Original article

The role of intercellular mediators in the regulation of reparative processes of chronic wounds when using photobiomodulation therapy

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Abstract:

Objective: The treatment of chronic wounds is one of the actual problems in medicine. Photobiomodulation (PBM) has gained considerable recognition among treatment modalities for various medical problems including wound repair processes. This research aims to investigate the role of intercellular mediators in the regulation of reparative processes of chronic wounds with the PBM therapy in the experiment. Methods: Studies were performed on 12 Wistar rats weighing 250 ± 30g. Rats were used for modeling a chronic wound. Animals were divided into two groups: control and experimental. The animal wounds from the experimental group were treated with low-intensity laser radiation from “Lika-therapist М” 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. On the 14th day after the application of wounds, 6 rats from each group were removed from the experiment. PBM efficiency was evaluated with intercellular mediators analysis, as well as with histological examination. ELISA serum analysis was done for quantifying interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10) and granulocyte macrophage colony stimulating factor (GM-CSF). Statistical processing of the results was performed using the Statistica 6.0 analysis package. Result: PBM effect on the expression of intercellular mediators in blood serum of animals with chronic wounds after 14 days after wound modeling, showed: increase of cytokine IL-1β (p > 0,05) and IL-4 (p < 0,05) levels; decrease of TNF-α and IL-10 levels (p < 0,05); without significant changes were concentrations IL-6 and GM-CSF. The histological study showed decrease vascular count and better organization of collagen fibers in experimental group. Conclusion: Histological studies of wound healing revealed an earlier transition of the process to the remodeling phase with the use of PBM therapy. PBM therapy can be an instrument for optimizing the reparative process by correcting the regulation by intercellular mediators.

Keywords: photobiomodulation; chronic wound; intercellular mediators; histological examination.

Wound healing is a complex, dynamic, biological process that includes the coordinated actions of various types of cells, carried out and regulated by numerous growth factors and cytokines¹. Intercellular mediators play an important role at all stages of wound healing: inflammation, the formation of granulation tissue, remodeling². Impaired regulation of the reparative process leads to the formation of chronic wounds³.

The treatment of chronic wounds is one of the urgent problems in medicine. Given the high prevalence of long-term non-healing wounds⁴, increased antibiotic resistance and economic treatment costs³, it became necessary to find new approaches to stimulate the reparative process. Methods such as microcurrent dressing, application of magnetic surgical tools, treatment with mild vacuum⁵-⁷ are being put into practice. Of interest is one of the modern methods

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– photobiomodulation therapy (PBM), also known as low-level laser therapy (LLLT). In recent years, PBM has gained considerable recognition and importance among treatments for various medical problems, including wound healing processes, musculoskeletal complications and pain control. However, the cellular-molecular mechanisms underlying tissue repair using PBM are still not well understood. As a result of this, the efficiency and safety of using low-intensity laser radiation in medicine do not correspond to the optimal sets of action characteristics (for example, wavelength and radiation dose) and individual characteristics of the patient. This makes it difficult to predict treatment outcomes for patients with chronic wounds.

Materials and methods

Animals

The studies were conducted on 12 white Wistar rats weighing 250 ± 30 g at the age of 9 months.

Ethical clearence:

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 (Strasbourgh, 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 Ethics Bioethics Committee of Kharkiv Medical Academy of Postgraduate Education. Kharkiv, Ukraine (Protocol No. 2 dated June 26, 2020).

Wound Surgery.

The rats were reproduced with a model of a trophic wound with local hypoxia and microcirculation disturbances. The choice of a wound model is due to the hypoxicity of chronic wounds, since blood flow and, consequently, oxygen delivery to tissues is impaired. To simulate a trophic wound in an animal under general anesthesia (zoletil mononarcosis at the rate of 10 mg/kg body weight), the hair was depilated, after which the skin was cut with surgical scissors until superficial fascia in the form of a circle with a diameter of 20 mm, after which a purse string suture was applied along the edges of the wound. Formed skin-fascial nodal sutures. On the surface of the bottom of the wound, the fascia was dissected by perpendicular sections with the formation of cells 5 × 5 mm in size. The wound remained open throughout the entire period of the experiment.

Study Design

After operations, animals were divided into two groups: one group of animals was exposed to a wound defect by low-intensity laser radiation (experimental group), animals of the second group were wounded irradiated fictitiously (control group). Animals were taken out of the experiment on the 14th day, 6 animals from each group. Animals were euthanized by inhalation of chloroform in a confined space. The 14th day was chosen, since by this time inflammatory processes should have resolved and, probably, the process of transition from the proliferation phase to the remodeling phase is taking place.

PBM

The wounds of the experimental group animals were exposed to low-intensity laser radiation using the “Lika-therapist M” apparatus (Ukraine) in continuous mode at a wavelength of 660 nm, an output power of 50 mW, an energy density of 1 J/cm² for 5 days. The selection of parameters of PBM was carried out according to the analysis of previously published studies.

