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THE INFLUENCE OF PHOTOBIOMODULATION THERAPY ON CHRONIC WOUND HEALING

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Abstract. The treatment of chronic wounds is one of the main therapeutic and economic problems of contemporary medicine. Photobiomodulation (PBM) stands out among the contemporary methods of wound treatment. The photobiostimulation effects of 660 nm laser irradiance on chronic wound were investigated. Effects of low-level laser on cytokines concentrations in the serum were evaluated using immunosorbent assay kits. Histological studies conducted here showed that application of PBM therapy accelerates wound repair during early stages of healing. Application of PBM therapy facilitated the reduction of inflammation and faster wound healing.

Key words: photobiomodulation, chronic wound, inflammation, cytokines, histological examination.

1. INTRODUCTION

The treatment of chronic wounds is one of the main therapeutic and economic problems of contemporary medicine. The prevalence of slow-healing wounds, expensive treatment, increasing antibiotic resistance and drug side effects necessitated a search for alternative treatment methods. Methods such as microcurrent dressing, application of magnetic surgical tools, treatment with mild vacuum, cryo medicine applications [1–4] are being put into practice.

Photobiomodulation (aka Low Level Laser Therapy – LLLT) stands out among the contemporary methods of wound treatment. Photobiomodulation (PBM) typically uses electromagnetic radiation with a wavelength between 500 and 1100 nm and the power within 1–500 mW that corresponds to the energy density 0.05–50 J/cm² [5]. The unique properties of laser radiation – monochromaticity, coherency, polarization and collimation have generated great interest for the laser's biological effects.

Since the laser invention in 1960 by Theodore Maiman, and Endre Mester's publications about the influence of laser radiation on the mouse hair growth and

human wound healing [6, 7], there was an enormous growth in corresponding publications and methodologies for disease treatment including chronic wounds. There is a fair share of work, however, that showed insignificant effect of low-intensity laser radiation on biological subjects, or even negative side effects. It could be the case that PBM parameters were suboptimal, in particular the dosage that should be tuned to the properties of biological tissues according to the Arndt-Schulz rule [8, 9]. Sometimes negative results may originate from dosage calculations [10]. Hence the search for optimal low-intensity laser radiation parameters is still ongoing; these include the laser type, the wavelength, radiation power, energy density, and duration of treatment as a function of the biological type of tissue being treated.

Wound healing is a natural reaction of tissues to any kind of damage. Physiologically, the healing process is achieved *via* the interaction (in space and in time) of four factors: hemostasis, inflammation, proliferation and remodeling [11]. Chronic wounds do not consistently follow all the stages of repair, with healing often being stuck in the inflammation stage [12]. A more detailed understanding of the mechanisms governing the inflammatory response and its resolution is needed. The success of the wound healing process depends on growth factors, cytokines and chemokines, participating in complex signal integration that coordinates cell processes. The regulation of pro- and anti-inflammatory states is vital for wound healing, while inability to balance these subtle processes can lead to a long term chronic inflammatory state [13].

Of interest is studying the efficiency of PBM therapy in treatment of wound defects during early stages of the repair process simulated *in vivo*. The investigation of cell-molecular mechanisms of healing wounds with complications when treated with low-level laser radiation will improve our understanding of the pathophysiology of repairing processes, which will enable further improvement of treatment for patients with chronic wounds.

2. MATERIALS AND METHODS

2.1. ANIMALS

Research was conducted on 24 white laboratory rats of the Wistar breed, weighing 250 ± 30 g, and 9 months old. The experiments were carried out in accordance with the principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and the General Principles of Animal Experiments, approved by the First National Congress on Bioethics (Kiev, 2001) and the rules for working with experimental animals approved by the Bioethics Committee of Kharkiv Medical Academy of Postgraduate Education. All experiments were designed to keep the number of animals to the minimum that was required to achieve statistical significance.

2.2. WOUND SURGERY

Rats from control (n = 12) and experimental (n = 12) groups were used for modeling a chronic wound [14]. To model a trophic wound, the anesthetized animal (general anesthesia with zoletil 10 mg/kg of body mass) was depilated in withers area and the skin was excised with surgical scissors to subcutaneous tissue in the form of a circle with a diameter of 20 mm; then a purse suture was applied on the periphery of the wound. Fasciocutaneous skin sutures were formed. Subcutaneous tissue was excised on the surface of the wound bottom by perpendicular cuts to form cells 5 × 5 mm. The wounds remained open for full duration of the experiment.

