtained in tensile testing: The load and elongation of each skin specimen were measured, and these data were plotted as a load-elongation curve as shown in Fig. 3. The load of each specimen at the time of primary breakage was determined as the rupture strength of the operative wound.

Histological examination: A skin specimen with an operative wound was harvested from the dorsal lumbar region on the 7th day after surgery, and fixed in 10% formalin solution, embedded in paraffin, and thin sections were prepared. The operative wound and surrounding skin tissues (epidermis, immediately below the epidermis, dermis, the fat layer of the deep dermis, and cutaneous muscle) were stained with hematoxylin-eosin, and observed with a light microscope, and collagen fibers were analyzed with a polarization microscope. The skin specimens from the buttock region were stored in 10% formalin solution for additional histological examination.

Statistical analysis: In each group, the mean and the standard deviation of the rupture strength were calculated. The effects of the irradiation setting, 8.5 mW vs 17.0 mW, and the irradiation frequency on the mean rupture strength were examined by a two-way analysis of variance (ANOVA). Holm's multiple comparison test was performed as a post hoc analysis.

RESULTS

Tensile testing: In the tensile tests, the rupture strengths of the operative wounds were measured. The results are summarized in Fig. 4. Two-way ANOVA demonstrated that both the irradiation setting and the frequency affected the mean values of the rupture strength ($p<0.01$).

In a multiple comparison test, the mean rupture strength of the operative wound in the 8.5 mW every other day irradiation group was significantly greater than that of the non-irradiation group ($p<0.05$). The mean rupture strength of the operative wound in the 17.0 mW every day, 17.0 mW every other day, and 17.0 mW on only the 1st day irradiation groups was significantly greater than that of the non-irradiation group ($p<0.01$). The 17.0 mW every other day irradiation group had significantly greater rupture strength than the 17.0 mW on only the 5th day irradiation group ($p<0.05$). No significant difference was observed between the other groups.

Histological findings: Although histological study was performed for all 11 groups, only the results of the non-irradiation group, the 8.5 mW on only the 5th day irradiation group, and the 17.0 mW every other day irradiation group

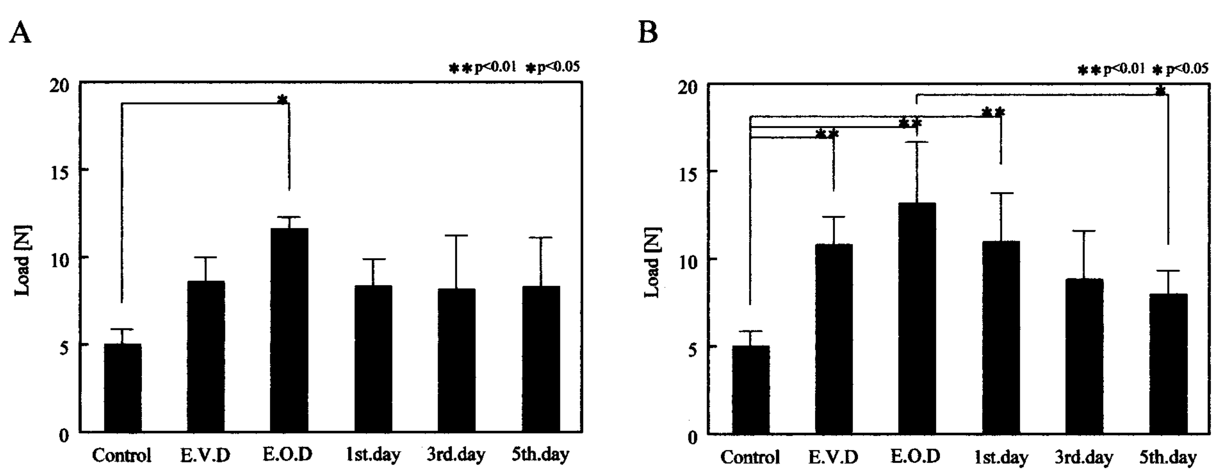

Fig. 4. Rupture strength of operative wounds. mean ± S.D., n=5. control: non-irradiation group, E. V. D.: daily irradiation (from the 1st to 6th day) group, E. O. D.: every other day (1st, 3rd, 5th day) irradiation group, 1st day: irradiation on only the 1st day groups, 3rd day: irradiation on only the 3rd day group, 5th day: irradiation on only the 5th day group. A: The 8.5 mW He-Ne laser irradiation groups and the non-irradiation group. B: The 17.0 mW He-Ne laser irradiation groups and the non-irradiation group.

