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ORIGINAL ARTICLE

Effect of equal daily doses achieved by different power densities of low-level laser therapy at 635 nm on open skin wound healing in normal and corticosteroid-treated rats

Peter Gál · Michal Mokrý · Boris Vidinský · Róbert Kilík · Filip Depta · Magdaléna Harakaľová · František Longauer · Štefan Mozeš · Ján Sabo

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Abstract Optimal parameters of low-level laser therapy (LLLT) for wound healing are still discussed. Hence, our study was aimed to compare effects of different power densities of LLLT at 635 nm in rats. Four, round, full-thickness, skin wounds were made on the backs of 48 rats that were divided into two groups (non-steroid laser-treated and steroid laser-treated). Three wounds were stimulated daily with a diode laser (daily dose 5 J/cm^2) each with different power density (1 mW/cm^2, 5 mW/cm^2, and 15 mW/cm^2), whereas the fourth wound served as a control. Two days, 6 days, and 14 days after surgery, eight animals from each group were killed and samples were removed for histological evaluation. In the non-steroid laser-treated rats, significant acceleration of epithelization and collagen synthesis 2 days and 6 days after surgery was observed in stimulated wounds. In steroid laser-treated rats, 2 days and 14 days after surgery, a decreased leucocyte/macrophage ratio and a reduction in the area of granulation tissue were recorded, respectively. In conclusion, LLLT, by the method we used, improved wound healing in the non-steroid laser-treated rats, but it was useless after corticosteroid treatment.

Keywords Daily dose of 5 J/cm^2 · Low-level laser therapy · Wound healing · Different power densities

P. Gál (✉) · M. Mokrý · B. Vidinský · F. Depta · M. Harakaľová · J. Sabo
Department of Medical Biophysics,
Pavol Jozef Šafárik University,
Trieda SNP 1,
040 11 Košice, Slovak Republic
e-mail: galovci@yahoo.com

R. Kilík
1st Department of Surgery, Pavol Jozef Šafárik University
and Louise Pasteur Faculty Hospital,
Košice, Slovak Republic

F. Longauer
Department of Forensic Medicine, Pavol Jozef Šafárik University,
Košice, Slovak Republic

Š. Mozeš
Institute of Animal Physiology, Slovak Academy of Sciences,
Košice, Slovak Republic

Introduction

Despite the use of many promising physical methods, such as vacuum assisted closure (VAC) [1], magnetic fields [2], light emitting diodes (LEDs) [3] or low-level laser therapy (LLLT) [4], delayed wound healing is still a significant problem in clinical practice. In general, it is well known that long-term application of corticosteroids is responsible for poor wound healing. Accordingly, in steroid-treated animals and humans, inhibition of the inflammatory phase, decrease of the wound strength, wound contracture, and inhibited epithelization and fibroblastic proliferation have been found [5–7].

LLLT belongs to modern experimental approaches used in wound healing therapy. In numerous clinical and experimental studies it has been proven that low-energy laser light reduces pain, positively influences inflammatory, proliferative and maturation phases of wound healing, and increases wound tensile strength [8–14]. Even if the importance of LLLT has been stressed by these results, this method has not yet been generally accepted for use in clinical practice. Nevertheless, several researchers have

shown that LLLT may have adverse effects on wound healing [15–20], although one of the reasons for its ineffectiveness may be associated with the use of extremely low doses [21].

At the present time there is no general agreement about the exact way how LLLT influences wound healing. Nevertheless, it has been well documented that the helium–neon (HeNe) laser, using a dose of 4 J/cm^2, accelerates wound closure and increases collagen deposition [22, 23]. Furthermore, in a previous study, we demonstrated a positive effect of HeNe laser irradiation at a dose of 3 J/cm^2 on the healing of primary sutured skin wounds [24]. On the other hand, Kana et al. revealed a deceleration in healing by increasing the dose to 20 J/cm^2 on open skin wounds [22]. Similarly, in wounded fibroblasts exposed to 5 J/cm^2 of HeNe laser irradiation, increased cell proliferation, migration, and viability have been observed, whereas higher doses, 10 J/cm^2 and 16 J/cm^2, have been found to be inhibitory [25, 26].

It was found that longer wavelengths are associated with a greater depth of penetration [27]. Hence, it may be hypothesized that different wavelengths of laser light stimulate wound healing variously. Accordingly, in an experiment in which the effects of 665 nm, 675 nm, and 810 nm laser light on the proliferation of fibroblasts and endothelial cells were compared, it was found that the highest proliferation activity was observed in 665 nm light-stimulated cultures, while 810 nm inhibited the proliferation of fibroblasts [28]. Since most of the target cells of LLLT are located in the epidermis and hair follicles (epidermal stem cells), as well as in the upper parts of the dermis (fibroblast, macrophages, endothelial cells), we selected a red laser to be tested in our study instead of an infra-red one [29, 30].

