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Histological Assessment of the Effect of Laser Irradiation on Skin Wound Healing in Rats

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ABSTRACT

Objective: **The purpose of this study was to evaluate, from the histological point of view, the effect of diode laser irradiation on skin wound healing in Sprague-Dawley rats.** ***Background Data:*** **Various biological effects have been described in different studies after low-level laser therapy (LLLT).** ***Methods:*** **Two parallel full-thickness skin incisions were performed on the back of each rat (n = 49) and immediately sutured. After surgery, one wound of each rat was exposed to laser irradiation (continuous mode, 670 nm, daily dose 30 J/cm^2), whereas the parallel wound was not irradiated and served as control. Both wounds were removed 24, 48, 72, 96, 120, 144, and 168 h after surgery and routinely fixed and embedded in paraffin sections, stained with hematoxylin and eosin, van Gieson, periodic acid Schiff + periodic acid Schiff diastase, Mallory's phosphotungstic hematoxylin, and azur and eosin, and histopathologically evaluated.** ***Results:*** **As compared to non-irradiated control wounds, laser stimulation shortened the inflammatory phase as well as accelerated the proliferative and maturation phase, and positively stimulated the regeneration of injured epidermis and the reparation of injured striated muscle.** ***Conclusion:*** **LLLT at 670 nm positively influences all phases of rat skin wound healing.**

INTRODUCTION

DURING PAST YEARS, low-level laser therapy (LLLT) has not been frequently used in clinical practice. It is hypothesized that the effect of LLLT at the cellular level is based on the absorption of monochromatic visible and near-infrared radiation by components of their redox properties and on the acceleration of electron transfer.[1] The laser affects cell proliferation, synthesis of ATP, and collagen. Decreasing the hypoxia elicited by injury and supporting the release of growth factors during wound healing has also been demonstrated.[3] At the present time, however, there is no general agreement about the exact way in which LLLT influences the process of wound healing. In numerous clinical studies, it has been proven that low-energy laser light reduces pain, accelerates wound healing, and positively influences inflammatory processes or the healing of diabetic tissue.[4–8] However, there also exist clinical studies in which healing after LLLT was not accelerated.[9–11]

Since rat skin contains three layers (i.e., epidermis, dermis, and striated muscle), it is a good model for studying the healing of three different tissue types. Only the epidermis has the capability to regenerate. Wound healing of injured dermis takes place in three basic phases: inflammation, proliferation, and maturation.[12] The phases are not strictly separated from each other; their processes freely blend together. In the healing of injured striated muscle, there are two concurrent processes, which are not only supportive but also competitive with each other.[13] The first process is the differentiation of new myofibers from satellite cells. After the injury, activated satellite cells differentiate to myoblasts and fuse with each other into multinucleated myotubes. Myonuclei are located centrally in the myotubes, and they are known as "centronucleated" cells.[14] However, the exact factors initiating the activation of satellite cells are poorly known. The second process of muscle healing is the formation of the granulation and scar tissues, which serve as a

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scaffold for regenerating myofibers. This process is very similar to the healing of injured dermis.

The purpose of our study was to evaluate, from the histological point of view, the influence of diode laser irradiation on all three injured layers (epidermis, dermis, and striated muscle) of skin wounds during the first week of the healing process in Sprage-Dawley rats.

METHODS

Animal model

Our experiment was performed in compliance with the requirements of the Ethic Committee of the Faculty of Medicine, Pavol Jozef Šafárik University, in Košice, and approved by the State Veterinary Administration of the Slovak Republic. Forty-nine male, 6–8-month-old Sprague-Dawley rats, weighing 450–550 g, were used for the experiment and randomly divided into seven groups of seven animals. The animals were housed in Plexiglas cages with free access to a standard laboratory diet and tap water. A combination of ketamine (Calypsol, Chemical Works of Richter Gedeon, Hungary) in dose of 40 mg/kg, xylazine (Rometar a.u.v., Czech Republic) in dose of 15 mg/kg, and tramadol (Tramadol-K, Krka, Slovenia) in dose of 5 mg/kg was used to anesthetize the rats. Atropin was used as premedication (Atropin, Hoechst-Biotika, Slovak Republic) in dose of 0.05 mg/kg. Two 3-cm-long parallel full-thickness skin incisions were performed under aseptic conditions on the left and right side of each experimental rat spine and immediately sutured by four simple sutures (Chiraflon 3/0, Chirmax, Czech Republic). The irradiated and control wounds were 4 cm distant from each other.

