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830-nm Irradiation Increases the Wound Tensile Strength in a Diabetic Murine Model

Istvan Stadler, PhD,* Raymond J. Lanzafame, MD, MBA, Ryan Evans, BS, Vikram Narayan, BS, Brian Dailey, MD, Norene Buehner, BA, and John O. Naim, PhD
The Laser Center and Department of Surgery, Rochester General Hospital, Rochester, New York

Background and Objective: The purpose of this study was to investigate the effects of low-power laser irradiation on wound healing in genetic diabetes.
Study Design/Materials and Methods: Female C57BL/Ksj/db/db mice received 2 dorsal 1 cm full-thickness incisions and laser irradiation (830 nm, 79 mW/cm^2, 5.0 J/cm^2/wound). Daily low-level laser therapy (LLLT) occurred over 0–4 days, 3–7 days, or nonirradiated. On sacrifice at 11 or 23 days, wounds were excised, and tensile strengths were measured and standardized.
Results: Nontreated diabetic wound tensile strength was 0.77 ± 0.22 g/mm^2 and 1.51 ± 0.13 g/mm^2 at 11 and 23 days. After LLLT, over 0–4 days tensile strength was 1.15 ± 0.14 g/mm^2 and 2.45 ± 0.29 g/mm^2 ($P = 0.0019$). Higher tensile strength at 23 days occurred in the 3- to 7-day group (2.72 ± 0.56 g/mm^2 LLLT vs. 1.51 ± 0.13 g/mm^2 nontreated; $P \leq 0.01$).
Conclusion: Low-power laser irradiation at 830 nm significantly enhances cutaneous wound tensile strength in a murine diabetic model. Further investigation of the mechanism of LLLT in primary wound healing is warranted. Lasers Surg. Med. 28:220–226, 2001.

Key words: biostimulation; collagen; diabetes; murine; fibroblast; impaired wound healing; laser; low-power laser therapy; wound; wound healing; wound tensile strength; experimental surgery; LLLT; type I diabetes

INTRODUCTION

Wound healing is a complex, well-choreographed response to injury characterized by inflammatory, proliferative, synthetic, and maturation phases. The inflammatory phase includes hemostasis, platelet degranulation, and activation of the complement and clotting cascades, which provide a scaffold for wound healing [1]. Platelet degranulation is responsible for the release of a series of potent cytokines [2–6]. Macrophages take the governing role during the inflammatory phase, being responsible for debridement of the injured area, matrix synthesis, angiogenesis, and the synthesis of nitric oxide, which regulates wound fibroblasts [7]. A reduction in the inflammatory response inhibits healing in clinical and experimental diabetes [8,9], whereas its prolongation results in the stigmata of the fibroproliferative diseases [10].

The appearance of endothelial cells and fibroblasts marks the start of the proliferative phase [11]. Fibroblasts are transformed into "wound fibroblasts" [12] that migrate from the surrounding tissue, become proliferative, and produce collagen. Regulation of excess collagen deposition by collagenase activity signals the start of the maturation and remodeling phases. The main feature of the maturation phase is the deposition of collagen. This is the most important phase of wound repair because the rate, quality, and total amount of the deposited matrix determines the strength of the scar. Collagen I is the predominant type of collagen (90%) in the matured scar, whereas collagen III comprises up to 30% of the collagen in granulation tissue and does not contribute to wound strength [13]. Increased collagen synthesis persists for at least 3–5 weeks after wounding and is due to the increased number of fibroblasts and a net increase in collagen production per cell.

Newly synthesized collagen is biochemically and physically different and has a smaller fiber diameter than unwounded skin [14]. Unwounded skin shows a basket weave arrangement of collagen fibers, whereas the scar's fibers are parallel to the skin. Fiber thickness and orientation with wound tensile strength are positively correlated [15]. The healed scar never becomes as organized as the nonwounded dermis. The progressive increase in the tensile strength of a normally healing wound is 3% of nonwounded tissue at 1 week after wounding, 20% at 2 weeks, and 80% at 4–6 weeks, with no subsequent increase thereafter [16,17].

