

Research Article

Effects of Low-Intensity Laser Irradiation on Wound Healing in Diabetic Rats

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Objective. The effects of low-intensity 630 nm semiconductor laser irradiation at 3.6 J/cm² (LISL) on wound healing in diabetic rats were studied in this paper. **Methods.** 36 diabetic rats with dorsal cutaneous excisional wounds were divided into three LISL groups and a control group randomly. The three LISL groups were irradiated with LISL at 5, 10, and 20 mW/cm² five times a week for two weeks, respectively. The process of wound healing was assessed by assessing blood glucose, calculating percentage of wound closure, histopathological evaluation, and immunohistochemical quantification. **Results.** Blood glucose of all groups remained at similar levels throughout the experiment. LISL could obviously promote wound contraction, fibroblasts proliferation, and collagen synthesis, alter bFGF and TGF- β 1 expression, and reduce inflammatory reaction in the early and middle phases of chronic wound-healing process. However, LISL could not shorten cicatrization time, and the treatment effects were not sensitive to illuminate parameters in the later phase of the experiment. **Conclusions.** LISL might have auxiliary effects in the early and middle phases of wound healing in STZ-induced diabetic rats, but the reciprocity rule might not hold. The wound-healing process of early-phase diabetes rats shows typical characteristics of self-limited disease.

1. Introduction

Diabetes is a complex metabolic disorder involving many body organs and systems and can devastate the lives of affected individuals [1]. It is estimated that global prevalence of diabetes gets to 6.6% (285 million people) in 2010, and the number of people with diabetes will have risen to 438 million or 7.8% of the world's population by 2030 [2]. Impaired wound healing is a complication of diabetes and a serious problem in clinical practice [3]. As many as 15% of people with diabetes will develop foot ulceration and wounds, and 3% will have a lower limb amputation [4, 5].

Photobiomodulation (PBM) is a modulation of laser irradiation (LI) or monochromatic/broad band light on biosystems, which stimulates or inhibits biological functions but does not result in irreducible damage. The LI used in PBM is always low intensity-LI (LIL), ~ 10 mW/cm². However, moderate-intensity LI (MIL), $10^2\sim 3$ mW/cm², is of

PBM if the irradiation time is not so long that it damages organelles or cells.

In recent years, PBM has gained considerable recognition and importance among treatment modalities for various medical problems including wound repair processes, musculoskeletal complications, and pain control [6–8]. Many literatures were reported that PBM can promote the healing process by reducing pain and inflammation, promoting cells proliferation, facilitating collagen synthesis, fostering immunity, and increasing wound tensile strength [4, 8–12].

A substantial amount of studies show that PBM with appropriate treatment parameters can promote the chronic wound healing in diabetic rats [13–15]. LI at 630 nm in visible red-light region was generally believed as the optimum wavelength, but significant beneficial effects have also been observed on diabetic rats for LI at 532, 810, and 980 nm [4]. Range of power density options varied considerably (from LIL to MIL). It's worth noting that

TABLE 1: Groups of experiment and treatment parameters.

	Power density (mW/cm ²)	Irradiation time (min)	Energy density (J/cm ²)	Treatment schedule (times/week)	Number of rats
Control group	0	0	0	0	9
5 mW/cm ² group	5	12	3.6	5	9
10 mW/cm ² group	10	6	3.6	5	9
20 mW/cm ² group	20	3	3.6	5	9

a lately study by Akyol and Güngörmüş [8] suggested that 808 nm MIL at 100 mW/cm² has a beneficial effect on the early recovery of skin incisions in female Wistar rats with streptozotocin (STZ)-induced diabetes. There were significant differences between the MIL group and control group in both reepithelialization and inflammation at 10th day. However, the differences disappeared both in inflammation and reepithelialization at 20th day. This is typical of self-limited disease, which indicates that the wound of early phase diabetes can heal completely by itself. Similar phenomenon had been mentioned in conventional wound-healing process [16, 17]. Recently, a research even suggested that diabetes may be also self-limited, and the abnormalities underlying diabetes are reversible [18].

The aims of our paper was to study the effects of low-intensity laser irradiation (LIL) on the wound healing of male Wistar rats with STZ-induced diabetes. With more detailed and comprehensive methods than Akyol et al.'s study, we tried to gain better insight into the healing process more deeply, to verify the characteristics of self-limited disease in the wound-healing process, and to offer an appropriate intensity at 3.6 J/cm² for further study.