PBM therapy was carried out at an early stage of wound healing, starting the day after modeling the wound for 5 days. A distant exposure method was used to avoid contact with an open wound. The distance from the emitter head to the wound was selected so as to illuminate the entire area of the wound. The laser tip beam was held perpendicular to the surface of the irradiated tissue.

Evaluation method

The effectiveness of PMB therapy was evaluated using: studies of the levels of intercellular mediators, as well as histological studies.

Blood for research was taken from the heart. Then a skin flap with a wound area was taken.

Studies of intercellular mediators were carried out by enzyme immunoassay in the blood serum of animals. Interleukin-1β (IL-1β), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α) levels were determined using reagent kits Vector-Best. GM-CSF levels were determined using an eBioscience kit (USA).

Histological evaluation

Histology of the skin was performed in samples fixed in 10 % neutral formalin, and then dehydrated in increasing strength of alcohols (50°, 70° and twice 96°), then alcohol with chloroform was used,
then chloroform, followed by paraffin embedding\textsuperscript{13}. Sections, 5-7 microns thick, were stained with hematoxylin and eosin. The sections were visualized using a “Primo Star” microscope (Carl Zeiss). Photomicrographs of the preparations were obtained using a Microocular digital camera.

**Statistical analysis**

Statistical processing of the results was performed using Statistica 6.0 statistical analysis package. To describe the results obtained, the data were presented as M ± SE, where M is the arithmetic mean, SE is the standard error of the arithmetic mean. The significance of 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.

**Results**

The concentrations of intercellular mediators in the blood serum of animals whose experimental wounds were exposed to low-intensity laser radiation compared with animals that did not receive PBM therapy are presented in the table.

**Table. Change in the level of intercellular mediators in groups of animals**

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>IL-1β</th>
<th>GM-CSF</th>
<th>IL-10</th>
<th>IL-4</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 14 days</td>
<td>3,563 ± 0.593</td>
<td>1,598 ± 0.330</td>
<td>212,344 ± 5,301</td>
<td>1,661 ± 0.227</td>
<td>6,419 ± 0,831</td>
<td>3,101 ± 0,050</td>
</tr>
<tr>
<td>Experimental 14 days</td>
<td>3,966 ± 0,373</td>
<td>1,781 ± 0,136</td>
<td>165,863 ± 6,718*</td>
<td>2,559 ± 0,201*</td>
<td>6,863 ± 0,906</td>
<td>1,772 ± 0,111*</td>
</tr>
</tbody>
</table>

* p < 0.05 in comparison with the control group 14 days

PBM effect on the expression of intercellular mediators in blood serum of animals with chronic wounds after 14 days after wound modeling, showed: increase of cytokine IL-1β (p > 0.05) and IL-4 (p < 0.05) levels; decrease of TNF-α and IL-10 levels (p < 0.05); without significant changes were concentrations IL-6 and GM-CSF.

Histological assessment showed that after 14 days in the animals of both groups there was a “creep” of the epithelial layer from the edges of the wound onto the granulation tissue. The newly formed epidermis had a different thickness in the areas. It was noted as a multilayer, with a clear differentiation into layers, and a thinned epithelium, with cells “spread out” on the surface of the regenerate. In areas adjacent to healthy skin, the formation of hair follicles and sebaceous glands was observed.

In the control group of animals, the wound cavity was filled with maturing connective tissue with a moderate number of vessels. Sites of young granulation tissue with thin-walled capillaries and vascular cavities, foci of inflammation and hemorrhage were preserved (Fig. 1a).

In animals of the experimental group, mature connective tissue with densely packed, thickened bundles of collagen fibers, single vessels and a few fibrocytes was observed in the marginal areas of the wound. In the main part of the wound, a maturing connective tissue with densely packed bundles of collagen fibers oriented parallel to the surface of the wound was located. The location of the blood vessels was not uniform in the areas. In layers close to the surface of the wound, a moderate number of capillaries, often dilated and full-blooded, was noted. In the layers adjacent to the bottom of the wound, the vessels were few in number (Fig. 1b).

a) b)

**Discussion**

Intercellular mediators play an important role in the mechanisms of protection and recovery from injury. The wound healing process is accurately performed and regulated by a number of intercellular mediators in space and time. The levels of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) in chronic wounds remain high at all stages of wound healing due to the prolonged inflammatory phase\textsuperscript{14}. As inflammation subsides, proliferation becomes a major theme with an emphasis on re-epithelialization, vasculature restoration and granulation tissue formation\textsuperscript{15}. Then comes the last phase – remodeling tissue. The resolution stage is essential for the restoration of functionality and the “normal” appearance of the lesioned tissue\textsuperscript{16}.