In the experiment we tested a diode laser that produces radiation at 635 nm, comparable to that of a HeNe laser (632.8 nm); thus, similar effects might be expected if analogous doses were to be used. However, the optimal power density is still unknown. Therefore, the aim of our study was to compare the influence of different power densities, achieving equal daily doses, on skin wound healing in non-steroid laser-treated and steroid laser-treated rats, using an excisional model and histological evaluation.

Materials and methods

Animal model

This experiment was approved by the Ethics Committee of the Faculty of Medicine of P. J. Šafárik University and by the State Veterinary Administration of the Slovak Republic.

Ten-months-old male Sprague-Dawley rats (n=48), weighing 500–550 g, were included in the experiment and randomly divided into two groups of 24 animals, i.e., non-steroid laser-treated group (N) and steroid laser-treated group (S). The rats were subjected to general anaesthesia [ketamine 40 mg/kg (Narkamon a.u.v., Spofa, Prague, Czech Republic), xylazine 15 mg/kg (Rometar a.u.v., Spofa, Prague, Czech Republic), tramadol 5 mg/kg (Tramadol-K, Krka, Novo Mesto, Slovenia)], and four, round, full-thickness, skin wounds, 4 mm in diameter, were made on the back of each rat. The wounds were placed at the corners of a quadrangle with 5 cm sides.

Steroid-treated animals were given a bolus dose of 20 mg/kg methyl-prednisolone (Depo-Medrol, Pharmacia and Upjohn, Puurs, Belgium), intramuscularly, shortly before surgery, whereas animals from the non-steroid group remained untreated.

Low level laser therapy

We irradiated three wounds on each rat daily (for a maximum of 6 days) with a commercially available gallium–aluminum–arsenium (GaAlAs) diode laser (Maestro/CCM, Medicom Praha, Prague, Czech Republic; λ=635 nm; shape of beam oval, r_1=5 mm, r_2=2.5 mm; time of treatment at 15 mW/cm^2=5 min 33 s, at 5 mW/cm^2=16 min 40 s, and at 1 mW/cm^2=83 min 20 s; probe distance to wound 10 cm) to administer the total daily dose of 5 J/cm^2, while the fourth wound was not irradiated and served as a control. One of the laser-treated wounds was irradiated at 1 mW/cm^2 power density, the second at 5 mW/cm^2 and the third at 15 mW/cm^2. The positions of the laser-treated and control wounds were rotated within the groups. During treatment, the rats were restrained in a Plexiglas cage with an oval opening over each currently stimulated wound, and the other wounds were protected from reflected laser light.

Histopathological evaluation

Eight animals from each group were killed by ether inhalation 2 days, 6 days or 14 days after surgery. The tissue specimens were processed routinely for light microscopy (fixing, dehydrating, embedding, sectioning, staining with hematoxylin and eosin (HE, basic staining) and van Gieson's stain (VG, non-specific collagen staining)).

We used a semi-quantitative method to evaluate the following histological structures/processes, i.e., polymorphonuclear leukocytes (PMNLs), re-epithelization, fibroblasts, new vessels, and collagen synthesis. The sections were evaluated in a blind manner, according to the scale 0, 1, 2, 3, 4 (Table 1).

We used QuickPHOTO MICRO 2.2 (Promicra, Prague, Czech Republic) software to quantify the following

Table 1 Scale for the semi-quantitative evaluation of histological changes/structures in healing skin wounds. *ST* surrounding tissue, i.e., tissue out of granulation tissue (*GT*)

Scale	Epithelization	PMNLs	Fibroblasts	New vessels	Collagen
0	Absent	Absent	Absent	Absent	Absent-GT
1	Thickness of cut edges	Mild-ST	Mild-ST	Mild-ST	Minimal-GT
2	Migration of cells	Mild-GT	Mild-GT	Mild-GT	Mild-GT
3	Bridging of the excision	Moderate-GT	Moderate-GT	Moderate-GT	Moderate-GT
4	Keratinization	Marked-GT	Marked-GT	Marked-GT	Marked-GT

parameters: (1) To determine the inflammatory phase, we calculated the ratio of the number of PMNLs to the number of tissue macrophages (TMs) in specimens removed from animals killed 2 days after surgery. The numbers of PMNLs and TMs were counted in one high-resolution field from each section. (2) The length of the epithelial sheet was morphometrically evaluated in sections removed from animals killed 6 days after surgery and expressed as the percentage covering the whole wound surface. (3) To determine the effect of LLLT on granulation tissue (GT) formation, we measured the area of GT sections made from animals killed 6 days and 14 days after surgery.