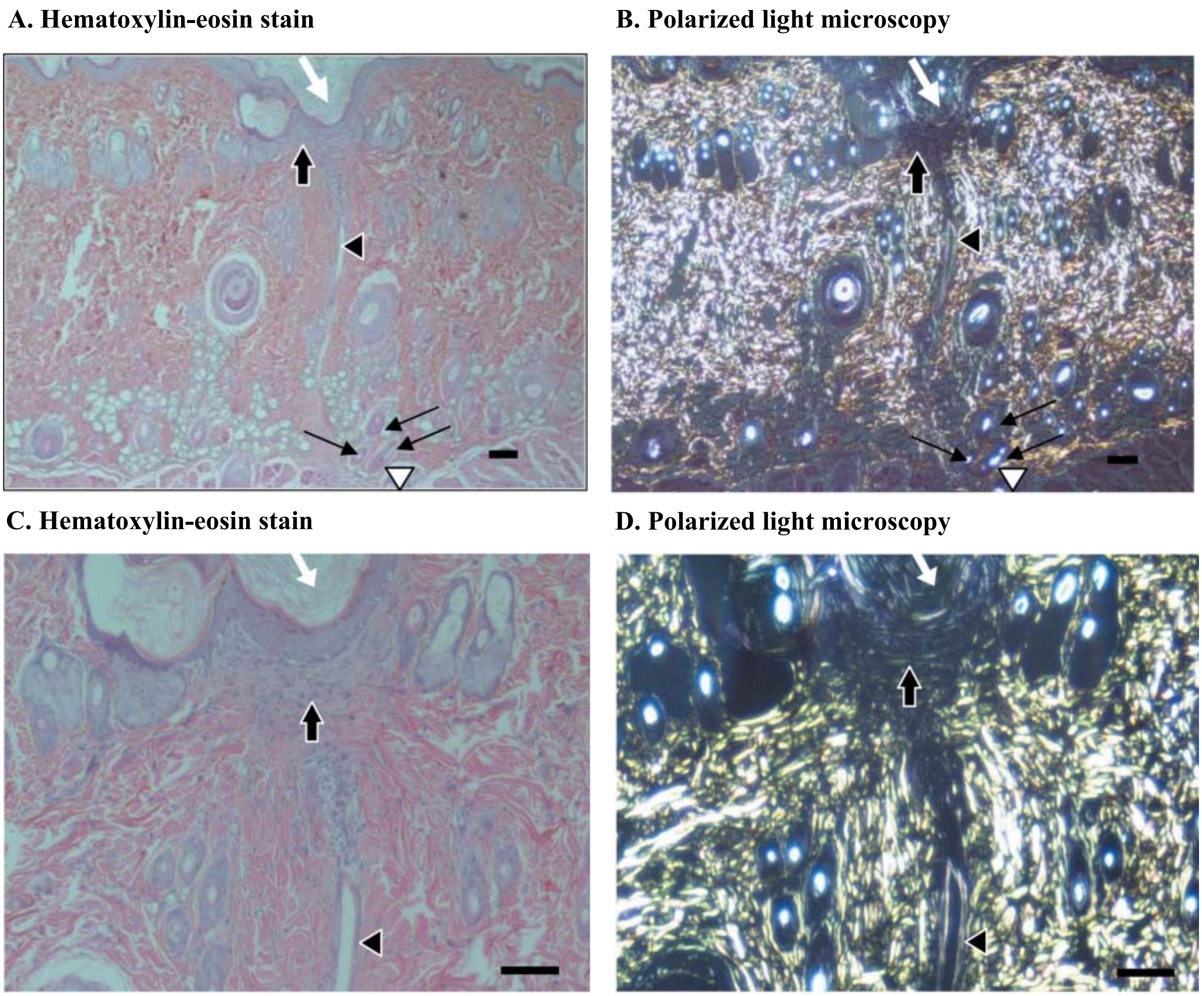

Fig. 5. A, B, C, D. Histological findings 7 days following surgery in the non-irradiation group. White arrow (⇨) indicates the position of the operative wound. Bar = 10 μm. Epidermis: The epidermis was uneven and severely pitted over a wide area. Keratinization and moderate thickening were observed around the position indicated by the white arrow (⇨). Vacuolar degeneration of prickle cells was observed for the full thickness of the epidermis. Infiltration of lymphocytes and mild infiltration of fibroblasts with enlarged nuclei were observed immediately below the epidermis (➡). Dermis: The actual incision was made obvious by rough and clear collagen fibers; however, formation of collagen fibers was not distinct in the surrounding region. A mild increase was observed in the number of fibroblasts with enlarged nuclei (▲). The continuity of the fat layer of the deep dermis was severely disrupted, and many small to medium-sized, undifferentiated hair follicles had formed. Significant infiltration of these cells into the disrupted muscle layer was observed (▽). Cutaneous muscle: The continuity of the muscle layer was severely disrupted around the incision, and the area appeared to be swollen. Collagen fibers had been deposited around the incision, and fibroblasts, lymphocytes, and newly developed capillary vessels were observed (△).

are presented here as the representative results.

The typical histology of the non-irradiation group is shown in Fig. 5, in which the same area in the histological sections was observed by bright-field microscopy (A and C) and by polarized microscopy (B and D). The inflammation was obvious as the epidermis was thickened and uneven, and infiltration of inflammatory cells was observed. Although the incision was distinctly visualized by the presence of collagen fibers, collagen fibers in the dermis were unclear. There were severe losses of continuity in the fat layer and cutaneous muscle, whose area appeared to be swollen.

The typical histology of the 8.5 mW on only the 5th day irradiation group is shown in Fig. 6. Mild inflammation was observed as the epidermis had thickened mildly, and mild infiltration of lymphocytes was observed immediately below the epidermis. The formation of collagen fibers in the dermis was not significant, but it was more obvious than in the non-irradiation group. The continuity of the fat layer of the deep dermis and the muscle layer was disrupted.

The typical histology of the 17.0 mW every other day irradiation group is shown in Fig. 7. There was no inflam-