2.3. PHOTOBIOMODULATION (PBM)

The animal wounds from the experimental group were treated with low-intensity laser radiation from "Lika-therapist M" (Ukraine) in continuous mode using a wavelength of 660 nm, output power at 50 mWt, energy density at 1 J/cm² once per day for 5 days. Since direct contact with the wound is undesirable, we used a remote application method. Taking into account the beam divergence of the diode laser (0.5 rad ± 20%) [recommended by "Lika-therapist M"], the distance between the laser head and the wound was determined such that the full wound area was exposed to the radiation. To minimize reflections, the laser beam was positioned orthogonally to the tissue. Animals in the control group underwent a similar manipulation as the ones in the experimental group, with the exception of the laser being turned off.

2.4. EVALUATION METHOD

LLLT efficiency was evaluated with: planimetric methods and cytokine analysis, as well as with histological examination.

Planimetric analysis. To assess the dynamics of wound area reduction, digital macro photography of the wound surface was performed. The area of the wound surface was measured in photographs using the ImageJ program (NIH, USA). The relative area of the wounds (S) was calculated by the following formula:

$$S = S_t / S_o \cdot 100\%,$$

where S_o is the area of the wound immediately after its application, and S_t is the area of the wound surface at a given healing period.

On the 3rd and the 7th day after inducing the wounds, 6 animals from the control and experimental groups were singled out. The animals were euthanized by inhalation of chloroform in a confined space. Their hearts were used to obtain the blood samples; a biopsy was performed in the wound area.

Cytokines were analysed via enzyme immunoassay analysis of blood serum. The levels of interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α),

interleukin-4 (IL-4), and interleukin-10 (IL-10) were measured using reagent kits Vector-Best (Russia).

Histological examination. Histology of the skin was performed in samples fixed in 10% neutral formalin, and then dehydrated in increasing strength of alcohol (50°, 70° and twice 96°), then alcohol with chloroform was used, then chloroform, followed by paraffin embedding [15]. Sections, 5–7 microns thick, were stained with hematoxylin and eosin, or picric acid/acid fuchsin, following the Van Gieson method. The sections were visualized using a "Primo Star" microscope (Carl Zeiss). Photomicrographs of the preparations were obtained using a Micro-ocular digital camera.

2.5. STATISTICAL ANALYSIS

Statistical processing of the results was performed using the Statistica 6.0 analysis package. To describe the results obtained, the data were presented as M ± SE, where M is the arithmetic mean, and SE is the standard error of the arithmetic mean. The significance of the differences between groups (statistical significance) was determined using the non-parametric Kruskal-Wallis ANOVA test for independent samples. Differences were considered statistically significant at $p < 0.05$.

3. RESULTS

Measuring the relative area of the wounds showed increasing area reduction in the experimental group that was subject to low-level laser radiation compared to the PBM group that received no active treatment (Fig. 1).

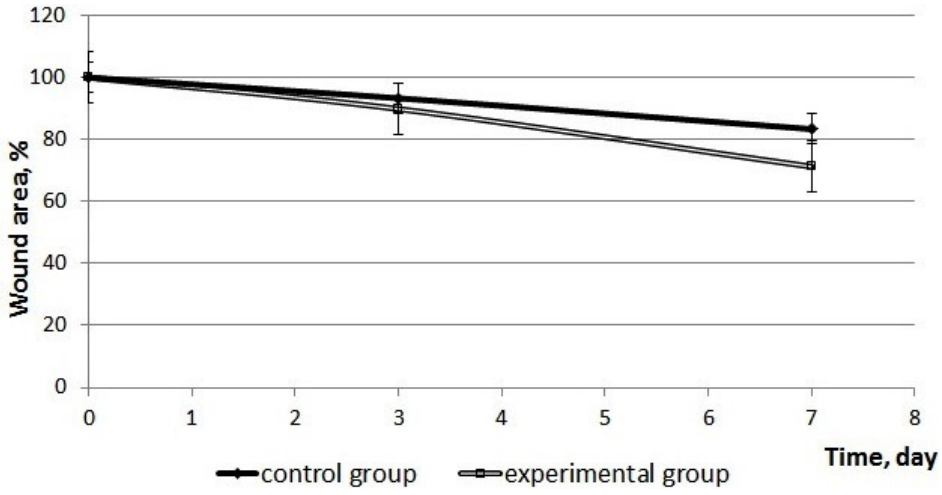


Fig. 1 – Wound area dynamics in the control and experimental groups.

Cytokine concentration in animal blood serum for animal wounds in control and experimental groups during day 3 and 7 are shown in Table 1.