Statistical analysis

For each evaluated parameter mean values ± standard deviations (SDs) were calculated. Data obtained from the semi-quantitative evaluation were compared, using the non-parametric Kruskal–Wallis test. Analysis of variance (ANOVA), followed by Tukey–Kramer's multiple comparison test, was used for the comparison of the PMNL/TM ratios and the GT areas. Significance was accepted at $P<0.05$.

Results

During the post-surgery period, the animals remained healthy, with no clinical evidence of infection. The results of our histological investigation are summarized in Figs. 1, 2, 3 and 4.

Day 2

Non-steroid laser-treated group

Formation of the demarcation line was the most typical histological change in this time period. There were no significant differences in the numbers of the most common acute inflammatory cells (PMNLs) between wounds (Fig. 1). In contrast, significant difference in the process of re-epithelization was shown (Figs. 1 and 5). In comparison with non-stimulated wounds, in wounds stimulated by 15 mW/cm^2 accelerated migration and proliferation of keratinocytes was observed [0 mW/cm^2 and 15 mW/cm^2, respectively ($P<0.05$)]. Comparable numbers of new vessels and fibroblasts near the excisions were recorded (Fig. 2). At the edges of the excisions, significant differences in the creation of new collagen fibers was recorded between

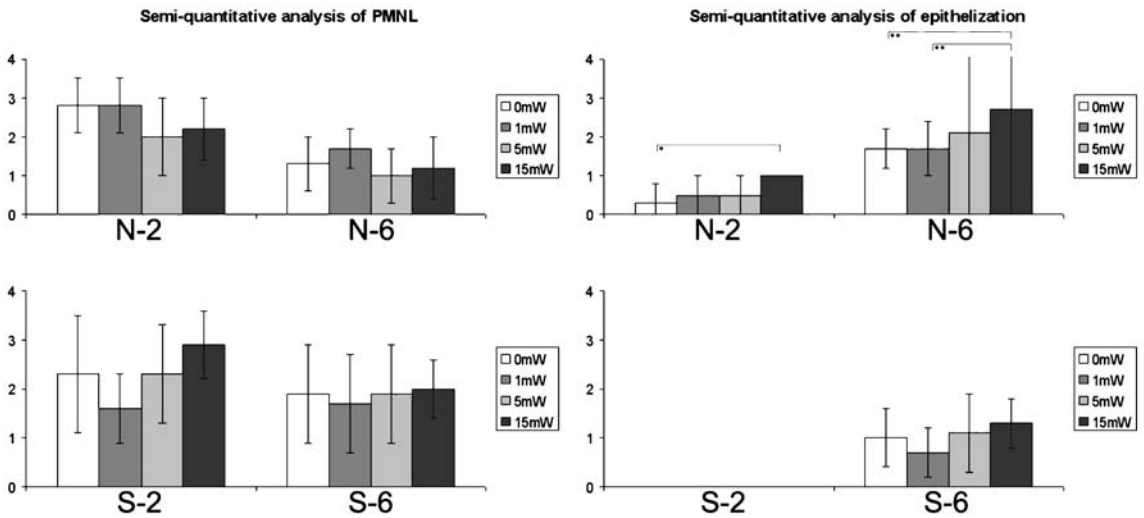

Fig. 1 Semi-quantitative analysis of polymorphonuclear leucocytes (PMNL) and re-epithelization 2 days and 6 days after surgery (data are presented as means ± SDs; *$P<0.05$, **$P<0.01$). *N* non-steroid laser-treated group, *S* steroid laser-treated group. In the S group the epithelization has not yet started