Severely impaired wound repair occurs in diabetes mellitus, regardless of the type. Diabetics tend to heal much more slowly than normal individuals, and the resultant wounds are typically of poorer quality. The exact mechanism of impaired healing in diabetes is not clear, and the underlying causes may vary. The shorter duration of the inflammatory phase may contribute to impaired wound healing. Decreased collagen synthesis and deposition resulting from a poor nutritional supply to the fibroblasts are thought to be primary causes of the impairment observed. Healed skin incisions in an insulin-

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*Correspondence to: Istvan Stadler, PhD, The Laser Center, Rochester General Hospital, 1425 Portland Avenue, Rochester, NY 14621. E-mail: Istvan.Stadler@viahealth.org
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deficient animal model are 35% lower in tensile strength at 6 weeks than nondiabetic controls [16–18].

Low-level laser therapy (LLLT) has been used as an alternative therapy in wound management since Mester and associates [20] reported the beneficial effect of He-Ne and argon lasers in 1,120 clinical cases of ulcers and nonhealing wounds. The use of LLLT in the treatment of impaired wound healing has been used more extensively in Europe than in the United States. Despite the extensive worldwide use of LLLT, it is not yet a universally accepted clinical tool in wound healing management. The parameters necessary for successful treatment are uncertain [20,21]. Recent studies showed that LLLT at 890 nm and at 660 nm did not improve the wound closure rate in a radiation-impaired mouse skin wound-healing model [22,23]. Yu and coworkers [24] reported that LLLT at 630 nm enhanced the rate of wound closure in genetically diabetic mice. Photodynamic therapy, using benzoporphyrin derivative monoacid ring and chloroaluminium sulfophtalocyanin, did not modulate wound healing in rats [25]. He-Ne laser irradiation enhanced collagen production in the healing of rabbit Achilles tendon [26]. Sliney reported [27] that irradiation with a diode laser at 904 nm or a He-Ne laser enhanced wound tensile strength in rabbits with linear incisions at 21 days after wounding. He also reported that low-level laser irradiation at 830 nm and at a fluence of 3 J/cm^2 was partially effective in diabetic patients with nonhealing leg ulcers and was more effective than the He-Ne laser.

This study was undertaken to show the effect of low-energy level laser irradiation at 830 nm on wound tensile strength in genetically determined diabetes (type I diabetes) in a murine model. The efficacy of LLLT treatment delivery at different time points in the healing process (i.e., 0–4 or 3–7 days after wounding) was compared.

MATERIALS AND METHODS

Animals

Homozygous, genetically diabetic C57BL/Ksj/db/db mice (Jackson Laboratory, Jackson, ME) were used for the experimental groups. Heterozygous littermates were used as nondiabetic controls. Animals were fed standard chow (LabDiet; PMI Nutrition International, Brentwood, MO), water ad libitum, and were housed in caging located in a controlled environment animal room for the duration of the study. Animals were caged individually after undergoing wounding to avoid tampering with the wound by other animals. Homozygous animals develop the stigmata of type I juvenile diabetes and develop marked obesity, hyperglycemia, and are less active than the heterozygotes. Heterozygous animals do not show any traits of diabetes.

Wounding Procedure

Animals were divided into groups of 10 animals per group in each experimental and control study regimen. Animals were anesthetized with ketamine-xylazine mixture IP, and the dorsum of each subject was shaved. The shaved surface was first cleaned with an alcohol pad, followed by the application of Betadine for skin disinfection. Two, full-thickness, 1-cm longitudinal incisions were made with a no. 15 blade scalpel on the dorsal skin. The incisions were placed 2 cm apart by using a template to ensure consistent placement of the wounds on each animal. Two simple 6.0 Polypropylene sutures were placed to hold the wound edges in apposition. Nonwounded animals were handled in a similar fashion, except for the absence of an incision, to serve as sham-operated controls.