Low-level laser therapy

The left wound of each experimental rat was stimulated by a commercially available AlGaInP diode laser (Maestro/CCM, Medicom Praha, Czech Republic; wavelength 670 nm, power density 25 mW/cm^2, continuous mode), whereas the right wound served as a control. During wound treatment, the rats were restrained in the Plexiglas cage with an oval opening over the stimulated wound, while the control wound was protected from reflected laser light. The area of the treated wound was divided into three sections, which were irradiated by punctual method step-by-step, spot size approximately 0.4 cm^2 (shape of beam—oval; $r_1 = 0.5$ cm, $r_2 = 0.25$ cm), the length of wound 3 cm. Each section was irradiated daily 8 min to achieve the total daily dose of 30 J/cm^2.

Histopathological evaluation

The skin wounds were removed from the body 24 h (group 1), 48 h (group 2), 72 h (group 3), 96 h (group 4), 120 h (group 5), 144 h (group 6), and 168 h (group 7) after surgery. The tissue specimens were processed routinely for light microscopy (fixating, dehydrating, embedding, cutting, and staining with hematoxylin and eosin [HE], van Gieson [VG], periodic acid Schiff + periodic acid Schiff diastase [PAS + PSD], Mallory's phosphotungstic hematoxylin [WF], azur and eosin [AZ]; Table 1). Seven animals (14 wounds) in each group were evaluated. We were interested in observing the histological structures and changes in the following three layers: epithelization and keratinization of the epidermis (first layer), creation of fibrin network, presence of inflammatory cells (polymorphonuclear leucocytes [PMNL], tissue macrophages), migration, proliferation and orientation (vertically, horizontally) of fibroblasts, creation of new extracellular matrix (ECM, especially new collagen fibers), neoangiogenesis of the dermis and muscle (second and third layer), and presence of centronucleated cells in the muscle only (third layer). As the major marker of the progress and regress of the inflammatory phase, the presence of PMNL was considered. The amount of fibroblasts, new collagen and vessels served as markers of the proliferative phase. The degree of the wound maturation depended on the reorganization of the ECM. The histological sections were in blinded form given to the observer performing the evaluation using a semi-quantitative method according to the following scale: 0, 1, 2, and 3 (Table 2).

During the post-surgery period, the animals remained healthy, without clinical evidence of infection. The results of our histological investigation are summarized in Table 3.

Statistical analysis

The statistical differences between the control and stimulated wounds were analyzed by using the unpaired Student *t*-test; significance was accepted at $p < 0.05$ (Table 3).

RESULTS

Group 1

Histological evaluation of the animals killed 24 h after surgery showed necrosis of skin tissue on the surface of the inci-

TABLE 1. BRIEF DESCRIPTION OF HISTOLOGICAL STAININGS

Staining	*Mainly stained structures*
Hematoxylin and eosin (HE)	Basic staining
Van Gieson (VG)	Collagen fibers
Periodic acid Schiff + periodic acid Schiff diastase (PAS + PSD)	Extracellular matrix (ECM)
Mallory's phosphotungstic hematoxylin (WF)	Fibrin
Azur and eosin (AZ)	Erythrocytes (visualization of vessels)

TABLE 2. EXPLANATION OF SCALE USED IN THE SEMI-QUANTITATIVE EVALUATION OF HISTOLOGICAL SECTIONS

Scale	*Epithelialization*	*PMNL*	*Tissue macrophages*	*Fibroblasts*	*New collagen*	*Neo-angiogenesis*	*Centro-nucleated cells*
0	Thickness of cut edges	Absent	Absent	Absent	Absent	Absent	Absent
1	Migration of epithelial cells	Mild	Mild	Mild	Mild	Mild	Mild
2	Bridging of the incision	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
3	Complete regeneration	Marked	Marked	Marked	Marked	Marked	Marked

sions as a consequence of mechanical damage. The epidermis of each wound was thickened at its cut edges as a result of the mitotic activity of basal cells. In control wounds, under the tissue necrosis, the beginning of the formation of the demarcation line consisted of PMNL (2.0 ± 0.6) was observed (Fig. 1). However, in laser-stimulated wounds, the forming of the demarcation line was almost finished (2.6 ± 0.5), and the line completely separated the necrosis from the vital tissue (Fig. 2). In control and stimulated wounds, the tissue macrophages concomitantly invaded the wound area (1.1 ± 0.4 vs. 1.3 ± 0.5). WF staining showed the fibrin network in the incisional space between the stumps of injured myofibers and in the dermis. The network contained blood cells and created a scaffold for migrating fibroblasts (0.9 ± 0.4). As compared to controls in stimulated wounds, a higher number of migrating fibroblasts (1.9 ± 0.4) was described. The striated muscle of control and stimulated wounds showed necrotic myofibers in the deepest part of the wound.