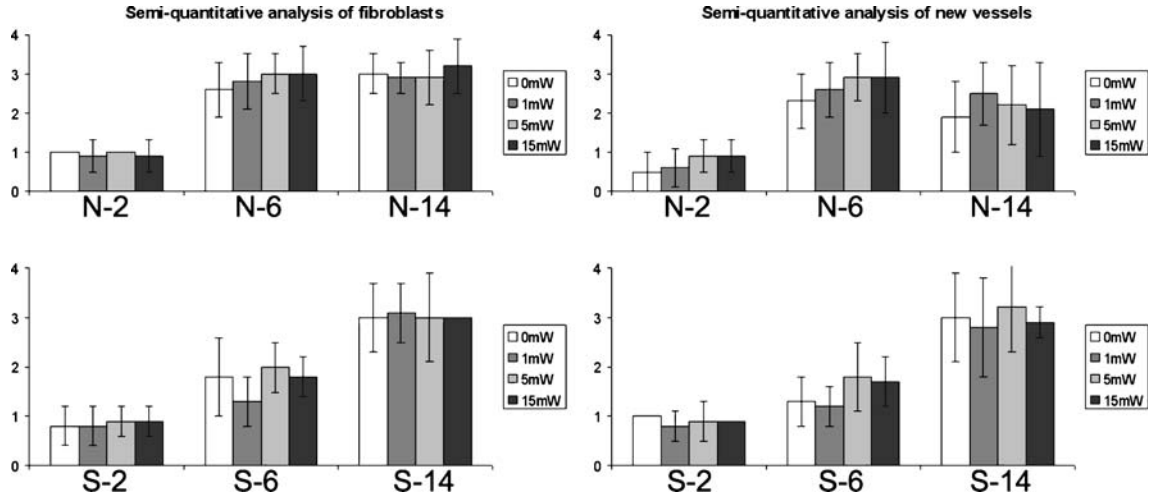

Fig. 2 Semi-quantitative analysis of fibroblasts and new vessels 2 days, 6 days and 14 days after surgery (data are presented as means ± SDs). *N* non-steroid laser-treated group, *S* steroid laser-treated group

control and stimulated wounds [0 mW/cm^2 vs 5 mW/cm^2 (P<0.01); 0 mW/cm^2 vs 15 mW/cm^2 (P<0.01); 1 mW/cm^2 vs 5 mW/cm^2 (P<0.01); 1 mW/cm^2 vs 15 mW/cm^2 (P< 0.01] (Fig. 3).

Steroid laser-treated group

In general, the wound healing process in steroid laser-treated animals was slowed (Figs. 1, 2 and 3). Migration of keratinocytes and creation of new collagen fibers had not started. The ratio of PMNL/TM was significantly higher in non-stimulated wounds than in all stimulated wounds [PMNL/TM: 0 mW/cm^2 vs 1 mW/cm^2 (P<0.01); 0 mW/cm^2 vs 5 mW/cm^2 (P<0.01), 0 mW/cm^2 vs 15 mW/cm^2 (P<0.01)]; thus, the inflammatory phase was accelerated after LLLT (Fig. 4).

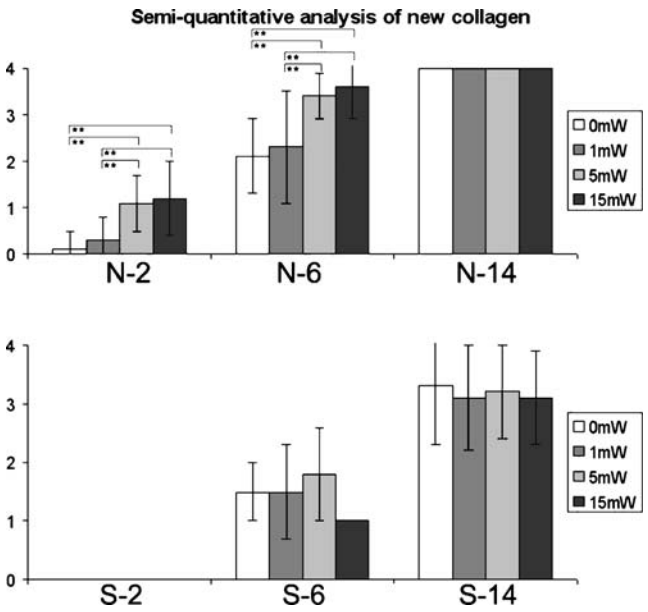

Fig. 3 Semi-quantitative analysis of new collagen 2 days, 6 days and 14 days after surgery (data are presented as means ± SDs; **P<0.01. *N* non-steroid laser-treated group, *S* steroid laser-treated group). In the S group 2 days after surgery collagen formation had not yet started