Laser Irradiation

A class IIIb 830-nm diode laser (model LAS-300-830-100; LaserMax, Inc., Rochester, NY) was used for wound irradiation. The laser output had a nominal power of 85 mW at 149.3 mA. The spot size of the beam was a 0.5 mm $\times$ 10 mm oval. The actual irradiance delivered to the wound was 79 mW/cm^2 at a distance of 2.5 cm.

The animals were irradiated daily to achieve a total fluence of 5 J/cm^2/wound during each treatment over five consecutive treatment days. The animals were divided into two equal treatment groups to undergo daily treatment at either 0–4 days or 3–7 days after injury.

Both wounds on each animal were irradiated in the same fashion. The control group animals underwent sham irradiation.

Tensile Strength Measurements

Wound-breaking strength was measured by using a custom-built tensiometer (Fig. 1), which contained the following components: a Burleigh 600, a piezo-electric stepper motor with control unit, a Mark IV gauge on a walking holder, and a clamp system. (LaserSurg Inc, Rochester, NY). The moving part (i.e., the gauge on the walking holder) pulls the sample vertically at a constant speed of 20 mm/min. Increasing resistance is recorded in grams and is displayed in the window of the gauge, which halts when the sample tears apart (Fig. 1). The value obtained is considered to be the maximal wound-breaking strength. Tensile strength was calculated by using the following formula:

$$\mathrm{TS} = \frac{\mathrm{MBS}}{\mathrm{Area}} \tag{1}$$

where TS = tensile strength (g/mm^2), MBS = maximal breaking strength (g), and area = actual surface area of the sample (mm^2).

Each tensile strength measurement was performed in a blinded fashion after an individual, who was not involved with this experimental work, coded each animal sample. The code was only broken after finishing the tensile measurements on all samples. This coding technique eliminated any potential investigator bias in performing and recording the tensile strength measurements.

Equal numbers of animals from each group were euthanized by pentobarbital overdose on day 11 or day 23 after injury. The wounds were carefully were excised, and a 15 $\times$ 10 mm strip from the wounded area was prepared by using a template to help obtain uniform