Table 1

Cytokines' levels in two groups of animals

Animal Group	**Indicators**			
	IL-1β	IL-4	IL-10	TNF- α
Control 3 days	2.241 ± 0.289	2.936 ± 0.173	265.456 ± 22.774	2.152 ± 0.021
Experimental 3 days	2.816 ± 0.152	1.419 ± 0.228*	166.143 ± 21.361*	2.199 ± 0.216
Control 7 days	3.405 ± 0.554	2.982 ± 0.397	204.986 ± 15.795	2.257 ± 0.465
Experimental 7 days	3.621 ± 0.482	2.622 ± 0.176	262.051 ± 17.024	1.440 ± 0.221**

* $p < 0.05$ in comparison with the control group 3 days
** $p < 0.05$ in comparison with the control group 7 days

PBM effect on the expression of pro- and anti-inflammatory cytokines in blood serum of animals with chronic wounds showed: a tendency of increased expression of anti-inflammatory cytokine IL-1β for the duration of healing ($p > 0.05$); statistically significant decrease of TNF-α level after 7 days after wound modeling ($p < 0.05$); a significant decrease in concentration of IL-4 and IL-10 after 3 days since skin damage ($p < 0.05$).

Histological examination of skin samples revealed the formation of a dense scab in the wound area by the third day in both groups of animals; it consisted of fibrin deposits and damaged as well as degenerated cell elements, mostly neutrophil white blood cells. Under the scab, there was a narrow leukocyte shaft underlying the scap throughout the defect. Moreover, microscopic examination revealed the proliferation of epithelial cells along the edges of the wound in both groups.

Animals from the control group had a wound cavity filled with fibrin fibers, polymorphonuclear leukocytes and macrophages, as well as with a few fibroblasts. Regions with fibroblast proliferation, collagenogenesis and neoangiogenesis were noticeable at the bottom and on the edges of the wounds near intact blood vessels (Fig. 2a).

Wound defects in animals from the experimental group mostly consisted of young granulation tissue with numerous fibroblasts and macrophages. Neutrophilic white blood cells and lymphocytes were observed in small amounts. Numerous newly formed capillaries were overfilled with blood to the degree of erythrostasis and small to moderate scale focal hemorrhages. Collagen fibers in some areas looked quasi-random, and in others showed horizontal orientation (parallel to the wound surface corresponding to the mechanical load) (Fig. 2b).

In 7 days the animals from both groups showed a growing epithelial layer under the scab and leukocyte-necrotic layer of granulation tissue.

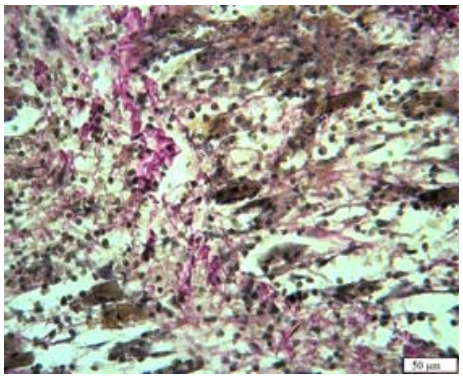
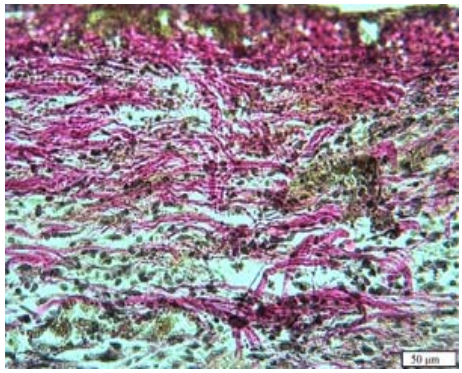
a)  b) 

Fig. 2 – The region of the wound of an animal after 3 days: a) the control group has a lot of polymorphonuclear leukocytes, individual fibroblasts, and collagen fibers; b) the experimental group has numerous collagen fibers and capillaries. Van Gieson.

Animals from the control group had wound cavities filled with young granulation tissue with moderate vascular content. Cells were represented by fibroblasts, macrophages and few polymorphonuclear leukocytes. Collagen fiber orientation was mostly random, with only few parallel patterns (relative to the wound surface). The central and surface regions of the wound showed the beginning of inflammatory infiltration, fibrin deposits, and hemorrhages (Fig. 3a).