Group 2

In the control wounds of animals sacrificed 48 h after surgery, necrotic debris on the surface was almost separated and the scab was forming. Migration of the epithelial cells beneath the scab (0.6 ± 0.5) was observed (Fig. 3). However, the control incisions were not completely bridged by a layer of epithelial cells yet, while the stimulated wounds were completely bridged by two to three layers (2.0 ± 0.6; Fig. 4). In control wounds, PMNL (2.4 ± 0.5) were largely replaced by macrophages (1.9 ± 0.7), whereas in laser-stimulated wounds a regress of the inflammatory phase was observed (PMNL 1.4 ± 0.5; tissue macrophages 2.0 ± 0.6). Tissue macrophages participated in the process of the degeneration of the necrotic myofibers. In non-treated wounds, proliferation of fibroblasts (1.9 ± 0.4) and endothelial cells (1.3 ± 0.5), which forms granulation tissue, was recorded. As compared to the control, a higher number of fibroblasts (2.1 ± 0.4) and new vessels (1.6 ± 0.5) were present in laser-stimulated wounds, showing progress of the creation of the granulation tissue.

Group 3

Histological sections of the 72-h healed control wounds demonstrated that the incisions were completely bridged by two to three layers of new synthesized epithelial cells (2.1 ± 0.4), with the beginning of keratinization (Fig. 5). As compared to controls, the stimulated wounds were completely bridged by five layers of new synthesized epithelial cells (2.7 ± 0.5; Fig. 6). The inflammatory phase in control and stimulated wounds was almost completed (PMNL 0.9 ± 0.4 vs. 0.3 ± 0.5; tissue macrophages 1.9 ± 0.7 vs. 1.4 ± 0.5). In comparison with stimulated wounds, lower numbers of new vessels and fibroblasts were present in control tissues (vessels 1.9 ± 0.7 vs. 2.7 ± 0.5; fibroblasts 2.3 ± 0.5 vs. 2.9 ± 0.4). The incisional space at the layer of striated muscle and dermis in controls contained

FIG. 1. Control wound at 24 h after surgery (hematoxylin and eosin, 100×). Tissue necrosis (**a**) over the incision; demarcation line (**b**) which consists mainly of polymorphonuclear leukocytes (PMNL), still incomplete.

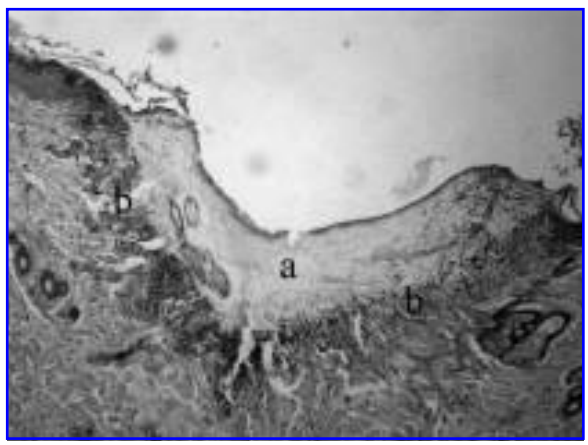

FIG. 2. Laser-treated wound at 24 h after surgery (hematoxylin and eosin, 100×). Tissue necrosis (**a**) over the incision; forming of the demarcation line (**b**) completed.