2. Material and Methods

2.1. Animals. Thirty-six male Wistar rats weighing 220–250 g from the animal house of Institute of Radiation Medicine Chinese Academy of Medical Sciences were used in this study. During the study, the rats were housed 9 per cage, maintained under controlled environmental conditions (12-hours light/dark cycle, temperature 23°C), and provided with standard laboratory food and water *ad libitum*.

2.2. Induction of Diabetes. Diabetes was chemically induced using STZ (Sigma Co., USA), 40 mg/kg, dissolved in citrate buffer (pH 4.4) and administered as tail vein injection in all rats. Seven days after STZ injection, blood glucose levels were measured using a glucometer and test strips (One Touch Ultra; LifeScan Co., USA), and all STZ-injected rats with a blood glucose of 16.5 mmol/L or more were included in the protocol.

2.3. Wound Surgery. Before surgery, the blood glucose level of each rat was checked again. Each rat was anesthetized with 10% chloral hydras (300 mg/kg) intraperitoneally. The hair on the dorsum of all rats was shaved using an electric clipper. The operative site was prepared aseptically. Two incisions (10 mm * 10 mm square) were made on the dorsum of each

rat using a steel scalpel (Rockwell number 15). One incision was performed on the left side of the dorsum and the other was on the right.

2.4. Groups of Experiment and Treatment Parameters. Thirty-six rats were randomly divided into 4 groups: 5 mW/cm² group, 10 mW/cm² group, 20 mW/cm² group, and control group, 9 rats per group. The study was performed using a 630 nm continuous semiconductor laser system designed by Laser Medical Laboratory of Institute of Biomedical Engineering, Chinese Academy of Medical Science & Peking Union Medical College. The output power was measured using a laser power meter (SOLO PE; Gentec-EO Inc., Canada). The laser treatment parameters are listed in Table 1. PBM was started immediately after surgery and repeated 5 times/week for two weeks. The laser beam was aligned to cover the entire wound area, including the boundaries.

2.5. Blood Glucose Level. Before surgery and 14 days after wounding, the blood glucose level of each rat was checked.

Average blood glucose was presented as mean blood glucose \pm SEM and compared with Matching *T* test.

2.6. Percentage of Wound Closure. At 3, 6, 9, and 12 days after wounding, the areas of wounds on all rats were recorded with standardized photography, and calculated the percentage of wound closure by ImageJ (<http://rsb.info.nih.gov/ij/>). Percentage of wound closure was calculated using the following formula [19]: [(Area of 1 Day – Area of X Days)/Area of 1 Day] \times 100%. Average wound closure percentage was presented as mean percentage \pm SEM and compared with one-way ANOVA with Tukey posttest.

2.7. Histopathological Evaluation. At 4, 8, and 14 days after wounding, 3 rats were chosen from each group randomly and killed by ether inhalation. The tissue specimens were stained with hematoxylin and eosin, examined with a semiquantitative method [10] to evaluate following histological features: polymorphonuclear leukocytes (PMNLs), reepithelialization, fibroblasts, new vessels, and collagen synthesis. The sections were studied by two independent observers and evaluated on a scale of 0–4. Observers were blinded for the study of the specimens. Average semiquantitative evaluation score was presented as mean score \pm SEM and compared with the nonparametric Kruskal-Wallis test.

2.8. Immunohistochemical Quantification. At 4, 8, and 14 days after wounding, the tissue specimens were fixed for 24

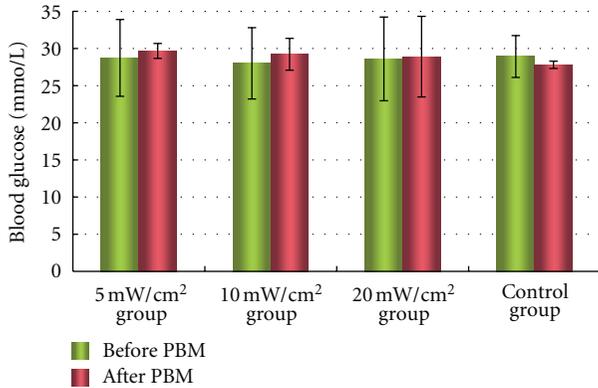


FIGURE 1: Blood glucose levels of all groups throughout the experiment.

hours in 4% paraformaldehyde prior to being embedded in paraffin and sectioned (longitudinal section perpendicular to wound surface, 5 μ m). The tissue was deparaffinized, rehydrated, and blocked with an appropriate blocking solution. Tissue was incubated at 4°C overnight with an antibody to basic fibroblast growth factor (bFGF) (SC-79; Santa Cruz Inc., USA) or transforming growth factor β 1 (TGF- β 1) (SC-146; Santa Cruz Inc., USA) followed by incubation with secondary antibody at 37°C for 20 minutes. Choose 5 sections per slide, analysis and read the immunohistochemical score (Image-Pro Plus; Media Cybernetics Inc., USA) using a method modified from that described by Soslow et al. [20]. Average immunohistochemical score (IHS) was presented as mean IHS \pm SEM and compared with one-way ANOVA with Tukey posttest.