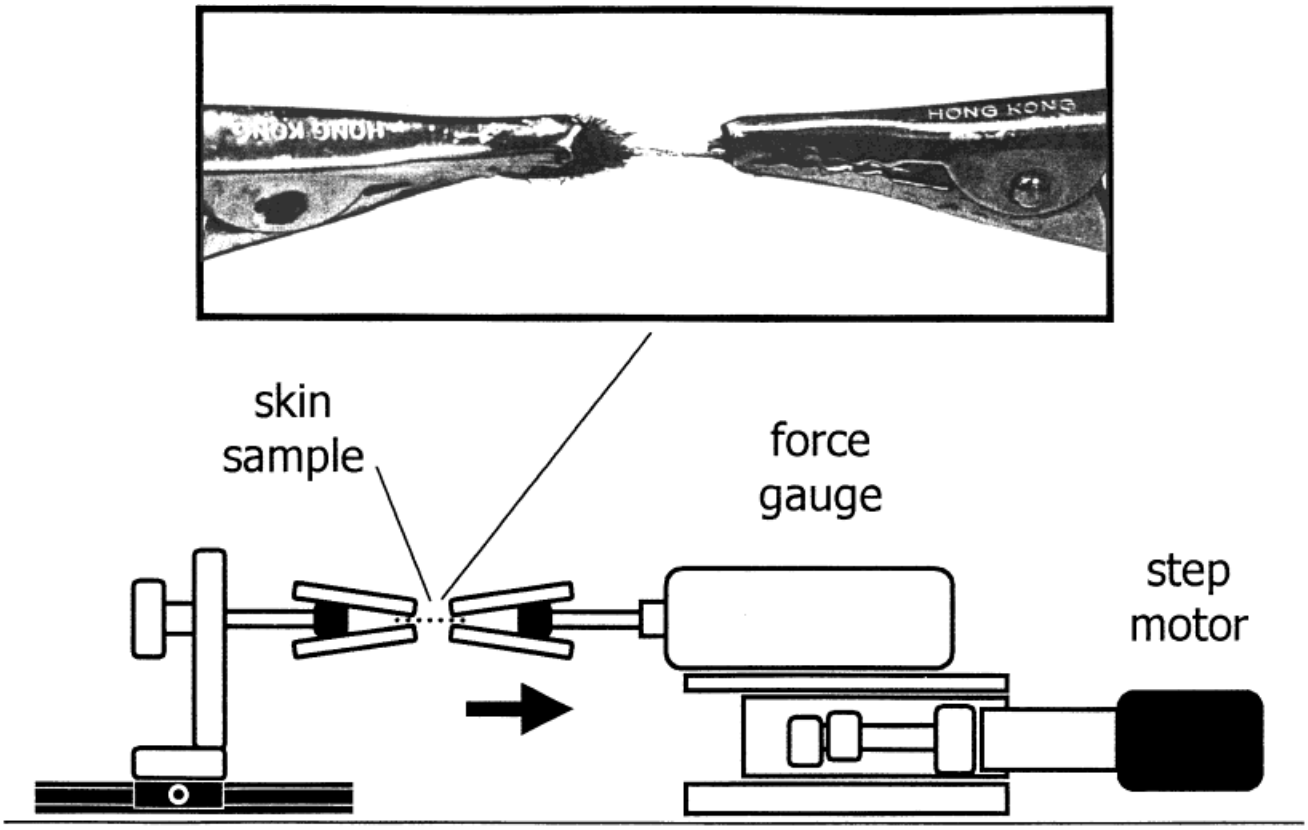

Fig. 1. The schematic drawing of the custom tensiometer used for tensile strength measurements. The photo insert shows the clamping of skin sample. (Photo was made after tearing the sample.). The moving part (i.e., the gauge on the walking holder driven by the stepper motor) pulls the sample vertically at a constant speed of 20 mm/min. Increasing resistance is recorded in grams and displayed in the window of the gauge.

samples. Each skin strip was prepared perpendicularly to the original incision. The actual surface area obtained was calculated for each sample obtained after exact measurement of the skin strip. The strip was placed between the two clamps of the tensiometer, and the clamps were secured to avoid any slippage of the sample. Pulling was performed vertically and perpendicularly to the original direction of the incision. Maximal breaking strengths were registered for each sample, and wound tensile strength values were calculated by using the above formula. All measurements were performed on fresh samples. The skin samples used to measure the tensile strength on intact or unwounded skin were handled and prepared in the same fashion.

Statistical Evaluation

The mean and standard deviation were calculated for each experimental group. A two-tailed Student's t-test was calculated to examine for significant differences at the $P \leq 0.05$ level.

The independent parameters were the treatment time schedule of laser treatment (0–4 days, 3–7 days), the age of the wound (11, 23 days), and the type of mouse (nondiabetic, diabetic). The measured tensile strength was taken to be the independent parameter.

RESULTS

The findings from our current investigation show that LLLT, using irradiation at 830 nm with a 5 J/cm^2 fluence enhanced the overall wound strength in both nondiabetic and diabetic animals compared with sham controls. The degree of the positive effects obtained after LLLT varies with timeline of the treatment (i.e., whether treatment was delivered at 0–4 or 3–7 days after injury).