Day 6

Non-steroid laser-treated group

The histological analysis in this group demonstrated a remission of the inflammatory process in all wounds (Fig. 1). The keratinocytes had migrated beneath the scab and mostly completely bridged the excisions. However, different progress of re-epithelization between certain stimulated and non-stimulated wound was shown [0 mW/cm^2 vs 15 mW/cm^2; 1 mW/cm^2 vs 15 mW/cm^2 (P<0.01)] (Fig. 1). Results of semi-quantitative analysis were confirmed by morphometrical analysis (0 mW/cm^2 vs 15 mW/cm^2 [P<0.01); 1 mW/cm^2 vs 15 mW/cm^2 (P<0.05)] (Fig. 4). This time period showed a typical histological picture of the proliferative phase, with expressive representation of fibroblasts and new vessels in all wounds, with no significant differences between wounds (Fig. 2). On the other hand, a greater number of new collagen fibers was seen in wounds stimulated by laser radiation at higher power densities [0 mW/cm^2 vs 5 mW/cm^2 (P<0.01); 0 mW/cm^2 vs 15 mW/cm^2 (P<0.01); 1 mW/cm^2 vs 5 mW/cm^2 (P< 0.05); 1 mW/cm^2 vs 15 mW/cm^2 (P<0.01)] (Figs. 3 and 6). No significant differences between wounds were observed when the GT area was measured (Fig. 4).

Steroid laser-treated group

The typical steroid-induced impairment of wound healing characterized this group. However, no significant differ-

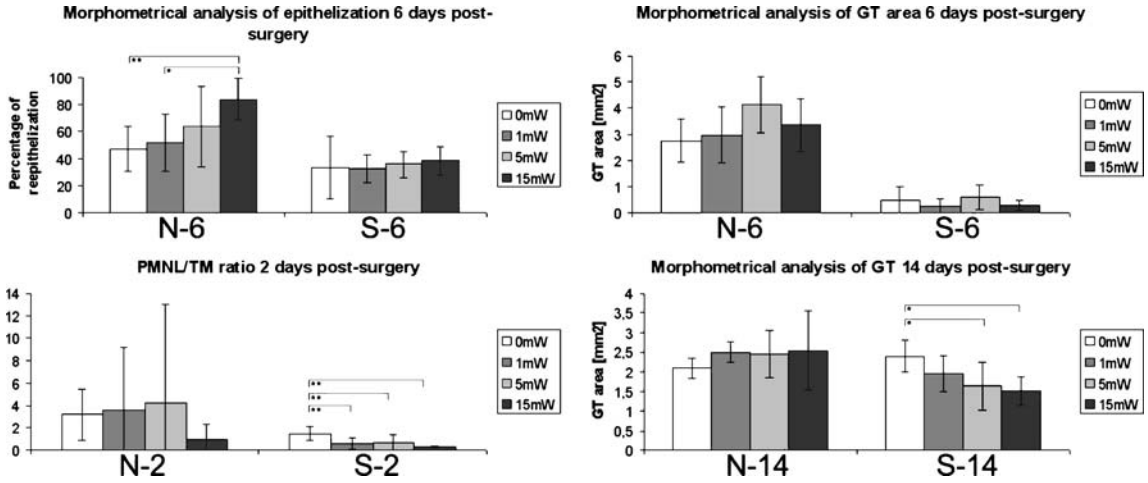

Fig. 4 Morphometric analysis of epithelization (expressed as percentage) of absolute re-epithelization) 6 days after surgery and granulation tissue (GT) area (in square millimeters) 6 days and 14 days after surgery; cell counting from one high-resolution field (×1,000), PMNL/TM ratio 2 days after surgery (data are presented as means ± SDs). *$P<0.05$, **$P<0.01$) *N* non-steroid laser-treated group, *S* steroid laser-treated group

ences in the evaluated histological parameters between the stimulated and non-stimulated wounds were observed (Figs. 1, 2, 3 and 4).

Day 14

Non-steroid laser-treated group

For this time period remodeling and reorganization of extracellular matrix (ECM) was characterized; thus, the scar was formed (Fig. 3). In addition, a mild regress of vessels in the granulation tissue was shown in this group (Fig. 2). However, significant histological differences between wounds were not seen, either in the semi-quantitatively evaluated structures or in those quantitatively evaluated (Figs. 2, 3 and 4).