TABLE 3. SEMI-QUANTITATIVE EVALUATION (MEAN ± STANDARD DEVIATION) OF HISTOLOGICAL STRUCTURES AND CHANGES AFTER LOW-LEVEL LASER THERAPY (LLLT; CONTROLS/LLLT)

Group	*Epithelialization*	*PMNL*	*Tissue macrophages*	*Fibroblasts*	*New collagen*	*Neo-angiogenesis*	*Centro-nucleated cells*
1	0.0 ± 0.0/0.0 ± 0.0	2.0 ± 0.6/2.6 ± 0.5*	1.1 ± 0.4/1.3 ± 0.5	0.9 ± 0.4/1.9 ± 0.4*	0.0 ± 0.0/0.0 ± 0.0	0.0 ± 0.0/0.0 ± 0.0	0.0 ± 0.0/0.0 ± 0.0
2	0.6 ± 0.5/2.0 ± 0.6*	2.4 ± 0.5/1.4 ± 0.5*	1.9 ± 0.7/2.0 ± 0.6	1.9 ± 0.4/2.1 ± 0.4	0.0 ± 0.0/0.0 ± 0.0	1.3 ± 0.5/1.6 ± 0.5	0.0 ± 0.0/0.0 ± 0.0
3	2.1 ± 0.4/2.7 ± 0.5*	0.9 ± 0.4/0.3 ± 0.5*	1.9 ± 0.7/1.4 ± 0.5	2.3 ± 0.5/2.9 ± 0.4*	0.9 ± 0.4/1.9 ± 0.7*	1.9 ± 0.7/2.7 ± 0.5*	0.0 ± 0.0/0.0 ± 0.0
4	2.4 ± 0.5/3.0 ± 0.0*	0.1 ± 0.4/0.0 ± 0.0	1.4 ± 0.5/1.1 ± 0.4	2.4 ± 0.5/3.0 ± 0.0*	1.9 ± 0.7/2.1 ± 0.7	2.4 ± 0.5/2.9 ± 0.4	0.0 ± 0.0/0.0 ± 0.0
5	2.7 ± 0.5/3.0 ± 0.0	0.1 ± 0.4/0.0 ± 0.0	1.0 ± 0.0/1.1 ± 0.4	2.9 ± 0.4/3.0 ± 0.0	2.1 ± 0.7/2.9 ± 0.4*	2.7 ± 0.5/3.0 ± 0.0	0.1 ± 0.4/0.7 ± 0.5*
6	3.0 ± 0.0/3.0 ± 0.0	0.0 ± 0.0/0.0 ± 0.0	1.3 ± 0.5/1.1 ± 0.4	3.0 ± 0.0/3.0 ± 0.0	2.7 ± 0.5/2.9 ± 0.4	2.7 ± 0.5/2.4 ± 0.5	0.7 ± 0.5/0.9 ± 0.4
7	3.0 ± 0.0/3.0 ± 0.0	0.0 ± 0.0/0.1 ± 0.4	1.1 ± 0.4/1.0 ± 0.0	3.0 ± 0.0/3.0 ± 0.0	3.0 ± 0.0/3.0 ± 0.0	2.4 ± 0.5/1.4 ± 0.5*	1.0 ± 0.0/1.0 ± 0.0

*$p < 0.05$.

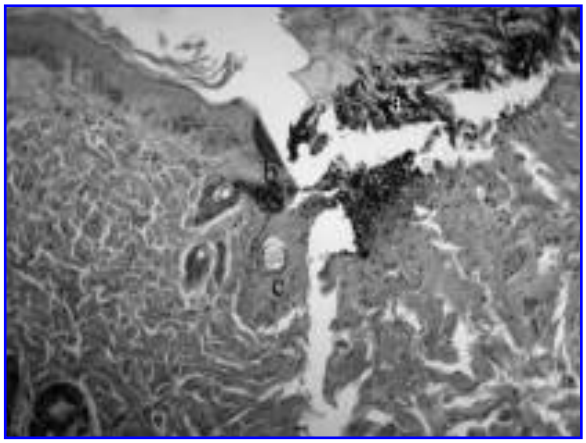

FIG. 3. Control wound at 48 h after surgery (hematoxylin and eosin, 200×). Forming of demarcation line finished (**a**); migration of epithelial cells (**b**). Hair follicle (**c**) as an alternative center of reepithelialization.