We studied the concentrations of intercellular
mediators at the stage of transition of the proliferative phase to the remodeling phase. Despite the anti-inflammatory effects of PBM therapy\textsuperscript{17}, there were no significant differences in the levels of pro-inflammatory cytokines IL-1\(\beta\) and IL-6 after application of low-intensity laser radiation. Which, apparently, is associated with systemic inflammation after damage. Because it is known that IL-1\(\beta\) participates in a pro-inflammatory positive feedback loop that sustains the pro-inflammatory macrophage phenotype observed in poorly healing wounds and blocks the induction of a healing-associated macrophage phenotype observed during normal healing\textsuperscript{18}. An important role in wound healing is played by interleukin 6. IL-6 regulates the hypothalamic-pituitary-adrenal axis and is involved in monocyte chemo-taxis, angiogenesis, and collagen accumulation\textsuperscript{19}.

The literature mainly presents works in which the role of intercellular mediators was studied with low-intensity laser exposure to a wound defect in the early stages of wound healing. Data on the expression of cytokines and growth factors during wound healing during the transition of proliferation to remodeling during PBM therapy are scarce and contradictory. In studies of burn wounds in rats, a decrease in serum IL-6 and TNF-\(\alpha\) levels was observed 14 days after low-intensity laser therapy\textsuperscript{20}. However, in another study, an increase in IL-6, TNF-\(\alpha\), and IL-10 levels was observed at the proliferation stage in the treatment of skin wounds in He-Ne rats with a laser\textsuperscript{21}.

Chronic TNF-\(\alpha\) overexpression negatively affects skin regeneration\textsuperscript{22}. The decrease in the pleiotropic cytokine TNF-\(\alpha\) observed in our study with the use of PBM therapy is consistent with literature\textsuperscript{11}. In the cited study, the anti-inflammatory effect of low-intensity laser radiation, resulting in a decrease in the concentration of TNF\(\alpha\) cytokines, was demonstrated after two weeks of treatment with pressure sores with laser radiation at a wavelength of 658 nm. It is also known, that decreased TNF-\(\alpha\) expression promotes a better arrangement of collagen fibers\textsuperscript{23}, which in our work is confirmed by a histological evaluation of experimental wounds.

Cytokines influence each other, forming the so-called “cytokine networks” with negative and positive feedbacks. Thus, the impact on any link in this network leads to the involvement of all other components in this process. Thus, the IL-10 studied by us is a regulatory cytokine produced by various types of cells that can inhibit the production of pro-inflammatory cytokines, such as IL-6 and TNF-\(\alpha\)\textsuperscript{24}. IL-10 is often considered the dominant anti-inflammatory and antifibrotic player in active inflammation and in wound healing. The observed a decrease in the level of multifunctional cytokine IL-10 with PBM is apparently due to the fact that IL-10 signaling is pleiotropic, and its effects are dependent on its cellular source, the organ system involved, and the local milieu\textsuperscript{25}.

Also in our work, the anti-inflammatory cytokine IL-4 was studied. Animal models show the
involvement of IL-4 in normal wound healing, where it activates fibroblasts and stimulates the synthesis of extracellular matrix. In the present study, when using PBM therapy for the healing of chronic wounds, the levels of the anti-inflammatory cytokine IL-4 increased at the stage of proliferation transition to the remodeling phase. Perhaps these trends are associated with compensatory reactions that occur in response to changes in the levels of pro-inflammatory cytokines.

We also considered granulocyte macrophage colony stimulating factor (GM-CSF). It is a pleiotropic cytokine that activates cell lines of granulocytes and macrophages. GM-CSF is a potential target in many inflammatory/ autoimmune conditions. In our study, there were no statistically significant differences in GM-CSF levels after exposure to low-intensity laser radiation. Apparently, this is due to the multidirectional effect of granulocyte-macrophage colony-stimulating factor in inflammation. GM-CSF not only promotes macrophage survival, but also supports their differentiation towards a pro-inflammatory phenotype. However, despite the pro-inflammatory role, GM-CSF is also involved in the elimination of inflammation.

Thus, in our study, the multidirectional effect of PBM therapy on the levels of intercellular mediators during the transition of the proliferative phase to the remodeling phase was demonstrated.

Further research is needed on to explain the mechanisms involved in the healing process, analyzing the relationship of radiation parameters and the subsequent effect on all phases of wound healing.

Conclusions
Histological studies of wound healing revealed an earlier transition of the process to the remodeling phase with the use of PBM therapy. PBM therapy can be an instrument for optimizing the reparative process by correcting the regulation by intercellular mediators.

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Conflict of interest
The authors have no conflicts of interest to declare.

Authors’ contribution
SP, NB, MK have given substantial contributions to the conception and the design of the manuscript. NB, MK, OL, NS, MV took part in the acquisition, analysis, and interpretation of the data. All authors have participated in drafting the manuscript, SP revised it critically. All authors contributed equally to the manuscript and read and approved the final version of the manuscript.

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