Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 4, 2021, Pages. 2226 – 2234
Received 05 March 2021; Accepted 01 April 2021.

Effects of Low-Level Laser Therapy on Reactive Oxygen Species, Platelet Aggregation Activity, and the Expression of Growth Factors in the Process of Regeneration of Chronic Wounds

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ABSTRACT

An understanding of the cellular and molecular mechanisms underlying wound healing with photobiomodulation (PMB) therapy will allow the influence on repair processes, which will lead to new and effective therapeutic strategies for many pathological conditions. The aim of our work was to study the effect of PBM therapy on the regulation of reparative processes in chronic wounds. Studies were performed on 30 Wistar rats. 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 in continuous mode using a wavelength of 660 nm, an output power of 50 mW, an energy density of 1 J/cm², and 60 s exposure time. The animals were removed from the experiment on the 3rd and 7th days, with 6 animals from each group. The study of the levels of reactive oxygen species (ROS), platelet-derived growth factor (PDGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and basic fibroblast growth factor (bFGF) was carried out. Induced platelet aggregation was studied. Changes in the expression of the studied parameters in the blood serum of animals with chronic wounds when using PBM therapy were found: a decrease in ROS levels and an increase in bFGF concentrations at the initial stages of wound healing. The multidirectional effect of PBM therapy on GM-CSF and PDGF levels was observed in the studied time frame. The use of PBM therapy makes it possible to regulate disturbances in reparative processes by modulating ROS, platelet aggregation activity, and the expression of endogenous growth factors.

Keywords
Photobiomodulation,wound healing, reparative processes, growth factors

Introduction

Wounds with impaired healing (chronic wounds) pose a serious health problem worldwide. Such wounds are widespread and cause significant morbidity, mortality and medical costs (Avishai et al., 2017).Currently, various methods of stimulating the reparative process are used to heal wounds (Abdurakhmonov et al., 2021; Veligotskiy et al., 2016). The search for new approaches to wound healing continues

Wound healing involves the spatial and temporal synchronization of different cell types with different roles in the phases of hemostasis, inflammation, growth, re-epithelialization, and remodeling (Rodrigues et al., 2019).

After trauma, the restoration of hemostasis occurs due to the activation of platelets – the first responders, which are of decisive importance in the pathophysiology of thrombosis (Dhall et al., 2016). Platelets are also identified as relevant modulators of inflammation and tissue regeneration (Etulain, 2018). After activated, platelets release over 300 active substances (Golebiewska & Poole, 2015), including numerous growth factors, which stimulate both the inflammatory cascade and the healing process. Disruption of the activation processes and aggregation of platelets can lead to chronic inflammation and disruption of the subsequent repair process.

The development of the inflammatory phase leads to the recruitment of leukocytes, neutrophils, and macrophages; the production of growth factors; and the activation of dermal and epidermal cells (Demidova-Rice et al., 2012). Inflammatory cells produce reactive oxygen species (ROS), which play a key role in the early stages of wound healing, eliminating infectious threats

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(Percival et al., 2015). ROS act as important physiological regulators of intracellular signaling pathways (Finkel, 2011). In chronic conditions, there is an increased production of free radicals, which leads to a prolonged inflammatory state (Schafer & Werner, 2008).
Targeting and eliminating the cellular and molecular causes of prolonged inflammation in chronic wounds can be an effective strategy for wound prevention and treatment.
The inflammatory response is complex and modulated by many internal and external factors (Wilkinson&Hardman, 2020). Photobiomodulation (PBM) therapy, also known as low-level laser therapy (LLLT), can modulate inflammation, stimulate fibroblast proliferation and angiogenesis, and therefore can be used to improve wound healing (Otterço et al., 2018). The biological effect of LLLT also includes the activation of various growth factors, the induction of reactive oxygen species, the modification of the components of the extracellular matrix, the suppression of inflammatory cytokines (Kushibiki et al., 2013). A better understanding of the cellular and molecular mechanisms underlying wound healing with PMB therapy will allow the influence on repair processes, which will lead to new and effective therapeutic strategies for many pathological conditions.
The aim of our work was to study the effect of photobiomodulation therapy on the regulation of reparative processes in chronic wounds (on the example of reactive oxygen species (ROS), platelet aggregation activity, platelet-derived growth factor (PDGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), basic fibroblast growth factor (bFGF)).