Steroid laser-treated group

With regard to the impaired process of wounding in the steroid group, the decrease in the number of vessels and the increase in collagen fibers was not apparent by any

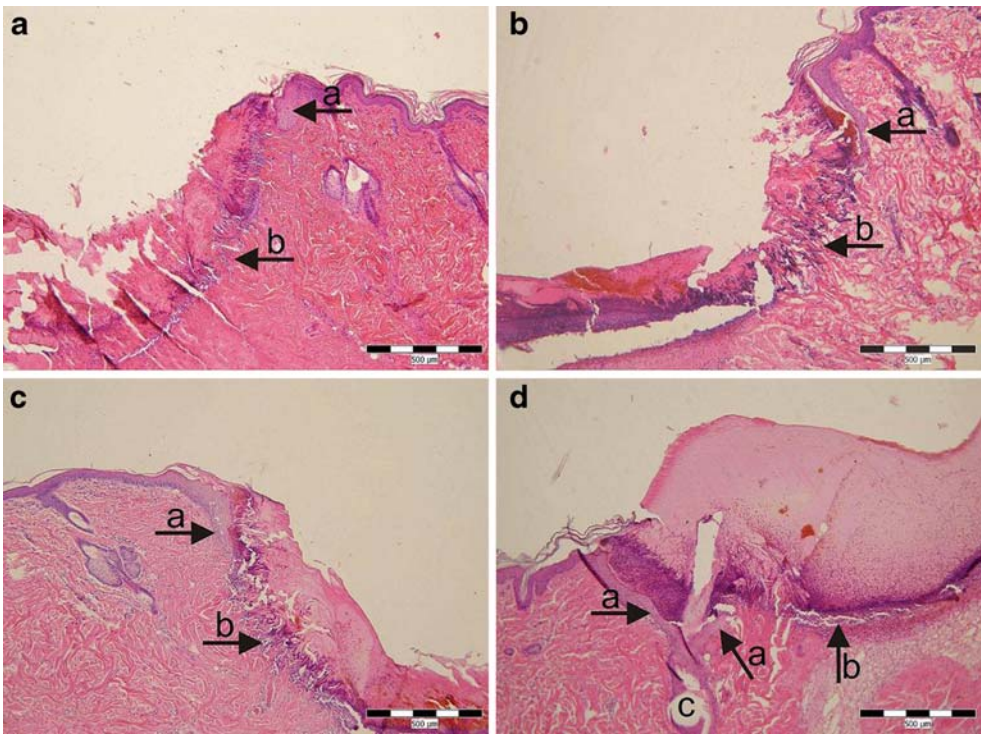

Fig. 5 Skin wounds 2 days after surgery in non-steroid laser-treated animals. **a** Control wound (*a* thickness of cut edges of the epidermis, *b* demarcation line separating necrosis from the vital tissue); **b** 1 mW/cm^2 LLLT (*a* the beginning of epithelial cell migration, *b* demarcation line); **c** 5 mW/cm^2 LLLT (*a* migration of epithelial cells, *b* demarcation line); **d** 15 mW/cm^2 LLLT (*a* most prominent migration of epithelial cells, *b* demarcation line, *c* hair follicle). HE; *scale bar* represents 500 μm