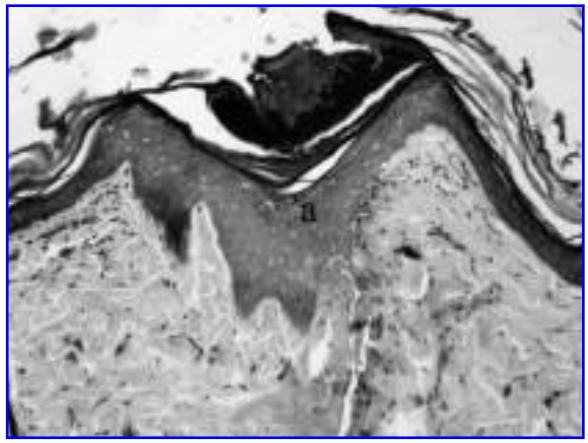

FIG. 6. Laser-treated wound at 72 h after surgery (azur and eosin, 200×). Incision bridged by five layers of epithelial cells (**a**)

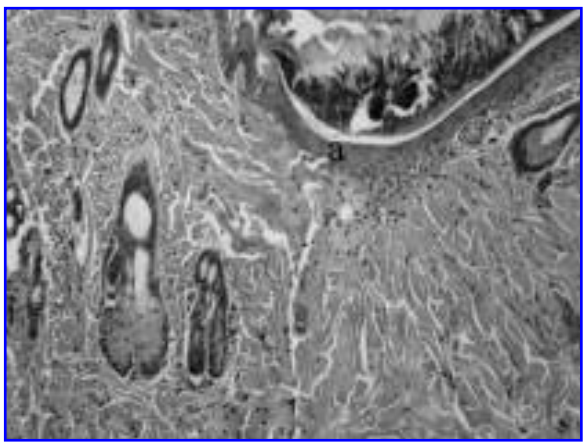

FIG. 4. Laser-treated wound at 48 h after surgery (hematoxylin and eosin, 200×). Bridging of the incision with two layers (**a**) of new synthesized epithelial cells.

ECM without a significant quantity of collagen (0.9 ± 0.4), while in stimulated wounds an increase in the amount of new ECM and collagen was found (1.9 ± 0.7).

Group 4

The control wounds of animals killed 96 h after surgery showed epithelial thickening over the incisions (2.4 ± 0.5) and almost finished surface keratinization of the epidermis. The thickness of the keratin layer in laser-stimulated wounds was similar to that of the intact epidermis, so regeneration of the epidermis was completely finished (3.0 ± 0.0). The inflammatory phase in control wounds was completely finished (PMNL 0.1 ± 0.4). However, tissue macrophages were always present (1.4 ± 0.5). Fibroblasts were vertically oriented to the incisions in control and stimulated wounds (2.4 ± 0.5 vs. 3.0 ± 0.0). In control wounds (Fig. 7), fewer vessels and less new collagen in granulation tissue in comparison with stimulated wounds were

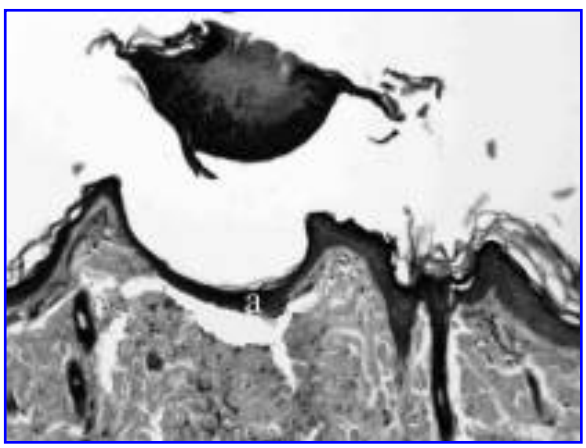

FIG. 5. Control wound at 72 h after surgery (azur and eosin, 200×). Bridging of the incision with two to three layers of epithelial cells (**a**) with the beginning of stratification.

FIG. 7. Control wound at 96 h after surgery (hematoxylin and eosin, 200×). Granulation tissue in the layer of striated muscle (**a**).

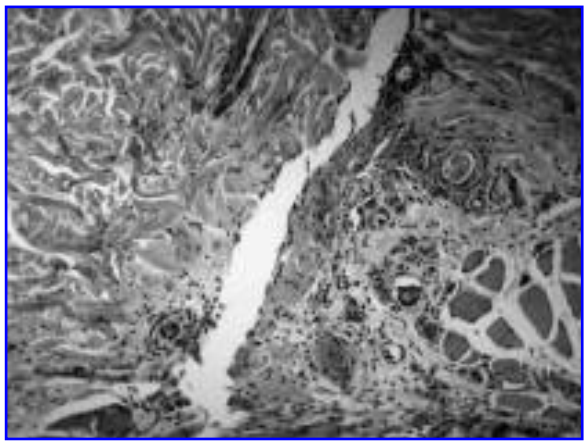

FIG. 8. Laser-treated wound at 96 h after surgery (Hematoxylin and eosin, 200×). Greater number of vessels and fibroblasts in granulation tissue of irradiated wounds (**a**).