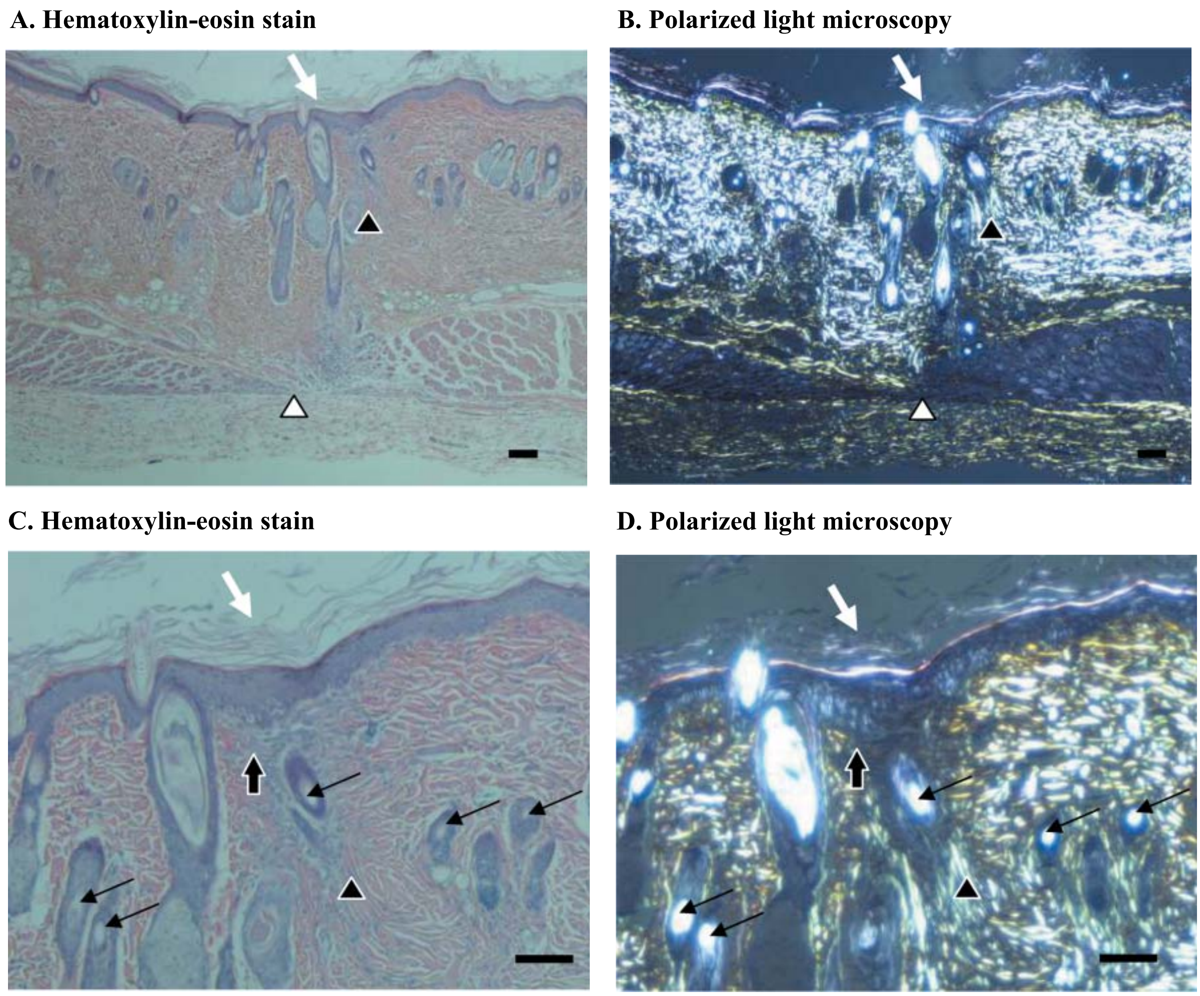

Fig. 6. A, B, C, D. Histological findings 7 days following surgery in the 8.5 mW on only the 5th day irradiation group. White arrow (⇨) indicates the position of operative wound. Bar = 10 μm. Epidermis: The epidermis has thickened mildly. The surface was uneven and moderately pitted. Moderate keratinization was observed around the position indicated by the white arrow (⇨). Vacuolar degeneration of prickle cells was observed from the middle layer and through to the basal layer. Mild infiltration of lymphocytes and fibroblasts was observed immediately below the epidermis. The number of fibroblasts increased moderately and their nuclei were enlarged (➡). Dermis: Newly developed clear collagen fibers were present in irregular locations, and they had varying thicknesses and no structure (▲). The nuclei of fibroblasts were enlarged. A mild increase was observed in the number of fibroblasts. The continuity of the fat layer of the deep dermis was moderately disrupted, and multiple medium-sized hair follicles had newly developed (→). The infiltration of a significant number of these cells into the disrupted muscle layer was observed. Cutaneous muscle: The continuity of the muscle layer was moderately disrupted around the incision. Collagen fibers had been deposited around the incision to a moderate extent, and fibroblasts, lymphocytes, and newly developed capillary vessels were observed (△).

mation, since neither thickening of the epidermis nor infiltration of inflammatory cells was observed. Remarkably, the formation of collagen fibers was observed in the gap of the operative wound. The continuity of the fat layer of the deep dermis had recovered, and the structure of the cutaneous muscle layer had also recovered.

Histological findings in the other groups were similar to the findings observed in the 17.0 mW every other day irradiation group and in the 8.5 mW only 5th day irradiation group.

DISCUSSION

Tensile testing of the operative wounds was performed in this study to reveal the functional effect of LLLT on wound healing. Allendorf *et al.* [3] similarly conducted tensile tests of wounds in a rat and irradiated them with a He-Ne laser, but found no significant difference in strength between the irradiated and non-irradiated groups. However, in their study the rats underwent general anesthesia at each irradiation session, which was carried out each day following surgery. It is commonly observed that repeated general