Figure 2 illustrates the wound tensile strength at 11 and 23 days after LLLT at 0–4 days and 3–7 days in a nondiabetic mouse model. Tensile strength was significantly increased after 11 days in the irradiated group compared with the control group (sham irradiated). A two-tailed t-test produced the following values comparing the treated and control groups ($t = 3.29$, $P = 0.0094$, $df = 9$). The tensile strength of wound was also tested at 23 days after wounding. Laser treatment significantly enhanced the tensile strength of the wounds compared with control at 23 days after injury. ($t = 2.661$, $P = 0.0099$, $df = 10$) Wound tensile strength in the irradiated group and in the

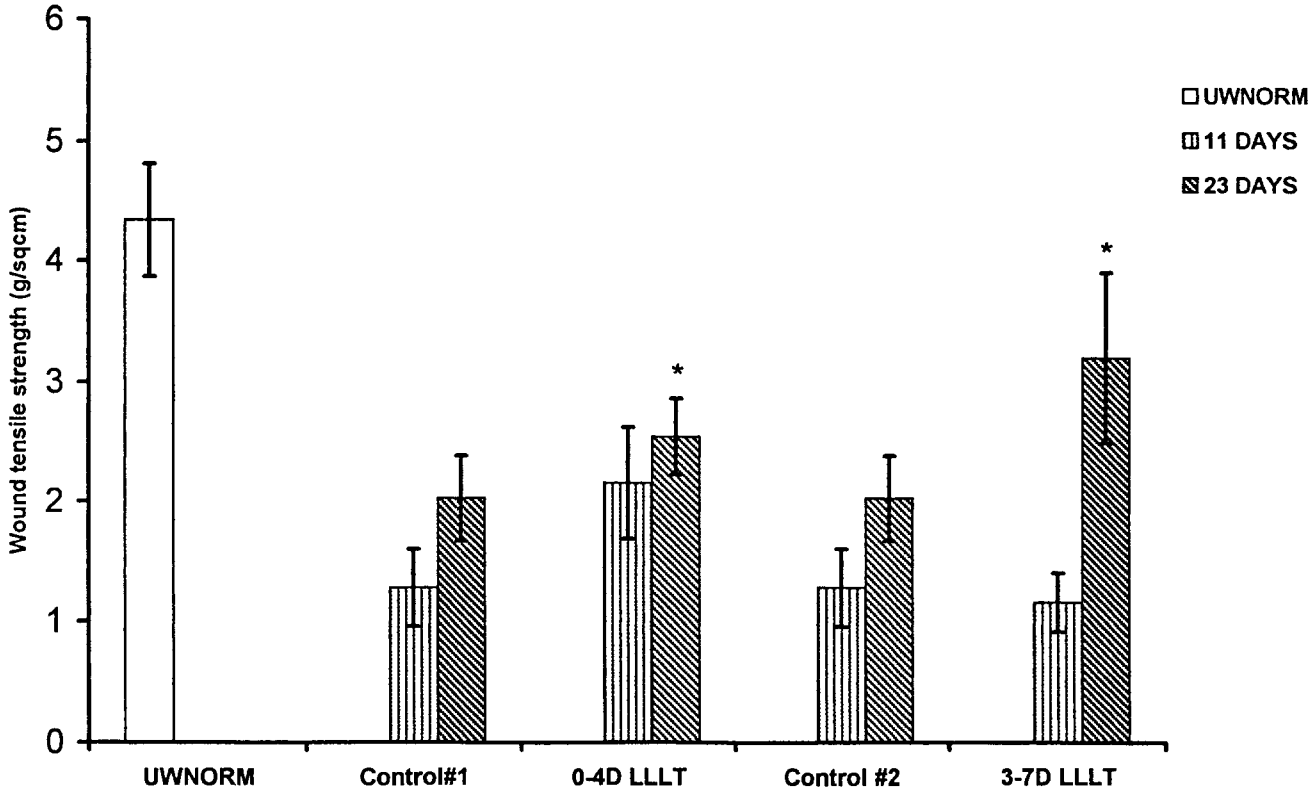

Fig. 2. The effect of laser treatment on wound tensile strength at 11 and 23 days after wounding in nondiabetic mouse model. Laser treatment was performed daily from 0 to 4 days (0–4 days LLLT) or between 3 and 7 days (3–7 days LLLT) after wounding. Sham-irradiated animals served as controls in both cases. (control 1: animals were sham irradiated between 0 and 4 days after wounding; control 2: group animals were sham irradiated between 3 and 7 days after wounding.) Open column (UWNORM) represents the wound tensile strength from the nonwounded skin samples. Means $\pm$ SD were calculated. *Statistically significant difference ($P \leq 0.05$) in comparison with the control.