3. Results

3.1. Blood Glucose Level. There was no significant difference among diabetic rats that had been randomly placed into the 4 groups at the beginning of the experiment ($P > 0.05$). Blood glucose remained at similar levels throughout the experiment, with no significant difference between values before and after PBM ($P > 0.05$). At the end of the experiment, blood glucose of all rats (both control group and three PBM groups) were still in pathological blood glucose range (more than 16.5 mmol/L) with no significant difference ($P > 0.05$) (Figure 1).

3.2. Percentage of Wound Closure. At 3 days after wounding, only wounds of 20 mW/cm² group closed significantly faster than control group ($P < 0.05$). Wound closure percentages of other groups showed no significant difference ($P > 0.05$).

At 6 and 9 days after wounding, wounds of all three PBM groups closed significantly faster than control group ($P < 0.05$), but there was no significant difference among 5 mW/cm² group, 10 mW/cm² group, and 20 mW/cm² group ($P > 0.05$).

At 12 days after wounding, percentage of wound closure in all groups remained at similar levels ($P > 0.05$) (Figure 2).

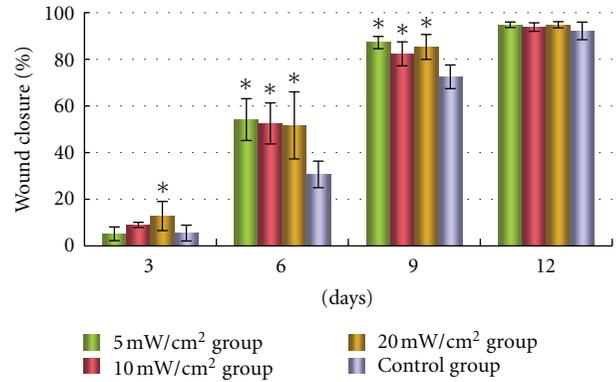


FIGURE 2: Percentage of wound closure throughout the experiment. Asterisk means that this group has statistical difference with control group ($P < 0.05$).

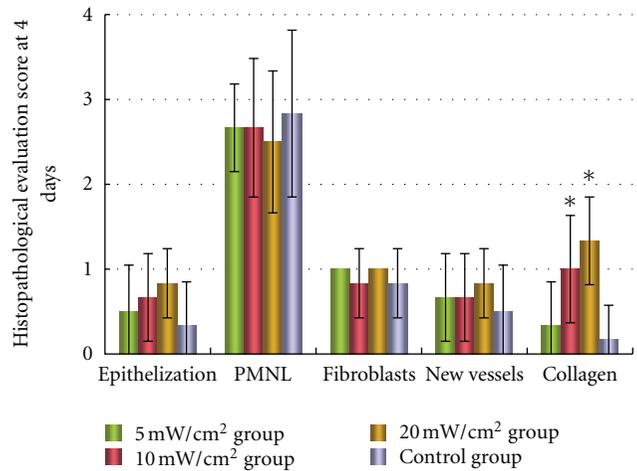


FIGURE 3: The semiquantitative histopathological evaluation score at 4 days after wounding. Asterisk means that this group has statistical difference with control group ($P < 0.05$).

3.3. Histopathological Evaluation

3.3.1. 4 Days. Remarkable inflammatory exudation and necrotic tissue could be observed in all tissue specimens. Comparable numbers of PMNLs, reepithelization, new vessels, and fibroblasts were recorded in all groups. However, there were significant differences in the creation of new collagen fibers between control group and 10 mW/cm² group ($P < 0.05$) or 20 mW/cm² group ($P < 0.05$) (Figure 3).

3.3.2. 8 Days. Attenuated inflammation, mature granulation tissue, extensive collagen deposition, and greater reepithelization can be observed in three PBM groups. Control group had more inflammatory exudates and fresh granulation tissue (Figure 4).