Materialsandmethods

Animals

30 Wistar rats weighing 250 ± 30 g at the age of 9 months were involved in the experiment. The experimental study was conducted after approval from the Bioethical Committee of Kharkiv Medical Academy of Postgraduate Education (Protocol No. 2 dated June 26, 2020). 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 (Kyiv, 2001).

Wound formation

After the induction of anesthesia (general anesthesia with zoletil 10 mg/kg of body mass), the chronic wounds was modeled in the proximal part of the back of rats (Zinatullin et al., 2014). The operations were performed under aseptic conditions by one surgeon. After depilation, skin wounds were created in the form of a circle with a diameter of 2 cm. Flap subcutaneous tissue was then dissected including the panniculus carnosus (the subcutaneous structure providing vascularization). Further, a purse-string suture was applied along the wound edges, and fascial cutaneous interrupted sutures were formed. On the surface of the bottom of the wound, the superficial fascia was dissected by perpendicular incisions with the formation of cells measuring 5 × 5 mm.
After the operation, the animals were randomly divided into two groups of 12 animals each. Wound defects of animals in the experimental (Exp) group were exposed to low-intensity laser radiation, and the wounds of animals in the control (Con) group were irradiated fictitiously. The

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intact (Int) group had six healthy rats.

PBM

The rats in the Exp group received PBM therapy once a day for 5 days, starting 24 hours after wound formation. The laser device Lika-therapist M (Ukraine) was used in continuous mode at a wavelength of 660 nm, an output power of 50 mW, an energy density of 1 J/cm², and 60 s exposure time. The laser tip was held perpendicular to the surface of the irradiated tissue, while the beam covered the entire area of the wound.

Evaluation method

The effectiveness of PBM therapy on wound healing was assessed by assessing the functional activity of platelets, as well as studies of ROS levels and growth factors.
The animals were removed from the experiment on the 3rd and 7th days, with 6 animals from each group. The animals were euthanized by inhalation of chloroform in a confined space. To collect blood, the chest of the rat was opened by surgery, and blood was collected by cardiac puncture.
The study of the levels of ROS, GM-CSF, bFGF, PDGFwas carried out by the method of enzyme-linked immunosorbent assay in blood serum using eBioscience kits (USA).

Functional activity of platelets

Induced platelet aggregation was studied in platelet-rich plasma by the turbidimetric method using a computerized analyzer of platelet aggregation SOLAR 2110 (Belarus). Sodium citrate (3.2%) was used as a blood stabilizer in a 9:1 ratio. Adenosine diphosphate (ADP) Technology-Standard (Russia) was used as an inductor at a concentration of 2.5 μmol/l, 5 μmol/l, and 10 μmol/l. Aggregatogram recording was carried out at 37°C for 10 minutes. The subsequent analysis of the aggregation curve included the assessment of the type of aggregatogram and the determination of the following indicators: 1) the degree of aggregation – the maximum percent of plasma light transmission; 2) the time to reach the maximum aggregation rate – the time to reach the maximum percent of light transmission; 3) the rate of aggregation, calculated 30 seconds after the start of platelet aggregation.

Statistical analysis

Statistical processing of the results was performed using Statistica 6.0 (StatSoft, USA) 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 ANOVAtest for independent samples. Differenceswereconsideredstatisticallysignificantatp < 0.05. Histograms used in ROS, GM-CSF, bFGF, PDGF examinations were plotted by GraphPad Prism 7 software (GraphPad Software, USA).

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Results

The concentrations of ROS and growth factors in the blood serum of animals whose experimental wounds were exposed to low-intensity laser radiation compared to animals that did not receive PBM therapy are presented in Figure.