control group was significantly lower at 23 days than the tensile strength value obtained for nonwounded intact skin, indicating that healing was not as yet complete. No significant difference in tensile strength was observed between the controls and experimental groups at 11 days when treatment was applied between 3–7 days after wounding. Greater differences in wound tensile strength were observed at 23 days comparing the control and experimental groups. Based on statistical analysis these differences are significant ($t = 3.29$, $P = 0.002$, $df = 14$).

To verify that the maturation and remodeling phases of wound healing reached their end stage, the tensile strength of the sample from the wounded area was compared with the tensile strength of a sample taken from an unwounded area. Based on our analysis, the tensile strength of intact skin differs significantly from the value obtained for the laser-treated area. The tensile strength value was 2.55 ± 0.32 g/mm^2 for the laser-treated experimental group and 4.36 ± 0.47 g/mm^2 for the nonwounded area, when the treatment was applied between 0 and 4 days, and the wound burst strength was measured at 23 days ($t = 8.2$, $P \leq 0.001$, $df = 13$). The difference was statistically significant comparing the experimental group treated between 3 and 7 days after wounding and the nonwounded control (3.2 ± 0.71 g/mm^2, $t = 4.15$, $P \leq 0.001$, $df = 17$). The wound did not achieve the maximal strength observed in unwounded skin at 23 days after injury.

In the second part of our study, we performed experiments on diabetic mice. Samples from an unwounded area showed a decreased tensile strength in diabetic skin relative to the values obtained for nondiabetic skin. The influence of LLLT at 830 nm with a 5 J/cm^2 fluence is depicted in Figure 3.

No significant change was detected in the value of tensile strength obtained at 11 days after wounding when the treatment was applied between 0 and 4 days or 3 and 7 days after wounding relative to the control group (i.e., no laser treatment). Wound healing does proceed with time even in the groups that did not receive any light therapy. The value of tensile strength at 11 days was 2.16 ± 0.47 g/mm^2 for the light-treated group and 1.28 ± 0.32 g/mm^2 for the sham-treated group when treatment was delivered between 0 and 4 days after wounding. This difference is statistically significant ($P = 0.004$, $df = 10$, $t = 3.716$.). In