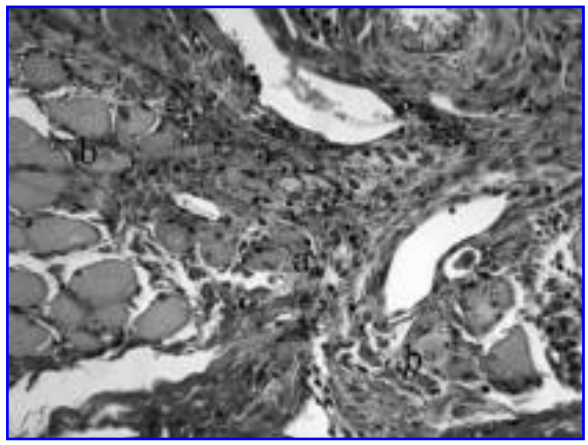

FIG. 10. Laser-treated wound at 120 h after surgery (van Gieson, 400×). Greater amount of collagen in granulation tissue (**a**); centronucleated cells (**b**).

present (vessels 2.4 ± 0.5 vs. 2.9 ± 0.4; collagen 1.9 ± 0.7 vs. 2.1 ± 0.7; Fig. 8).

Group 5

The thickness of the keratin layer of control wounds was similar to the intact epidermis (2.7 ± 0.5) and showed that regeneration was finished. In control and stimulated wounds, the granulation tissue consisted of many new vessels (2.7 ± 0.5 vs. 3.0 ± 0.0) and fibroblasts (2.9 ± 0.4 vs. 3.0 ± 0.0). The granulation tissue was most significant at the layer of the striated muscle. In the incisional slit of each wound in this group, a great amount of PAS+PSD–positive substance was present; it was the new ECM. As compared to control wounds (Fig. 9) in laser-stimulated wounds, VG staining showed that the new ECM consisted most of new collagen fibers (2.1 ± 0.7 vs. 2.9 ± 0.4; Fig. 10). The reorganization of these fibers observed in laser-stimulated wounds meant the beginning of the maturation phase. In this group, for the first time, the presence of centronucleated cells in the healing of injured striated muscle was described (0.7 ± 0.5); it was observed in laser-stimulated tissues (Fig. 10).

Group 6

The assessment of histological sections of 144-h healed control wounds demonstrated that vertically oriented fibroblasts (3.0 ± 0.0) were still present in this evaluated time interval. In stimulated wounds, for the first time, some fibroblasts were horizontally oriented (3.0 ± 0.0). The reorganization of collagen fibers in controls was recorded (2.7 ± 0.5); thus, the maturation phase was initiated. However, no significant difference was found in the number of fibroblasts in control and stimulated wounds (Figs. 11 and 12). Interestingly, in the dermis and in the striated muscle layer of stimulated wounds, the decrease in the number of vessels in the granulation tissue compared to controls was described (2.7 ± 0.5 vs. 2.4 ± 0.5). For the first time in control

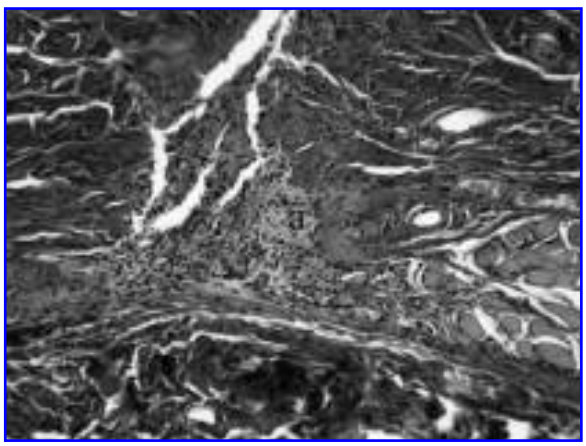

FIG. 9. Control wound at 120 h after surgery (van Gieson, 200×). New collagen fibers (**a**) in granulation tissue in the layer of striated muscle and the deepest part of dermis.