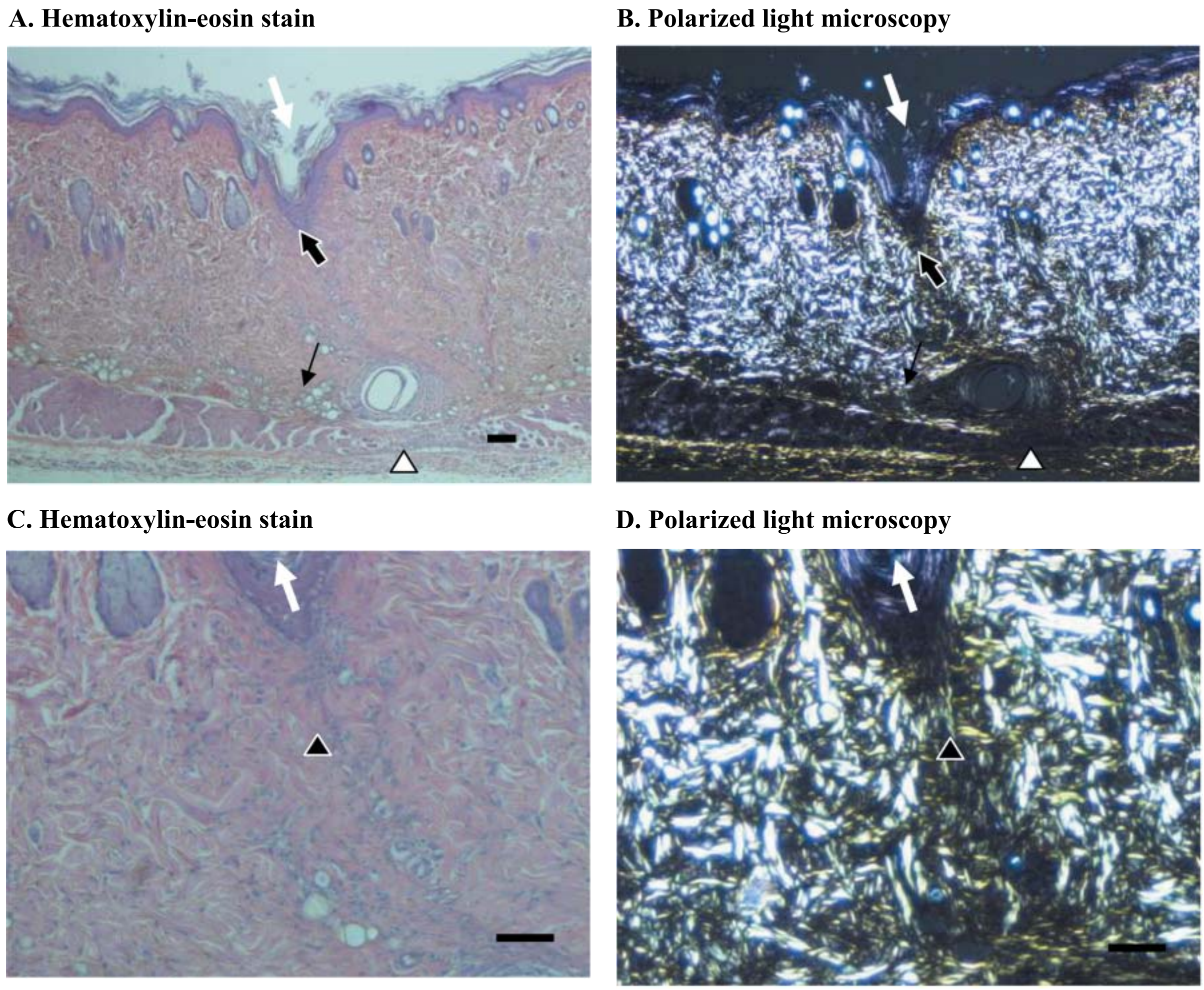

Fig. 7. A, B, C, D. Histological findings 7 days following surgery in the 17.0 mW every other day irradiation group. White arrow (⇨) indicates the position of the operative wound. Bar = 10 μm. Epidermis: The surface was uneven and moderately pitted. Mild keratinization was observed around the position indicated by the white arrow (⇨). No significant change was observed immediately below the epidermis (➡). Dermis: Dense and well-structured collagen fibers were aligned vertically along and inside the incision (▲). The fat layer of the deep dermis had re-formed well, except for the area of incision (→). Cutaneous muscle: The continuity of the muscle layer was completely restored (△).

anesthesia cause the suppression of cardiovascular, metabolic and immunological functions, leading to the delay of the wound healing. This delay of wound healing would possibly veil the effects of the LLLT on the results of tensile testing. Hence in this study, a Ballman cage was utilized to hold the rats, and laser irradiation was done without general anesthesia. Stadler *et al.* [29] also performed tensile tests of the operative wounds made in a diabetic murine model and irradiated the wounds with a diode laser, but found no significant difference between the irradiated and non-irradiated groups within the first 11 days following surgery. Delayed wound healing in diabetes might be responsible for this ineffectiveness of LLLT. Hence in this study, normal rats were utilized for experiments.