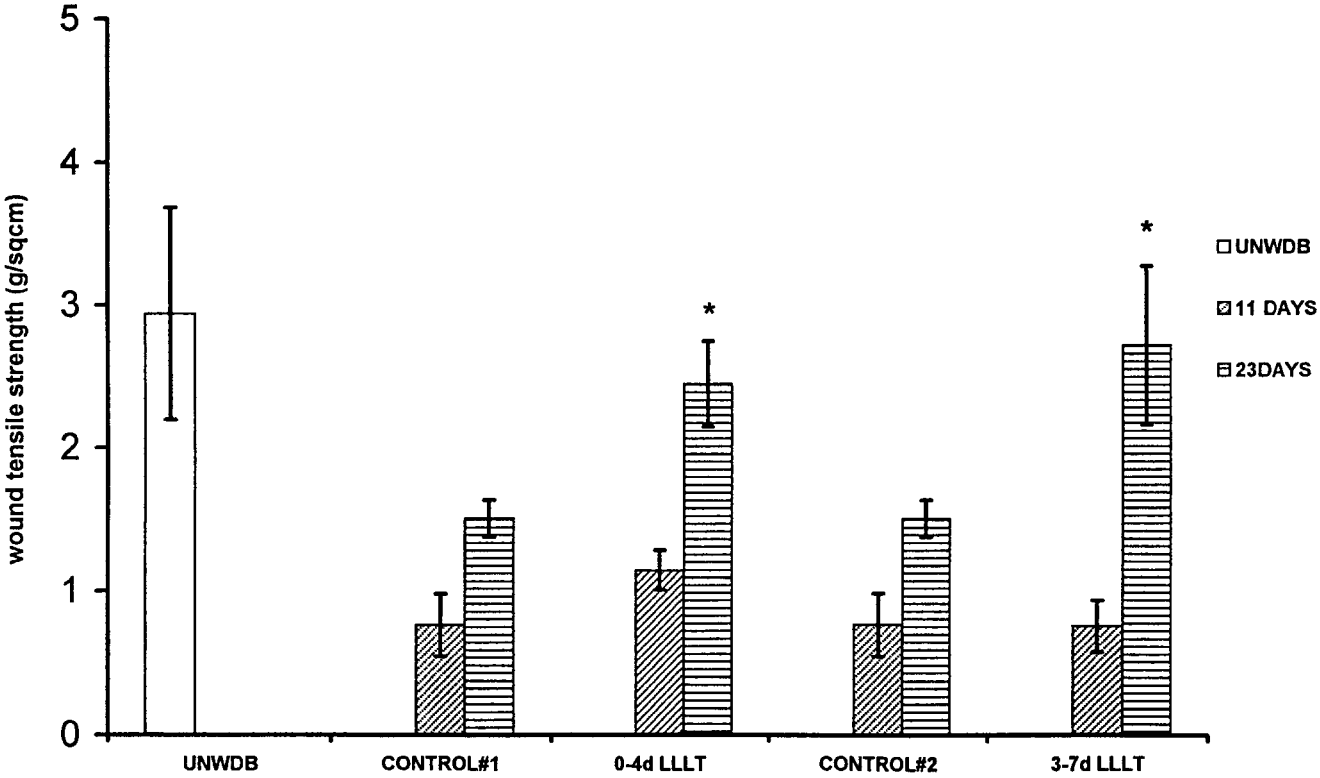

Fig. 3. The effect of laser treatment on wound tensile strength at 11 and 23 days after wounding in diabetic mice. Laser treatment was performed daily from 0 to 4 days (0–4 days LLLT) or between 3 and 7 days (3–7 days LLLT) after wounding. Sham-irradiated animals serve as controls in both cases. (control 1: animals were sham irradiated between 0 and 4 days after wounding; control 2: were sham irradiated between 3 and 7 days after wounding.) Open column (UNWDB) represents the wound tensile strength values obtained from the nonwounded skin area. Means $\pm$ SD were calculated. *Statistically significant difference ($P \leq 0.05$) in comparison with the control.

contrast, when the treatment was given between 3 and 7 days after wounding, a significant enhancement of healing at 11 days was not shown ($P = 0.95$, $df = 10$, $t = 0.61$).

Wound healing resulted in a further increase in tensile strength in both experimental groups (0–4 days LLLT; 3–7 days LLLT) over time as is shown by tensile strength measurements performed at 23 days after injury. The experimental values were statistically different from control ($P = 0.002$, $df = 6$, $t = 5.761$) for treatments applied between 0 and 4 days. The tensile strength for the laser-treated group was significantly lower than the value obtained for the nonwounded area (1.51 ± 0.30 g/mm^2 vs. 2.92 ± 0.74 g/mm^2; $P = 0.05$, $df = 13$, $t = 1.81$). The tensile strength at 23 days after wounding was significantly higher in the experimental group than in the control group (2.72 ± 0.56 g/mm^2 vs. 1.5 ± 0.3 g/mm^2; $P = 0.0015$, $df = 11$, $t = 4.198$).