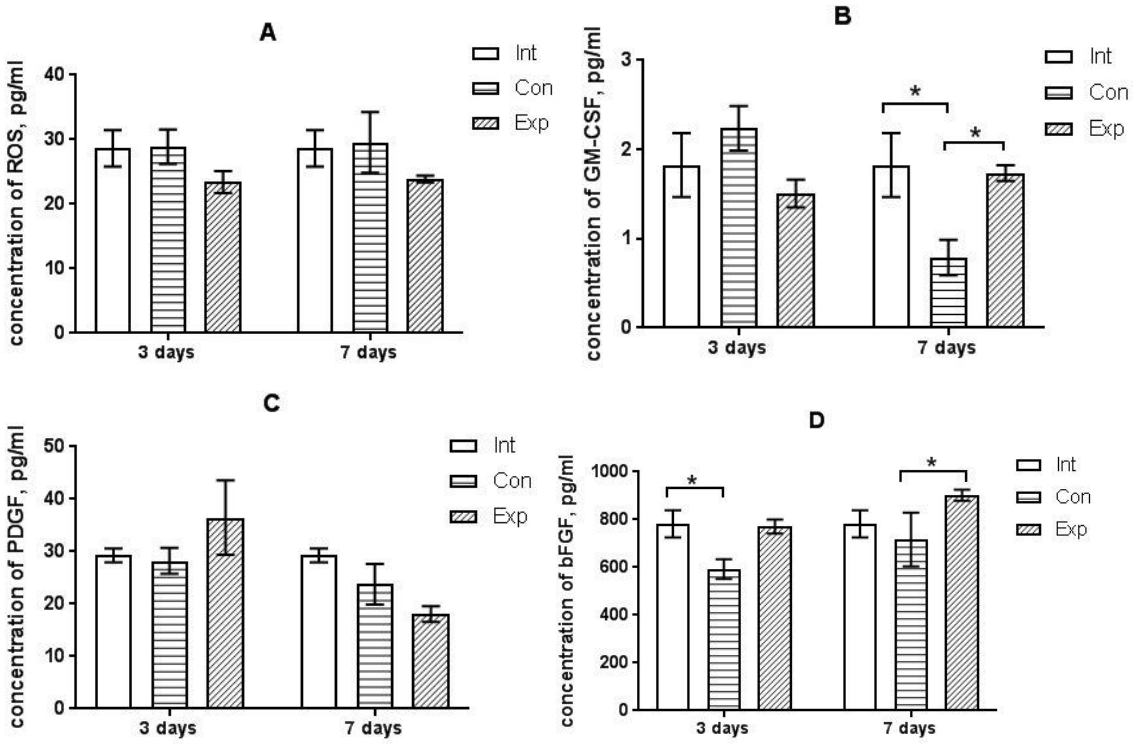

Figure. Changing the levels of the studied indicatorsin the blood serum of animals on the 3rd and 7th day: (A) ROS, (B) GM-CSF, (C) PDGF, (D) bFGF (*p < 0.05).

Changes in the expression of the studied parameters in the blood serum of animals with chronic wounds when using PBM therapy (Exp group) were found: a decrease in ROS levels and an increase in bFGF concentrations at the initial stages of wound healing. The multidirectional effect of PBM therapy on GM-CSF and PDGF levels was observed in the studied time frame (Figure).
When studying the effect of PBM-therapy on the functional activity of platelets in rats with wounds at various concentrations of the inducer of ADP aggregation on day 3 after surgery (Exp 3 days), a significant decrease in the degree and rate of platelet aggregation was observed (Table). The time to reach the maximum rate of aggregation, in this case, was shorter than this indicator in rats without PBM therapy (Con 3 days) for the wound defect. When studying the effect of low-intensity laser radiation on the functional activity of rat platelets on the 7th day after modeling wounds (Exp 7 days), a significant increase in the degree, speed, and time of reaching the maximum rate of platelet aggregation was observed (Table). The shapes of the aggregation curves (single-phase reversible aggregation) did not differ in the experimental groups at the studied concentrations of the inducer during the studied periods of wound healing.