In 7 days, animals from the group treated with low-level laser radiation showed signs of growth and development of granulation tissue in the form of numerous newly-formed capillaries and fibroblast proliferation. Collagen fibers in most regions were densely packed in bunches and had horizontal orientation. There were noticeable widened vessels overfilled with blood as well as the hemorrhages (Fig. 3b).

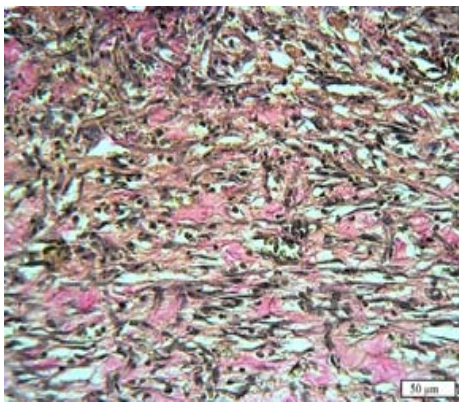
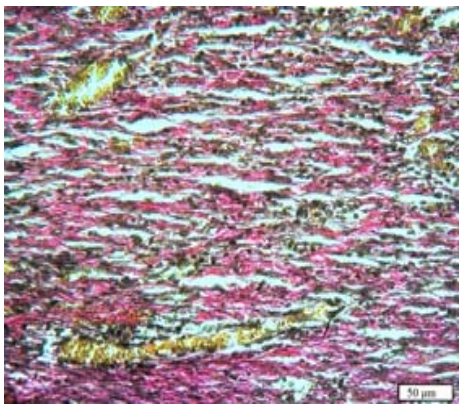
a)  b) 

Fig. 3 – The region of the wound of an animal after 7 days: a) the control group has fibroblast proliferation, chaotic, not packed collagen fibers; b) the experimental group has fibroblast proliferation, parallel packed collagen fibers, dilated capillaries. Van Gieson.

Thus, in the group of rats treated with PBM therapy for the duration of the experiment (3 and 7 days), wound healing was accompanied by less pronounced inflammation. Moreover, the proliferation stage seemed to be more intense and started faster, which led to intensive fibrillar and neoangiogenesis.

4. DISCUSSION

Taking into account that one of the main reason for slow wound healing is circulatory abnormalities [16], our research selected a model of trophic wound that reproduced local hypoxia and microcirculation disorders. Noting that chronic wounds tend to have persistent inflammation, our research of PBM therapy primarily investigated the inflammation stage.

The laser radiation used in our research had a wavelength of 660 nm. The low-intensity radiation in this red band of visible spectrum is most frequently used for skin wound healing since it penetrates the skin by 1–2 mm, reaching deep dermal layers [17].

Using visible radiation in the red band of spectrum proved to be more effective than using infrared radiation when it came to healing venous trophic ulcers [18]. To choose proper energy density (J/cm²), we examined available literature on PBM therapy applications to wound healing. We demonstrated positive effects of laser treatment with wavelength 660 nm, output power 60 mWt and energy density 1–4 J/cm² [19] and energy density 10 J/cm² [20]. The parameters used were consistent with most guidelines suggesting that energy density during the treatment session should be within 0.1–12 J/cm² [21]. Preliminary results from our lab demonstrated that using low intensity laser radiation with power of 50 mWt and energy density 10 J/cm² inhibits wound healing, while the one with energy density 1 J/cm² stimulates a healing process.

Thus, examining cellular-molecular mechanisms of chronic wound repair with PBM was conducted with wavelengths of 660 nm, output power 50 mWt and energy density 1 J/cm². In this research, we studied TNF-α, which is the pleiotropic cytokine produced by various types of cells, including keratinocytes, macrophages and mast cells.

TNF-α is often considered to be a pro-inflammatory cytokine. In combination with IL-1β and IL-6, it can stimulate acute phase response, act as a powerful neutrophil chemoattractant and stimulate classical macrophage activation through its binding to TNFR2, which activates MAPK and NF-κB signaling pathways [22]. TNF-α may have a positive or harmful effect on wound healing, and its increase leads to a decrease in the formation of granulation tissue, while its reduction contributes to a better arrangement of collagen fibers [23].

Our histological results showed a slow-down in development of granulation tissue in the animal skin samples with serum, which had high levels of TNF-α and better packaging and orientation of collagen fibers in most areas in animal skin

samples with low concentrations of TNF-α in the blood. Reduction in the level of TNF-α on the 7th day in our experiment was observed after application of PBM having an anti-inflammatory effect *in vivo* [24]. As a result we observed accelerated wound healing. These data were consistent with the literature. Cytokine TNF-α concentration reduction due to low-intensity laser radiation was demonstrated in studies of burn wounds in rats [25] and also in experimental studies of rat muscle injuries [26].