When the tensile strength of the wounded area at 23 days was compared with the tensile strength from an intact area, there was no statistically significant difference between them (2.72 ± 0.56 g/mm^2; vs. 2.92 ± 0.74 g/mm^2; $P = 0.50$, $df = 18$, $t = 0.683$). These results suggest that the wound-healing process was accelerated in the experimental groups as a result of low-level laser irradiation. The sham-radiated controls remain significantly different from unwounded skin at 23 days after injury (1.51 ± 0.3 g/mm^2 vs. 2.93 ± 0.74 g/mm^2; $t = 3.67$, $P \leq 0.005$, $df = 13$).

DISCUSSION

The ability to achieve successful wound healing is of paramount importance for both surgeon and patient alike. Several investigators have attempted to modify or accelerate wound healing in a variety of normal and impaired states [28]. Clinical and experimental assessment of wound healing based on direct visual observation or light microscopy of wound histology have failed to document any enhancement of healing in normal hosts, despite the application of cytokines and other agents to wounds [29,30]. However, our group and others have shown that LLLT and/or cytokines are capable of accelerating healing in genetic diabetes and other impaired states [24]. Others have published conflicting results when different treatments and parameters are applied to other injury models [22,23,25,31]. It is well known that wound tensile strength increases gradually after injury and achieves a value of 50% of uncut tissue strength at approximately 42 days in the normal host [32].

Understanding the discrete phases of wound healing and the likelihood of dehiscence have practical relevance that oftentimes determines the nature and strength of suture materials and wound management techniques that are applied clinically. Any form of treatment that is easily applied, inexpensive, and has a low incidence of associated morbidity could have far-reaching applications and could substantially reduce the disability and morbidity associated with surgery or trauma. Specifically, if a wound can be rendered sufficiently strong sooner, fewer sutures might be required and/or suture material can be removed earlier, perhaps reducing scarring and improving functionality or cosmesis. Enhanced healing of grafts and flaps would similarly result in fewer failures as a result of an improved ability to withstand excess shear forces applied to the immature wound.

The present study provides preliminary data that suggest that photostimulation is capable of enhancing wound tensile strength in normal (nondiabetic) and impaired (diabetic) littermates. Furthermore, the data show that there may be a temporal relationship between the age of the wound and the timeframe when the delivery of LLLT is most effective in accelerating the healing process. Our work suggests that collagen synthesis is affected by photoradiation at 830 nm and a fluence of 5 J/cm^2. However, much work must still be performed to verify these results and define the precise "driver" or photoacceptor in this phase of the healing process. The current study should not be construed to indicate that LLLT directly affects the chemical composition and strength of collagen itself. The apparent time dependence of the effect we observed would suggest that the appropriate millieu, such as cellular components, vascularity, and chemical substrates need to be locally present at the time of photoirradiation.

Several questions remain unanswered and will form the basis of future studies. Because the animals used in this study are dark skinned (i.e., black) and because light energy at this wavelength could result in some thermal effects, temperature-clamped and local cooling experiments are planned. Additional verification of these results on fair-skinned or albino rodents and with varying fluences should also be performed. Signal transduction studies and assessment of wound collagen levels and types are underway.

The reader should also be cautioned that although the results of this study, even if confirmed by other investigations, may not be directly applicable to chronic wounds or other tissues. The local environment and healing milieu of an acute, sutured wound in a normally perfused and oxygenated host presents a different challenge than a chronic nonhealing ulcer or other impaired wound state.

CONCLUSIONS

This preliminary investigation shows that LLLT at a fluence of 5 J/cm^2 with an 830-nm diode laser improves wound healing as measured by increases in wound tensile strength at 11 and 23 days after injury. The salutory effect of photoradiation was observed in linear incisions in both type I diabetic and nondiabetic animals. Daily treatment at 3–7 days after wounding appears superior to treatments delivered daily at 0–4 days after injury. A possible mechanism for these observations is a salutary effect of phototherapy on collagen synthesis. Further studies are warranted to differentiate between a true biologic or photothermal effect and to define the optimal parameters and timing of therapy.

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