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Table. Indicators of the functional activity of platelets in animals at various concentrations of ADP

Indicators	**Groups of animals**				
	Int	**Con 3 days**	**Exp 3 days**	**Con 7 days**	**Expl 7 days**
Aggregation degree,% 2.5 µmol/l	36,18 ± 5,97	57,73 ± 3,06	21,78 ± 4,12*	28,76 ± 1,61	68,10 ± 4,92**
Aggregation time, s 2.5 µmol/l	73,20 ± 5,78	102,33 ± 7,22	71,00 ± 3,04*	61,40 ± 2,86	101,67 ± 2,56**
Aggregation rate for 30 s, %/min 2.5 µmol/l	64,38 ± 8,86	88,00 ± 2,79	50,08 ± 4,25*	65,20 ± 2,77	109,27 ± 8,32**
Aggregation degree,% 5 µmol/l	63,07 ± 8,06	72,50 ± 0,44	42,95 ± 5,41*	45,34 ± 2,16	84,40 ± 3,57**
Aggregation time, s 5 µmol/l	121,55 ± 6,95	146,00 ± 5,25	122,00 ± 12,46	100,60 ± 8,33	152,67 ± 6,18**
Aggregation rate for 30 s, %/min 5 µmol/l	87,11 ± 9,24	100,07 ± 2,72	62,50 ± 6,23*	71,60 ± 3,38	125,73 ± 4,52**
Aggregation degree,% 10 µmol/l	75,61 ± 6,40	78,60 ± 1,18	59,55 ± 1,87*	57,86 ± 1,53	94,83 ± 4,27**
Aggregation time, s 10 µmol/l	176,63 ± 10,70	221,67 ± 7,86	206,50 ± 6,17	163,80 ± 6,84	209,00 ± 10,99**
Aggregation rate for 30 s, %/min 10 µmol/l	95,78 ± 7,71	104,87 ± 3,01	73,90 ± 3,91*	76,28 ± 2,19	128,27 ± 6,08**

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

Discussion

In our study, a chronic wound was modeled with the reproduction of the conditions of local hypoxia and microcirculation disorders. The result of hypoxia is an increase in the inflammatory state (Eltzschig & Carmeliet, 2011). Inflammation, in turn, is accompanied by an imbalance between free radical production and mechanisms of antioxidant control (Kovalov et al., 2017). In our study, PBM therapy has been shown to reduce ROS (Figure A). According to the literature, PBM can produce ROS in normal cells, but when used in cells with oxidative stress or in animal models of disease, ROS levels are reduced (Hamblin, 2017). Our results are consistent with the results of researchers on the effect of 635 nm LLLT on ROS homeostasis in the injured muscle. Malondialdehyde (MDA) – an indicator of lipid peroxidation, after exposure to low-intensity laser radiation, decreased after 1, 2, 3, and 7 days after injury (Luo et al., 2013).

Earlier, we have shown that the use of PBM therapy of the applied radiation parameters improved the process of wound healing on the 3rd and 7th days (Pavlov et al., 2020). According to the results of histological studies, a decrease in inflammation was observed. Also, advancing rapidly and actively proceeded proliferation phase, which was reflected in the intense fibrillo- and neoangiogenesis.

The anti-inflammatory effects of PBM therapy can explain the decrease in the levels of GM-CSF on the 3rd day and the increase in the levels of this cytokine on the 7th day after injury in the Exp groups of animals (Figure B). This multidirectional effect of PBM therapy on GM-CSF

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concentrations is due to the pleiotropic functions of this cytokine. The inflammatory activity of GM-CSF is primarily due to its role as a growth factor and differentiation of granulocyte and macrophage populations. However, in many situations, GM-CSF can act as an anti-inflammatory/regulatory cytokine. However, the proinflammatory and regulatory effects of GM-CSF seem to depend on the dose and the presence of other relevant cytokines in the context of the immune response (Bhattacharya et al., 2015). It is likely that PBM modulates various macrophage phenotypes during the wound healing process, which is reflected by changes in GM-CSF levels.