The histological evaluation score of reepithelization, numbers of PMNLs, fibroblasts, and new collagen fibers, except for new vessels, shown significant differences between control group and 20 mW/cm² group ($P < 0.05$). There were significant differences in extensive fibroblasts and new

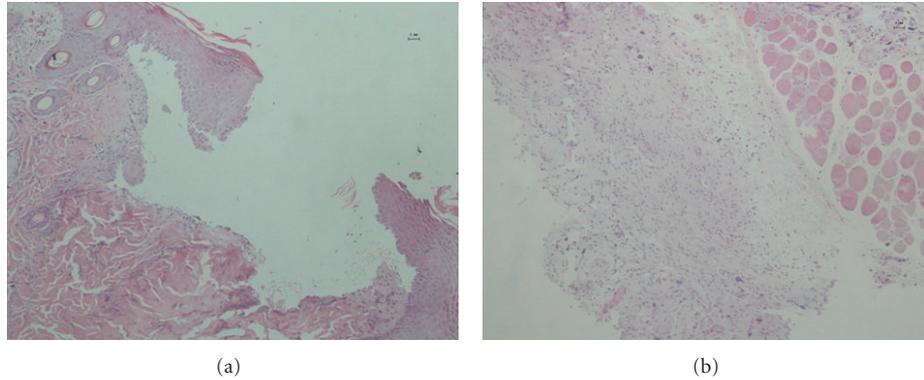


FIGURE 4: Photomicrograph of tissue samples from 20 mW/cm² group (a) and control group (b) at 8 days after wounding (HE stain × 100).

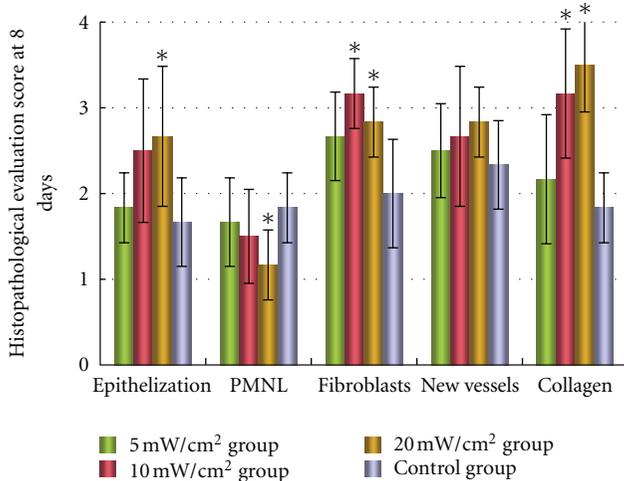


FIGURE 5: The semiquantitative histopathological evaluation score at 8 days after wounding. Asterisk means that this group has statistical difference with control group ($P < 0.05$).

collagen fibers between control group and 10 mW/cm² group ($P < 0.05$). The new vessels in all four groups were at similar level and had no significant differences ($P > 0.05$) (Figure 5).

3.3.3. 14 Days. New epithelial cells of all groups have covered almost all wounds, capillary gradually closed, granulation tissue gradually replaced with fibrous scar, fibroblasts decreased, and intercellular collagen content increased. The progress of wound healing in 4 groups was similar. However, there were significant differences in the evaluation score of new vessels between control group and 5 mW/cm² group or 20 mW/cm² group ($P < 0.05$), except 10 mW/cm² group (Figure 6).

3.4. Immunohistochemical Quantification. bFGF and TGF- β 1 protein expression at 4, 8, and 14 days after wounding revealed that growth factors were mainly located in the inflammatory cells (macrophages), granulation tissues (fibroblasts), and the surrounding newly formed capillaries.

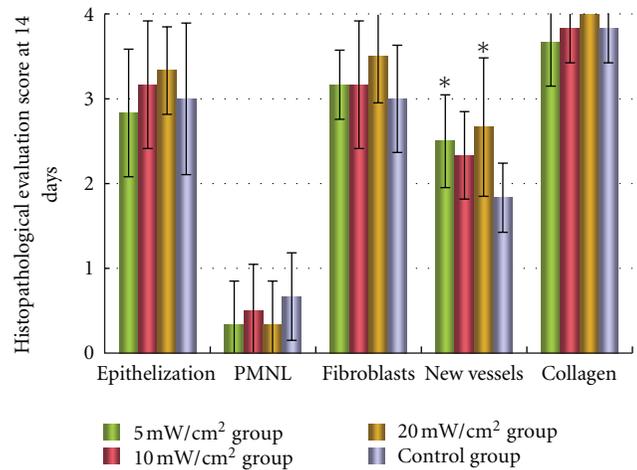


FIGURE 6: The semiquantitative histopathological evaluation score at 14 days after wounding. Asterisk means that this group has statistical difference with control group ($P < 0.05$).