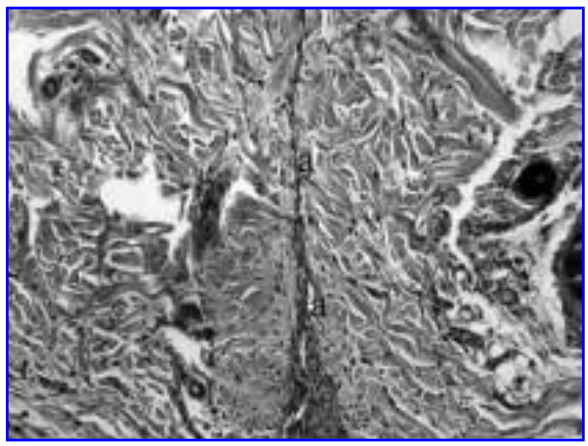

FIG. 11. Control wound at 144 h after surgery (periodic acid Schiff [PAS], 200×). In incisional gap, in dermis, vertically oriented fibroblast (**a**).

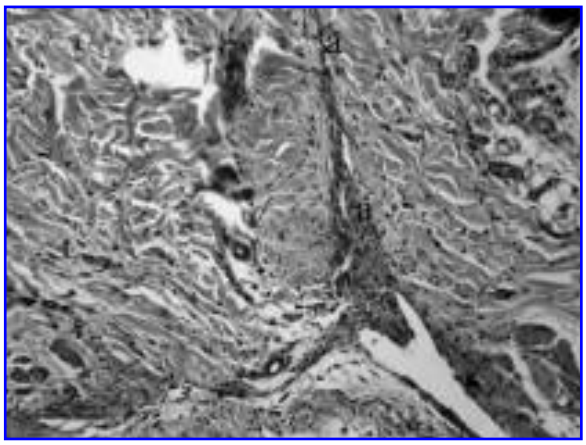

FIG. 12. Laser-treated wound at 144 h after surgery (periodic acid Schiff [PAS], 200×). Comparable number of fibroblasts in dermis (**a**).

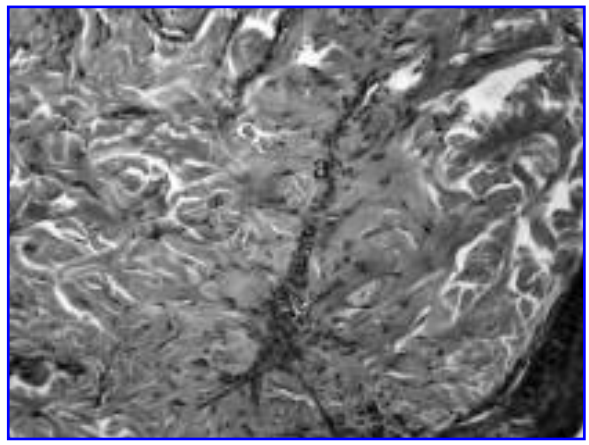

FIG. 14. Laser treated wound at 168 h after surgery (periodic acid Schiff [PAS], 200×). Horizontally oriented fibroblasts (**a**).

wounds, the presence of centronucleated cells was observed (0.7 ± 0.5).

Group 7

In this group, the analysis of the effect of LLLT on skin wound healing was performed 168 h after surgery. According to the VG- and PAS+PSD–stained slides, more glycoproteins, proteoglycans, and collagen fibers were synthesized by fibroblasts (3.0 ± 0.0). In control wounds, vertically oriented fibroblasts were present (Fig. 13), whereas in stimulated wounds most fibroblasts were horizontally oriented (Fig. 14). A small decrease in the number of new vessels (2.4 ± 0.5) was observed in control tissue. However, in laser-treated wounds, the number of vessels decreased to the minimum score (1.4 ± 0.5). The decrease of vessels and a predomination of cross-linked collagen fibers (3.0 ± 0.0) indicated more progressive tissue scar formation of stimulated wounds. Furthermore, an increase in the number of centronucleated cells at the limbs of damaged striated muscle was shown (1.0 ± 0.0 vs. 1.0 ± 0.0).