The shape of the test specimens is another important factor in the strength evaluation, although previous investigators did not pay much attention to it. We used experimented specimens that contained both the wound itself and the thin healthy skin at each end of the operative wound. It is considered important in the clinical point of view to conduct the strength evaluation at the 7th day when the primary closure of wound takes place [30]. In that case, it is necessary to set up certain width of the healthy skin region at each end of the operative wound for tensile testing. The thin healthy skin at each end of the operative wound would prevent the breakage during the preparation of specimens. However, the wider length causes the lower of sensitivity of tensile testing. Considering the above, we had conducted the tensile test of the specimens which had 1 mm, 2 mm or 3 mm width of healthy skin and demonstrated that the width of healthy skin made no significant difference in the rupture strength among these three types of specimens (unpublished data).

From this result, we judged that the width of healthy skin does not affect the tensile test results and this type of specimens could be favorably utilized for evaluation of the wound healing. In this study we used skin specimens with a 1-mm margin at each end of the operative wound for convenience of handling.

The rupture strength of the operative wound was increased by the irradiation of an He-Ne laser at irradiation settings of both 8.5 mW and 17.0 mW. The irradiation condition that enabled us to realize the maximum rupture strength was irradiation every other day rather than daily irradiation, although without statistical significance. Interestingly, the 17.0 mW on only the 1st day irradiation group demonstrated greater rupture strength compared with the 17.0 mW on only the 3rd day and 17.0 mW on only the 5th day irradiation groups. This suggests that irradiation on the 1st day following the operation might increase the rupture strength. However, a similar tendency was not observed in the 8.5 mW on only the 1st day, only the 3rd day and only the 5th day irradiation groups. Further study is necessary to elucidate the optimum irradiation settings.

Histological observations demonstrated the alleviation of excessive inflammation, the increased formation of collagen fibers, and the recovery of continuity of tissues in the 17.0 mW every other day irradiation group. On the other hand, there were indications of inflammation, poor formation of collagen fibers, and discontinuity of tissues in the non-irradiation group. The degrees of healing in other irradiation groups were intermediate between the two groups described above. These histological findings were inconsistent with the finding that the 17.0 mW every other day irradiation group had the highest rupture strength. The increased formation of collagen fibers would be responsible for the higher strength.

On the mechanism of LLLT in wound healing, two major hypotheses have been proposed. Kami [13] reported that laser irradiation induces a mild inflammatory reaction that upregulate angiogenesis around the wound with subsequent increase in blood flow, and thus promotes wound healing. On the other hand, Nagashima *et al.* [25] proposed that LLLT prevents harmful and severe inflammatory reactions, and hence leads to the increased formation of collagen fibers. Our histological findings supported the latter hypothesis; the 17.0 mW every other day irradiation group, which had the highest rupture strength, had less infiltration of inflammatory cells than the non-irradiation group. This hypothesis provides a reasonable explanation for the fact that the 17.0 mW on only the 1st day irradiation group had greater rupture strength; since inflammation is most severe on the 1st day following surgery, and the He-Ne laser irradiation of an operative wound at this stage is effective in alleviating inflammation as Aczick *et al.* [1] and Clark[5,6] have reported. However, our study has yet to clarify the alleviation mechanism of inflammation by either LLLT suppressing the level of inflammation or accelerating the process of inflammation in the early stage of wound healing. This should be elucidated in the future study.

The anti-inflammatory effect of LLLT has been confirmed by many studies. Reports on the therapeutic effect of lasers on rheumatoid arthritis [4, 11, 24, 27], which is a typical inflammatory disorder, stated that lasers significantly alleviate symptoms and improve conditions by means of an anti-inflammatory and analgesic effect. Laser irradiation of cultured synovial cells from patients with rheumatoid arthritis significantly decreased the production of IL-1β [11]. Laser irradiation inhibits inflammatory cells and cytokines [2, 10, 27]. Although molecular mechanism of LLLT has not been elucidated completely, the anti-inflammatory effect of laser irradiation may be one of the most important therapeutic effects on many injuries and disorders including wound healing.

In conclusion, our study demonstrates that LLLT with He-Ne lasers can promote the healing of operative wounds, by which the optimal condition was at least the 17.0 mW (PD of 281 mW/cm^2 and ED of 4.21 J/cm^2 a day) with a frequency of every other day.

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