In our work, the manifestation of the anti-inflammatory properties of PBM therapy can be a decrease in the degree, speed, and time to reach the maximum rate of platelet aggregation at the studied concentrations of the inducer on the 3rd day after exposure to the wound defect with low-intensity laser radiation (Table). However, on the 7th day after wound modeling and applicationof low-intensity laser radiation used parameters, an increase in the degree, speed, and time to reach the maximum rate of aggregation is observed. Perhaps the ability of platelets to increase or decrease inflammation, fight infection, and promote an adequate immune response should be taken into account. The ability of platelets to attach to the vessel wall, form aggregates, and promote fibrin formation, key elements of blood clotting, has been said to both favor and dampen inflammation, to fight infection, and to assure an adequate immune response (Nurden, 2018). The effect of PBM therapy on platelet aggregation activity in wound defects, as far as we know, is being investigated for the first time.

PDGF plays an important role in wound healing by stimulating cell proliferation, migration and angiogenesis (Raica&Cimpean, 2010). According to the literature, in a chronic wound, the levels of a number of growth factors, including PDGF, are reduced compared to an acute wound (Cooper et al., 1994). Our results demonstrate that PBM stimulates the production of PDGF in the early stages of wound healing (Figure C). The obtained data are consistent with the literature data on the increase in the expression of PDGF genes in the tissues of the gums and mucous membranes of rats after low-level laser exposure at the inflammatory stage of wound healing (Safavi, et al. 2008). Further use of laser therapy in our study contributes to a decrease in PDGF concentrations (Exp 7 days), which is apparently due to the fact that chronic wounds are delayed at the self-perpetuating inflammatory stage (Zhao et al., 2016). And high levels of proinflammatory cytokines at this stage are associated with a decrease in growth factors (Bennett & Schultz, 1993). Similar trends in the levels of the studied growth factor are shown in a study in which there was an increase in PDGF-BB levels in the wound fluid of the palate on the 7th day after laser irradiation, which then decreases on the 12th day (Keskiner et al., 2016). Thus, the increase PDGF levels observed in the present study in the Exp group on day 3 suggests that the PBM may accelerate wound healing by stimulating the production of this mediator.

In our study, when using PBM, an increase in the levels of bFGF was observed in the Exp group on the 3rd and 7th days (Figure D). These data are consistent with the results of studies in which PBM treatment leads to an increase in bFGF gene expression on the 4th and 7th days in a model of the excisional wound in rats with type 1 diabetes mellitus (Amini et al., 2019). Due to its mitogenic and angiogenic characteristics, bFGF can induce tissue remodeling, wound healing, and neovascularization (Akita et al., 2013). Prolonged inflammatory response decreases bFGF production (Pavlov et al., 2021). Thus, increased production of bFGF may be one of the mechanisms by which low-intensity laser radiation stimulates wound healing.

When using laser therapy, it is also necessary to take into account the dose-dependent effects of PBM described by the Arndt-Schultz curve (Dompe et al., 2020). For example, according to the

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literature, irradiation with a CO_2 laser at 1.0 J/cm^2 (the investigated laser energy densities are 0.1, 0.5, 1.0, 2.0, or 5.0 J/cm^2) is the most effective for activation fibroblasts (Shingyochi et al., 2017). Our study also used an energy density of 1.0 J/cm^2 but used different PBM parameters (wavelength, radiation power). Further research is needed to optimize the parameters used in PBM therapy for wound healing.

Conclusions

Photobiomodulation therapy modulates cellular processes to improve chronic wound healing.
The use of photobiomodulation therapy makes it possible to regulate disturbances in reparative processes by modulating ROS, platelet aggregation activity, and the expression of endogenous growth factors.
The mechanisms by which low-intensity laser radiation stimulates wound healing require further study.

Funding

This study was funded by the Ministry of Health of Ukraine from the state budget.

Conflict of interest

The authors have no conflicts of interest to declare.

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