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 M" in continuous mode using a wavelength of 660 nm, output power at 50 mWt, energy density at 1 J/cm^2 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.

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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 cytokines[1]. Intercellular mediators play an important role at all stages of wound healing: inflammation, the formation of granulation tissue, remodeling[2]**.** Impaired regulation of the reparative process leads to the formation of chronic wounds[3].

The treatment of chronic wounds is one of the urgent problems in medicine. Given the high prevalence of long-term non-healing wounds[4], increased antibiotic resistance and economic treatment costs[3], 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 vacuum[5-7] 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[8]. 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 (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 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[9]. 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[10]. 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[11,12].

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[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

Animal Group	Intercellular mediators					
	IL-1β	GM-CSF	IL-10	IL-4	IL-6	TNF- α
Control 14 days	3,563 ± 0,593	1,598 ± 0,330	212,344 ± 5,301	1,661 ± 0,227	6,419 ± 0,831	3,101 ± 0,050
Experimental 14 days	3,966 ± 0,373	1,781 ± 0,136	165,863 ± 6,718*	2,559 ± 0,201*	6,863 ± 0,906	1,772 ± 0,111*

* $p < 0{,}05$ in comparison with the control group14days

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).

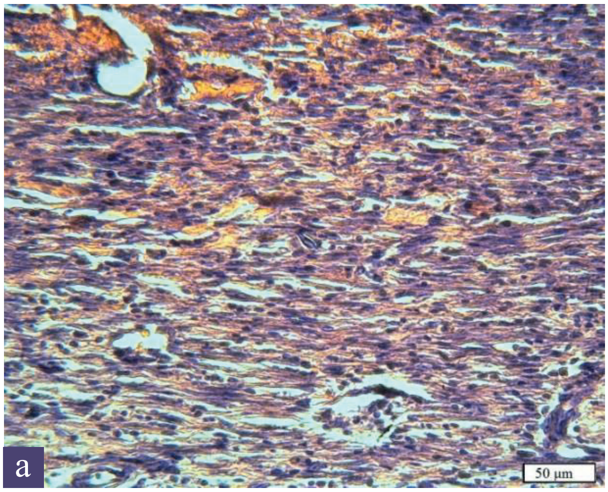
In animals of the experimental group, mature connective tissue with densely packed, thickened bundles of collagen fibers, single vessels and a few fibrocytes was observed in the marginal areas of the wound. In the main part of the wound, a maturing connective tissue with densely packed bundles of collagen 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[14]. As inflammation subsides, proliferation becomes a major theme with an emphasis on re-epithelialization, vasculature restoration and granulation tissue formation[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[16].

We studied the concentrations of intercellular



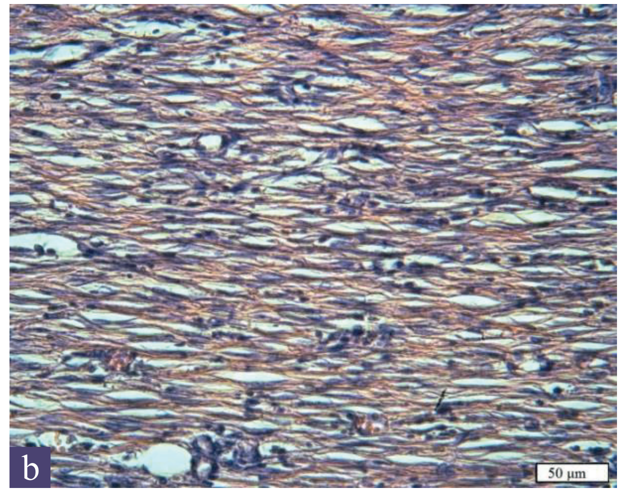


Fig.1. Plots of the central departments of wounds in rats after 14 days: a) in the control group – young granulation tissue with a large number of full-blooded capillaries, chaotic collagen fibers, proliferation of fibroblasts; b) in the experimental group – maturing granulation tissue with parallel bundles of collagen fibers and a few vessels. Hematoxylin and eosin.