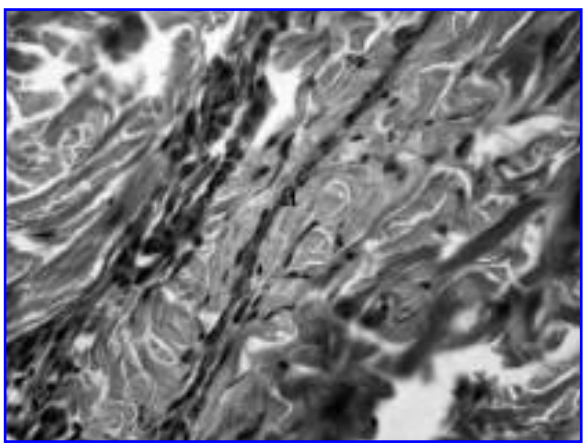

FIG. 13. Control wound at 168 h after surgery (periodic acid Schiff [PAS], 400×). Most of fibroblasts vertically oriented (**a**).

DISCUSSION

One potentially interesting aspect of the present study is that the most significant morphological changes occured during the first 7 days of wound healing. Similarly, the literature suggests the possibility of influencing this process by physical factors such as laser radiation. However, experiments in the literature vary in the parameters of laser irradiation used, wound models (excisional, incisional), and evaluated time intervals.[5,15–20] For example, in a study of the GaAIAs laser, at 904-nm wavelength, at 33 J/cm^2 on days 3, 7, and 14 using an excisional model, LLLT accelerated the inflammatory process and epithelization, and positively influenced collagen deposition in the wounds of steroid-treated rats as well as in non-treated rats.[21] Similarly, Stadler et al.,[18] using an incisional model, showed the positive effect of 830-nm irradiation at 5 J/cm^2 on the wound tensile strength tests in diabetic mice; however, they evaluated only 2 specific days, the 11th and the 23rd.

In the present study, in laser-treated wounds, we found an accelerated process of inflammation and proliferation that is in agreement with previous histomorphological studies.[16,22,23] Moreover, in our study, in treated wounds an increase in the amount of collagen fibers and more evident remodeling (maturation phase) of connective tissue were shown, and this corresponded with the results of experiment where radiation from the InGaAlP laser (685 nm, dose 2.5 J/cm^2) was used for acceleration of healing.[24] Kawalec et al.[25] showed a beneficial effect of treatment with the 980-nm GaAlAs diode laser at 18 J.cm^{-2} on wound healing in diabetic mice; however, treatment with 36 J/cm^2 seemed adverse and decreased the healing process.

In contrast to these results, in numerous studies, evidence has been provided on the inefficiency of LLLT.[21,26,27] In an experimental *in vivo* study trying to positively affect the healing of burns in rats after using laser irradiation of 635 and 690 nm at 1.5 J/cm^2, no macroscopic and microscopic differences among treated and control groups have been observed.[27] Simi-

larly, no significant differences in epithelization and contraction of skin wounds in horses after LLLT using GaAlAs laser at 2 J/cm^2 were demonstrated.[26] In addition, no significantly beneficial effects on the blood microcirculation in wound healing in laser-treated (HeNe laser at 1.5 J/cm^2) groups in comparison with controls were demonstrated.

It is generally accepted that the biological effect of LLLT depends on three major parameters: wavelength, dose, and power density. Because InGaAlP laser (670 nm) is commonly used for LLLT in clinical practice, we decided to asses its effect in our experimental study (excluding the placebo effect). One of the reasons for the negative effect of LLLT in numerous studies may be related to the use of extremely low doses.[28] For example, LLLT reduces PGE_2 levels in the joint capsule in animals, and this effect was reported within the range of 0.4–19 J/cm^2. While lower range limits for PGE_2 reduction were identified, upper range limits could not. At the present time, little is known about when or if this effect would level off.[29] It has been shown that power densities above 20 mW/cm^2 temporarily inhibit fibroblast metabolism.[30] However, we surmised that a dose at 30 J/cm^2 and power density at 25 mW/cm^2 would be capable of reducing inflammation without compromising fibroblast metabolism. The results from our study confirmed this assumption.

CONCLUSION

The results from our histopathological examination of the effect of LLLT using the AlGaInP diode laser show an accelerated process of regeneration of the epidermis, reparation of the dermis, and acceleration of the healing of injured striated muscle (Table 3). These findings extend and reinforce the theory of the positive influence of LLLT on the healing of skin wounds. However, readers should be cautioned that the results of our histomorphological study may not be directly applicable to chronic wounds or other healing tissues.

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