IHS analysis revealed that bFGF expression in all PBM groups increased significantly compared with the control group ($P < 0.05$) at 4 days after wounding. 5 mW/cm² group had a significant bFGF increase in comparison to control group at 8 days after wounding ($P < 0.05$). There were no significant difference between irradiated groups and control group at 14 days after wounding ($P > 0.05$) (Figure 7).

TGF- β 1 protein expression in 10 mW/cm² group at 4 days after wounding increased significantly compared with other groups ($P < 0.05$). However, there were no significant differences between PBM groups and control group at 8 and 14 days after wounding ($P > 0.05$) (Figure 8).

4. Discussion

The wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling [21]. It is widely accepted that chronic healing of diabetes wounds always accompanied with prolonged inflammation and decreased matrix accumulation [22].

TABLE 2: The differences of the animal models in two studies.

	Numbers of rats	The gender of rats	Wound surgery	Wound size in length (mm)	Position of control group and LLLT group
Akyol et al.'s study	18	Female	Diode laser	15	On the same rat
Current study	36	Male	Steel scalpel	10	On different rats

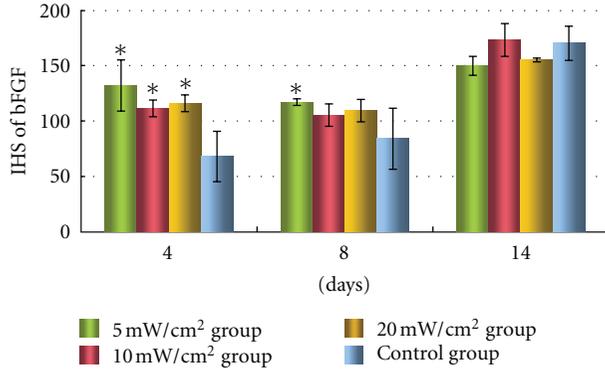


FIGURE 7: Immunohistochemical score (IHS) of bFGF protein expression throughout the experiment. Asterisk means that this group has statistical difference with control group ($P < 0.05$).

TABLE 3: The differences of the laser treatment parameters in two studies.

	Wavelength (nm)	Power density (mW/cm ²)	Energy density (J/cm ²)	Total treatment times
Akyol et al.'s study	808	100	2	5
Current study	630	5–20	3.6	10

Our histopathological study found that attenuated inflammation, greater reepithelization, mature granulation tissue (fibroblasts), and extensive collagen deposition can be observed in PBM groups at 8 days after wounding, especially in 20 mW/cm² group. At the same time, the control group had more inflammatory exudates and fresh granulation tissue. These phenomena are consistent with the analysis of wound closure percentage data that PBM can obviously promote wound contraction, especially among 6–9 days after wounding. Results from our investigation are also in agreement with some previous published studies [10, 23, 24] that PBM are able to promote wound healing by reducing inflammation without compromising the proliferation of fibroblasts and keratinocytes.

The process of wound healing is precisely executed and regulated by a number of growth factors spatially and temporally. As is known to all, bFGF has potent effects on wound-healing process. It plays a vital role in regulating the synthesis and deposition of various extracellular medium components, increasing keratinocyte motility during reepithelization, and promoting the migration of fibroblasts and collagenase production [25–29]. TGF- β 1 has increased expression from

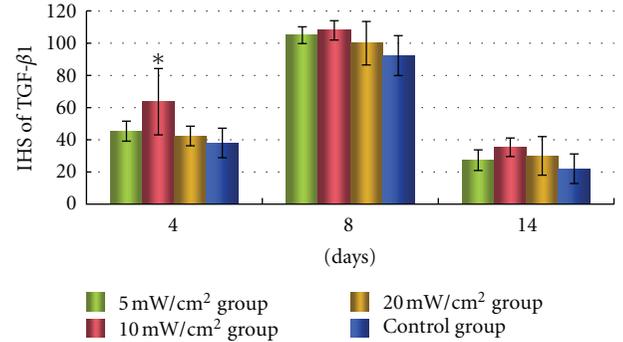


FIGURE 8: Immunohistochemical score (IHS) of TGF- β 1 protein expression throughout the experiment. Asterisk means that this group has statistical difference with control group ($P < 0.05$).

the very beginning of injury and is of particular importance to modulate events of wounding healing [25]. TGF- β 1 enhances the wound repair by influencing cells proliferation and migration, angiogenesis, extracellular matrix synthesis, and deposition [30, 31].