mediators at the stage of transition of the proliferative phase to the remodeling phase. Despite the anti-inflammatory effects of PBM therapy[17], there were no significant differences in the levels of pro-inflammatory cytokines IL-1β 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-1β 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[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[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-αlevels was observed 14 days after low-intensity laser therapy[20]. However, in another study, an increase in IL-6, TNF-α, and IL-10 levels was observed at the proliferation stage in the treatment of skin wounds in He-Ne rats with a laser[21]. Chronic TNF-α overexpression negatively affects skin regeneration[22]. The decrease in the pleiotropic cytokine TNF-α observed in our study with the use of PBM therapy is consistent with literature[11]. In the cited study, the anti-inflammatory effect of low-intensity laser radiation, resulting in a decrease in the concentration of TNFα 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-α expression promotes a better arrangement of collagen fibers[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-α[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[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[26]. 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[27]. 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[28]. However, despite the pro-inflammatory role, GM-CSF is also involved in the elimination of inflammation[29].

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.

Source of fund

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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.

References

1. Barrientos S, Brem H, Stojadinovic O and Tomic-Canic M. Clinical application of growth factors and cytokines in wound healing. *Wound Repair Regen* 2014;**22**(5):569-578. https://doi.org/10.1111/wrr.12205
2. Omar J, Shafii N, Zainan AE, Sirajudeen K and Abdullah M. Evaluation of Wound Healing Biomarkers of Interleukin 6 (IL-6), Vascular Endothelial Growth Factor (VEGF) and Matrix Metalloproteinases 9 (MMP-9) in Post Lower Segment Caesarean Section (LSCS) Patients Consuming Channa Striatus Extract. *BJMS* 2020;**19**(3):520-526. https://doi.org/10.3329/bjms.v19i3.45870
3. Broszczak DA, Sydes ER, Wallace D and Parker TJ. Molecular Aspects of Wound Healing and the Rise of Venous Leg Ulceration: Omics Approaches to Enhance Knowledge and Aid Diagnostic Discovery. *Clin Biochem Rev* 2017;**38**(1):35-55.
4. Hussain MA and F Huygens. Role of infection in wound healing. *BJMS* 2020;**9**(4):598-602. https://doi.org/10.3329/bjms.v19i4.46612
5. Arsita C, Nasihun T and Hussaana A. UVB Dose Optimization for Phototherapy in Vitamin D Deficiency: Profile Analysis of Vitamin D, TNF-α, Vascular Endothelial Growth Factor (VEGF) and Platelet Derived Growth Factor (PDGF) in Wistar Rats. *BJMS* 2020;**19**(4):749-754. https://doi.org/10.3329/bjms.v19i4.46636
6. Michaylusov RN and Negoduyko VV. Results of surgical magnetic instruments application for survey of wounds and removal of ferromagnetic foreign bodies. *Klin Khir* 2016;**7**:58-60.
7. Veligotskiy AN, Savitskiy RV, Pavlov SB and Leonov AV. Changes in the area of the wound surface on exposure low-dose vacuum. *Klin Khir* 2016;**7**:40-42.
8. Ma H, Li Y, Chen H, Kang M and Liu TCY. Effects of

Low-Intensity Laser Irradiationon Wound Healingin Diabetic Rats. *Int J Photoenergy* 2012;**2012**,838496. https://doi.org/10.1155/2012/838496