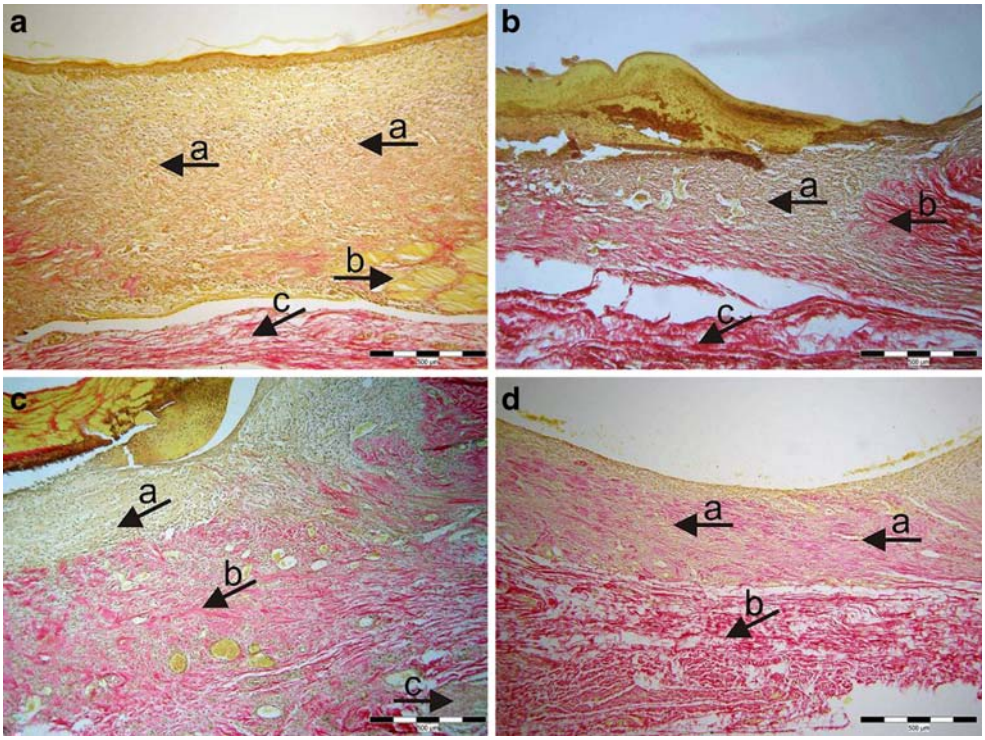

Fig. 6 Skin wounds 6 days after surgery in non-steroid laser-treated animals. **a** Control wound (*a* granulation tissue without a significant quantity of collagen, *b* striated muscle layer, *c* subcutaneous tissue); **b** 1 mW/cm^2 LLLT (*a* granulation tissue located in the middle of the wound, without collagen, *b* granulation tissue located near normal dermis contains new collagen, *c* subcutaneous tissue); **c** 5 mW/cm^2 LLLT (*a* upper part of granulation tissue without a significant quantity of collagen, *b* lower part of granulation tissue rich in new collagen and vessels, *c* striated muscle layer); **d** 15 mW/cm^2 LLLT (*a* whole granulation tissue contains new collagen, *b* subcutaneous tissue). VG stain; *scale bar* represents 500 μm)

significant differences between wounds (Figs. 2 and 3). Nevertheless, in this group, the scars of the laser-treated wounds were significantly reduced [0 mW/cm^2 vs 5 mW/cm^2 ($P<0.05$); 0 mW/cm^2 vs 15 mW/cm^2 ($P<0.05$)] (Figs. 4 and 7).

Discussion

The effectiveness of the various wavelengths and power densities of laser radiation on wound healing has still not been fully clarified. From a comparison of the effects

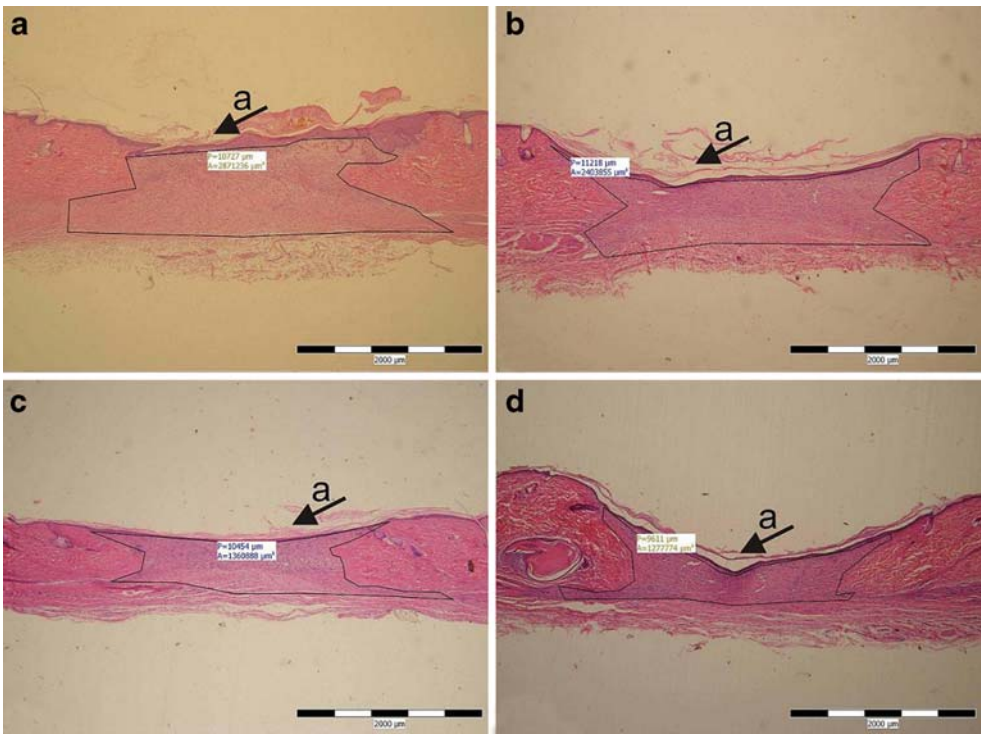

Fig. 7 Skin wounds 14 days after surgery in steroid laser-treated animals **a** control wound; **b** 1 mW/cm^2 LLLT; **c** 5 mW/cm^2 LLLT; **d** 15 mW/cm^2 LLLT; granulation tissue area decreases with increasing dose of LLLT (*a* indicates keratinization of epidermis). HE; *scale bar* represents 2,000 μm