Our current study investigated that the amount of growth factor productions was significantly altered in diabetic wounds with LIL, especially at 4 days after wounding. The results suggest that LIL promoted wound-healing process by influencing the levels of relevant growth factors, and then promoting cells proliferation and migration, regulating extracellular matrix synthesis and deposition. These are consistent with the aforementioned pathological analysis. It should point out that some growth factors protein expression always decrease in chronic healing wounds [25, 32]. It appeared that the LIL might be a candidate to replace growth factors in clinically treatment [33].

Some literatures have reported that PBM increases bFGF immunolabeling rather than gene expression [19, 34]. It suggested that the observed bFGF immunolabeling increase might derive from bFGF secretion or protein production, instead of de novo synthesis. LIL is capable of activating the latent TGF- β 1 complex in vitro, and its expression pattern in vivo suggests that TGF- β 1 plays a central role in mediating the accelerated healing response [35]. In this study, LIL at 5 mW/cm² increased bFGF protein expression at 4 or 8 days after wounding, LIL at 10 mW/cm² increased both bFGF and TGF- β 1 protein expression at 4 days after wounding, but LIL at 20 mW/cm² increased bFGF protein expression only at 4 days after wounding. The variation of bFGF in the 5 mW/cm² group at 8 days is much smaller compared to those of other groups. This may explain why only this group had a statistically significant increase in bFGF compared to the control group. However, the expression pattern of TGF- β 1

TABLE 4: The differences of the evaluate parameters in two studies.

	research methods	Evaluate parameters	Observe time (after wounding)
Akyol et al.'s study	Histopathological evaluation	Reepithelialization; inflammation	10 and 20 days
Current study	Morphology evaluation; histopathological semievaluation; immunohistochemical quantification	Wound closure percentage; PMNLs, reepithelization, fibroblasts, new vessels, and collagen synthesis; bFGF and TGF- β 1 protein production	3, 6, 9, and 12 days for morphology evaluation; 4, 8, and 14 days for histopathological and immunohistochemical evaluation

did not show any change after LIL at both 8 and 14 days after wounding in our study. The expression patterns in all PBM groups, both bFGF and TGF- β 1, were similar with control group at 14 days after wounding. In an attempt to further understand the impact of LIL on regulating relevant growth factors, further experiments with larger sample size and advanced technologies should be conducted.

According to the reciprocity rule, the same LI dose has the same PBM. It holds for MIL but does not for LIL. The latter was supported in this study. In addition, LIL at the same dose and different power densities exert different regulatory effects on bFGF (8 days after wounding) and TGF- β 1 (4 days after wounding) in our study. LIL at 20 mW/cm² performed more effective than the one at 5 or 10 mW/cm² in the evaluated histological parameters and wound contraction percentage. Moreover, taking the length of the treatment time into consideration, we may choose 20 mW/cm² in our further experiments with 630 nm 3.6 J/cm² continuous semiconductor laser and consider it as the optimum among the power densities we used.

It is worth noting that no matter what illuminate parameters we used (including control group), the wounds contracted mostly and various evaluated parameters were not sensitive to illuminate parameters (4 groups had similar pathologic and immunohistochemical scores) at 14 days after wounding. In other words, LIL cannot shorten cicatrization time, even though it performed positively in the early and middle phases of wound-healing process, which is similar with the study by Akyol and GÜngörmüş [8] using MIL at 100 mW/cm². This is typical of self-limited disease, which means that the wound of early-phase diabetes can heal completely by itself without the laser therapy. Similar phenomenon had been mentioned in conventional wound-healing process [16, 17], but rarely reported in healing of diabetes wound. Interestingly, there existed a lot of distinctions between the study of Akyol et al.'s and ours (Tables 2, 3, and 4). Anyway, both LIL and MIL have undeniable effects to facilitate wound healing as an auxiliary treatment by diminishing inflammatory exudation, enhancing wound contraction, avoiding infection, and ameliorating local pain and tumefaction in the early and middle phases.

5. Conclusions

630 nm LIL at 3.6 J/cm² might have auxiliary effects in the early and middle phases of wound healing of male Wistar rats with STZ-induced diabetes, but the reciprocity rule did not hold. The wound-healing process of early-phase diabetes rats

shows typical characteristics of self-limited disease, and the laser therapy cannot shorten the wound cicatrization time.

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