9. Zinatullin RM, Gizatullin TR, Pavlov VN, Kataev VA, Farhutdinov RR, Baymurzina JL, et al., inventor; Zinatullin RM, Gizatullin TR, assignee. A method for modeling a trophic wound in an experiment. Russian patent RF 2510083. 2014 March 20.
10. Sen CK. Wound healing essentials: let there be oxygen. *Wound Repair Regen* 2009;**17**(1):1-18. https://doi.org/10.1111/j.1524-475X.2008.00436.x
11. Taradaj J, Halski T, Kucharzewski M, Urbanek T, Halska U and Kucio C. Effect of laser irradiation at different wavelengths (940, 808, and 658 nm) on pressure ulcer healing: results from a clinical study. *Evid Based Complement Alternat Med* 2013;**2013**:960240. https://doi.org/10.1155/2013/960240
12. Nussbaum EL, Mazzulli T, Pritzker KP, Las Heras F, Jing F and Lilge L. Effects of low intensity laser irradiation during healing of skin lesions in the rat. *Lasers Surg Med* 2009;**41**(5):372-381. https://dx.doi.org/10.1515%2Fplm-2013-0049
13. Sarkisov DS and Perov JuL. Mikroskopicheskaja tehnika: rukovodstvo dlja vracheji laborantov. Moscow: Medicina, 1996.
14. Agyare C, Osafo N and Boakye YD. Biomarkers of Wound Healing. In Dogan KH, ed. Wound Healing – Current Perspectives. IntechOpen, 2018, DOI:10.5772/intechopen.80222. Available from: https://www.intechopen.com/books/wound-healing-current-perspectives/biomarkers-of-wound-healing
15. Landen NX, Li D and Stahle M. Transition from inflammation to proliferation: a critical step during wound healing. *Cell Mol Life Sci* 2016;**73**(20):3861-3885. https://doi.org/10.1007/s00018-016-2268-0
16. Shaw TJ and Martin P. Wound repair at a glance. *J Cell Sci* 2009;**122**:3209-3213. https://doi.org/10.1242/jcs.031187
17. Hamblin MR. Mechanisms and Applications of the Anti-Inflammatory Effects of Photobiomodulation. *AIMS Biophys* 2017;**4**(3):337-361. https://doi.org/10.3934/biophy.2017.3.337
18. Mirza RE, Fang MM, Ennis WJ and Koh TJ. Blocking Interleukin-1β Induces a Healing-Associated Wound Macrophage Phenotype and Improves Healing in Type 2 Diabetes. *Diabetes* 2013;**62**(7):2579-2587. https://doi.org/10.2337/db12-1450
19. Shen T, Kanazawa S, Kado M, Okada K, Luo L, Hayashi A, et al. Interleukin-6 stimulates Akt and p38 MAPK phosphorylation and fibroblast migration in nondiabetic but not diabetic mice. *PLoSONE* 2017;**12**(5):e0178232. https://doi.org/10.1371/journal.pone.0178232
20. Kim EJ and Kim SH. Anti-inflammatory effects of low-level laser in burn wound models in rats. *PTRS* 2017;**6**(4):170-175. https://doi.org/10.14474/ptrs.2017.6.4.170
21. Ma W, Fan Y, Zuo J, Chen Z, Yuan J and Song X. Complicated Effect of He-Ne Laser Therapy on Pro-/Anti-Inflammatory Cytokines from Serum in Rats. *AVS* 2018;**6**(5):88-94. https://doi.org/10.11648/j.avs.20180605.14
22. Nosenko MA, Ambaryan SG and Drutskaya MS. Proinflammatory cytokines and skin wound healing in mice. *Mol Biol* 2019;**53**(5):653-664. https://doi.org/10.1134/s0026898419050136
23. Serra MB, Barroso WA, da Silva NN, Silva SN, Borges ACR, Abreu IC, et al. From Inflammation to Current and Alternative Therapies Involved in Wound Healing. *Int J Inflam* 2017;**2017**:3406215. https://doi.org/10.1155/2017/3406215
24. Couper KN, Blount DG and Riley EM. IL-10: The Master Regulator of Immunity to Infection. *J Immunol* 2008;**180**(9):5771-5777. https://doi.org/10.4049/jimmunol.180.9.5771
25. Steen EH, Wang X, Balaji S, Butte MJ, Bollyky PL and Keswani SG. The Role of the Anti-Inflammatory Cytokine Interleukin-10 in Tissue Fibrosis. *Adv Wound Care* 2020;**9**(4):184-198. https://doi.org/10.1089/wound.2019.1032
26. Salmon-Ehr V, Ramont L, Godeau G, Birembaut P, Guenounou M, Bernard P, et al. Implication of interleukin-4 in wound healing. *Lab Invest* 2000;**80**(8):1337-1343. https://doi.org/10.1038/labinvest.3780141
27. Hamilton JA. GM-CSF-Dependent Inflammatory Pathways. *Front Immunol* 2019;**10**:2055. https://doi.org/10.3389/fimmu.2019.02055
28. Ushach I and Zlotnik A. Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage. *J LeukocBiol* 2016;**100**(3):481-489. https://doi.org/10.1189/jlb.3ru0316-144r
29. Dougan M, Dranoff G and Dougan SK. GM-CSF, IL-3, and IL-5 Family of Cytokines: Regulators of Inflammation. *Immunity* 2019;**50**(4):796-811. https://doi.org/10.1016/j.immuni.2019.03.022