Out study revealed a trend for increased levels of pro-inflammatory cytokine IL-1β in both control and experimental groups, which might indicate the retention of the wound process in the stage of persistent inflammation. IL-1β is multifunctional and one of the most powerful pro-inflammatory cytokines [27]. IL-1β can participate in the positive feedback loop that maintains inflammation in chronic wounds and contributes to poor healing [23].

In our study, we observed insufficient acute-inflammatory response, which might indicate chronic process. In contrast with TNF-α, we did not observe a decrease in IL-1β concentrations on the 3rd and 7th days after the application of PBM therapy. Similar results were described in another research [28], where after the skin wound was exposed to low-intensity laser radiation and despite good healing, there was no decrease in levels of IL-1β. On the other hand, the data obtained in our work contradicted the results described in the literature that observed decreased expression of pro-inflammatory cytokine IL-1β after PBM treatment [29, 30].

Nevertheless, histological studies conducted in our work demonstrated inflammation reduction in the experimental group. Similar results were obtained in another study [31].

It is possible that, depending on parameters, low-intensity laser radiation may have not only anti-inflammatory effect, but also activate pro-inflammatory pathways [32]. Along with pro-inflammatory cytokines in the immune response, anti-inflammatory cytokines were also expressed.

In our experimental study, the expression of IL-10 during wound healing reached the maximum on the 3rd day, then it declined through day 7. This finding was consistent with another study [33]. IL-10 is one of the main anti-inflammatory cytokines with late effects, which is released by activated macrophages, and serves to prevent excessive activation of macrophages during periods of inflammation [34]. IL-10 is most likely a powerful regulator of negative feedback loop, which affects the control and inflammation processes *via* autocrine and paracrine mechanisms [35]. It is possible that as a reaction to high levels of IL-1β due to PBM therapy, there was a trend of increasing levels of cytokine a week after wound infliction and despite reduction in IL-10 in 3 days.

There is little literature on the effects of low-intensity laser radiation on the expression of anti-inflammatory cytokines on the wound repair processes. The levels of IL-10 were decreasing ($p > 0.05$) at 1, 6 and 12 hours after inflicting skin

wounds to the rats, followed by low-intensity laser therapy at 670 nm [34]. In another study, the increase in levels of IL-10 were observed during treating of skin wounds in rats with He-Ne laser (25 mWt, 30 min, at a wavelength of 632.8 nm) for 10 days [29].

In the current study, when using PBM therapy to heal chronic wounds, anti-inflammatory cytokine IL-4 did not have a pronounced anti-inflammatory reaction: we observed the reduction of levels of IL-4 ($p < 0.05$) after 3 days, despite the trend for restoration of the initial level of this cytokine after 7 days ($p > 0.05$). The increased expression of anti-inflammatory cytokines during early stages of healing may possibly prevent or stimulate delaying the expression of anti-inflammatory cytokines, including IL-4.

Thus, influencing any part of so-called cytokine networks leads to the activation of all its other components. For instance, IL-4 inhibits the secretion of pro-inflammatory cytokines, such as IL-1, IL-6 and the TNF [36].

Histological studies in our work showed earlier formation of granulation tissue in skin samples of animals with a wound area that was exposed to laser radiation. A big component of tissue recovery and formation of granulation tissue, as well as activation and macrophage maturation, belongs to an IL-4 receptor (IL-4Rα) [37].

Hence the inflammation reaction is a normal part of the healing process after an injury, which helps to destroy microorganisms and damaged cells. Prolongation or delay of the inflammatory phase, however, adversely affects the remaining stages of wound healing. Growth factors and cytokines expressed by cells involved in healing allow the formation of positive and negative feedback loops to control cell concentrations and the ability to inhibit or initiate repair processes.

The mechanisms for restoring intercellular connections during the healing of chronic wounds with the PBM therapy are not fully understood. Further research is needed.

5. CONCLUSIONS

Application of photobiomodulation therapy facilitated the reduction of inflammation and faster wound healing. Histological studies conducted here showed that application of PBM therapy accelerates wound repair during early stages of healing: the proliferation phase proceeded faster, which was indicated by intense fibrillo and neoangiogenesis.

Application of photobiomodulation therapy allows the body to regulate the intricacies of repair processes by cytokine modulation. Thus, healing chronic wounds with PBM therapy proved to be useful and efficient during the initial stages of chronic wound healing.

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