of different wavelengths (670 nm vs 685 nm) and intensities (2 mW, 15 mW, and 25 mW), achieving a total dose of 10 J/cm^2, LLLT has been found to be more effective if higher intensity is combined with shorter wavelength or lower intensity with longer wavelength [31]. Nevertheless, from assessment of the effects of different wavelengths, i.e., 633 nm, 670 nm, 820 nm, on redox changes in HeLa cells, it has been found that cytochrome c oxidase becomes more oxidized, due to irradiation at each wavelength used [32]. These results support the suggestion that the mechanism of LLLT at the cellular level is generally based on the increase of oxidative metabolism in mitochondria.

Results from our investigation point to an inverse relationship between power density and the course of the inflammatory phase. Whereas the lowest power density slightly increased wound infiltration in the control rats with inflammatory cells 6 days after surgery, both higher power densities acted in a slightly anti-inflammatory manner during all the time intervals examined. This trend is in agreement with results from a study published by do Nascimento and co-workers [31], as well as from our previous study where we demonstrated that high doses of LLLT are able to accelerate wound healing by reducing inflammation without compromising the proliferation of fibroblasts and keratinocytes [13]. On the other hand, laser stimulation significantly accelerated the inflammatory phase only in the steroid laser-treated group. However, this effect was observed rather by the power density-independent decrease in PMNL/TM values than by the absolute amount of inflammatory cell infiltration.

By evaluating the effect of LLLT on both the proliferative phase of dermis repair and epidermis regeneration, we observed that epithelization and collagen synthesis were significantly accelerated only in non-steroid laser-treated animals in a power density-dependent manner. In addition, in this group, a typical picture of the proliferative phase with expressive representation of fibroblasts and new vessels in all wounds, without significant differences between wounds, has been shown. In contrast, in steroid laser-treated rats, a significant negative impact by methyl-prednisolone on the formation of the granulation tissue was demonstrated, with no significant differences between stimulated and control wounds. Hence, in our experiment, it was demonstrated that LLLT significantly accelerated the proliferative phase only in non-steroid laser-treated animals. However, another study has shown that LLLT at 904 nm and 33 J/cm^2 stimulated both the proliferative phase and granulation tissue formation in both non-steroid laser-treated and steroid laser-treated animals as well [6]. From this point of view, the exact effect of LLLT on the proliferation phase in steroid laser-treated rats remains to be answered.

In addition, scarring is a well-known side effect of skin laser treatment [33]. Hædersdal and co-workers investigated whether scarring after the acute inflammatory reaction following laser therapy could be reduced by administration of methyl-prednisolone and indomethacin. However, the results from their investigation point out that the use of the drug in the prophylaxis against scarring was not beneficial. On the other hand, our results demonstrate that LLLT, by our method, is able to accelerate wound healing without enlarging the area of the scar tissue. Furthermore, in contrast to non-steroid laser-treated rats, in steroid laser-treated rats the low-level laser therapy resulted in a statistically significant reduction in the granulation tissue 14 days after the rats had been wounded, which may indicate reduced scar formation. An explanation for this effect may be that laser-induced wound contraction by myofibroblasts [34] combined with methyl-prednisolone caused inhibition of GT formation [35]. Since, in non-steroid laser-treated rats, no effect was observed, it may be hypothesized that co-action of myofibroblasts and medication leading to GT reduction is inevitable for scar reduction.

Previously, evidence suggesting a possible systemic effect of LLLT was described [11, 36]. On the other hand, in numerous previous investigations in which control and laser-treated wounds were made on the same animal, a significantly positive effect of treatment has been observed [13, 24, 37–39]. Furthermore, results from our investigation, in which one control and three laser-treated wounds were made on the same animal, demonstrate differences between different power densities of LLLT. From this point of view, it can be hypothesized that the dominant effect of LLLT on skin wound healing is local. Therefore, the question of a systemic effect of laser therapy remains open and requires further research.

In conclusion, our results extend and reinforce the theory of the positive effect of LLLT on wound healing in normal conditions, but not after corticosteroid treatment. Moreover, our study demonstrates that LLLT at 635 nm effectively stimulates wound healing by using higher power densities. Since, by the use of the highest power density the shortest time is needed to achieve the daily dose of 5 J/cm^2, it can be suggested that the use of tested laser at 15 mW/cm^2 might be the optimal power density for further preclinical investigations.

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