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Features of the regulation of reparative processes of chronic wounds in response to the effect of photobiomodulation therapy on a wound defect. Photobiomodulation therapy for chronic wounds

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Abstract: Background: One of the promising methods of influencing the wound process is photobiomodulation (PBM) therapy. The optimal parameters of PBM therapy have not yet been found because the molecular mechanisms of light interaction with tissue are not fully understood.
Objective: Studying the influence of PBM of various parameters on the regulation of reparative processes of chronic wounds using the example of indicators of aggregation activity of platelets, platelet-derived growth factor (PDGF), interleukin-8 (IL-8), and amino-terminal propeptide of type III procollagen (PIIINP) at the remodeling stage. And also the study of the structural and functional features of chronic wound healing in an experiment under various parameters of PBM therapy.
Methods: Experiments were carried out on Wistar rats. Chronic wounds were simulated. Experimental animals were exposed to PBM at a wavelength of 660 nm and an energy density of 1 J/cm^2. In serum, PDGF, IL-8, and PIIINP levels were measured by enzyme-linked immunosorbent assay. The functional activity of platelets was measured using the turbidimetric method. Histological analysis was performed.
Results: The work noted the dose-dependent effect of PBM using the example of platelet aggregation at the remodeling stage during the healing of chronic wounds. The use of PBM therapy resulted in increased serum PDGF levels. Histological examination data indicate a positive effect of PBM therapy on the wound healing process.
Conclusions: The effectiveness of the use of PBM therapy for the healing of chronic wounds to regulate reparative processes has been proven.

Keywords: platelet aggregation, intercellular mediators, histological indicators, remodeling phase, optimization of therapy parameters.

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Chronic wounds are a significant burden on healthcare systems around the world. In Europe, 1.5–2 million people suffer from acute or chronic wounds [1]. Chronic wounds affect the quality of life of nearly 2.5% of the US population [2]. In Ukraine, patients with long-term non-healing wounds account for up to 15% of the number of hospitalized patients with wound defects of the skin. By origin, they are represented by post-traumatic infected wounds (31.0%), venous ulcers (28.6%), trophic diabetic ulcers (25%), and erysipelas (15.48%) [3].

Chronic wounds can be accompanied by many diseases and disorders, such as diabetes, chronic kidney disease, stress, etc. [4, 5]. To overcome the factors that play a decisive role in delaying wound healing, it is urgent to search for new approaches to stimulate the reparative processes of chronic wounds. Modern methods of chronic wound treatment include the use of hydrogel dressings [6], drugs of placental origin [7], and ultrasound therapy [8]. Artificial intelligence-based visual injury detection is being explored [9]. High-throughput sequencing technology is used to identify the diversity of the chronic wound microbiome [10]. One of the promising methods of influencing the wound process is photobiomodulation (PBM) therapy. Therapeutic benefits associated with PBM include increased tissue regeneration and repair, decreased inflammation, pain relief, and reduced oxidative stress [11]. Despite the positive results of using this method, the optimal parameters of PBM therapy have not yet been found because the molecular mechanisms of light interaction with tissue are not fully understood.

The purpose of the work is to study the influence of PBM of various parameters on the regulation of reparative processes of chronic wounds using the example of indicators of aggregation activity of platelets, platelet-derived growth factor (PDGF), interleukin-8 (IL-8), and amino-terminal propeptide of type III procollagen (PIIINP) at the remodeling stage. And also the study of the structural and functional features of chronic wound healing in an experiment under various parameters of PBM therapy.

Materials and Methods

Experimental protocol

This study included 24 Wistar rats, 8–9 months of age, weighing 220–250 g. The animals were randomized into four groups of 6 each: intact (Int), control (Con), and two experimental groups (EG1, EG2). Surgical wounds were created on the backs of animals in the control and experimental groups. Wound induction operations were performed under general anesthesia (Zoletil (Virbac, France) 10 mg/kg body weight). A model of a hypoxic chronic wound with impaired microcirculation was reproduced. The wound was a circle with a diameter of 20 mm. Perpendicular loop-shaped fasciocutaneous sutures were applied along the edges of the wound. On the surface of the wound bottom, the superficial fascia was dissected with mutually perpendicular transverse and longitudinal cuts in the form of a square measuring 5×5 mm. The formed cells were sutured with U-shaped sutures [12].

Parameters of PBM therapy

PBM therapy was used in the experimental groups. The device "Lika-therapist M" (Cherkasy, Ukraine) was used. The wound defect was affected in a distant way to illuminate the entire wound area. Given the divergence of the diode laser beam (0.5 rad ± 20%) and the aperture diameter of

the extension handle of the laser device (0.2 cm), the distance between the extension handle and the wound was 3.53 cm. The tip of the laser emitter was held perpendicular to the wound surface. The therapy was applied for 5 days, starting 24 hours after surgery. In two experimental groups, a continuous irradiation mode was used at a wavelength of 660 nm, energy density 1 J/cm^2, and radiation powers varied: 50 mW was used in the first experimental group (EG1) and 10 mW in the second experimental group (EG2).

Blood Collection

On day 28 after wound creation, the animals were euthanized. Blood was collected from the heart (open puncture method). In serum, PDGF, IL-8, and PIIINP levels were measured by enzyme-linked immunosorbent assay. IL-8 levels were determined using Vector-Best reagent kits (Ukraine). PDGF and PIIINP levels were determined using eBioscience kits (USA).

Determination of the functional activity of platelets

The functional activity of platelets was measured on a computerized platelet aggregation analyzer SOLAR 2110 using the turbidimetric method. Platelet aggregation has been studied in platelet-rich plasma. Sodium citrate (3.2%) in a ratio of 9: 1 was used as a stabilizer for blood samples. Adenosine diphosphate (ADP) was used as an aggregation inducer at concentrations of 2.5, 5, and 10 μmol/L. The recording of aggregation diagrams was carried out at 37°C for 10 minutes. The degree of aggregation (maximum % light transmission of the plasma), time to reach the maximum aggregation rate (time to reach the maximum % light transmission), and aggregation rate (calculated 30 seconds after the onset of platelet aggregation) were determined. The type of aggregation diagrams was also assessed.

Histological analysis

For histological examination, a section of the animal's wound was excised, including all its sections (central, main, edge). The material was embedded in paraffin and stained with hematoxylin and eosin, as well as van Gieson's picrofuchsin staining. The preparations were analyzed and photographed using a PrimoStar microscope (Zeiss, Germany) and a Microocular digital camera.

A semi-quantitative method was used to evaluate the following histological processes and structures: reepithelization stage, polymorphonuclear leucocytes (PMNL), fibroblasts, new vessels, and new collagen [13]. The sections were evaluated blindly, according to the scale: 0, 1, 2, 3, 4 (Table 1).

Statistical analysis

The data were processed using Statistica 12.0 (StatSoft Inc., USA). The results were expressed 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 was evaluated using a one-way analysis of variance (ANOVA) with the non-parametric Kruskal–Wallis test for independent samples ($p < 0.05$). Histograms were plotted by GraphPad Prism 8 (GraphPad Software, USA).

Table 1. Semiquantitative histological evaluation of healing skin wounds.

Scale	Reepithelization stage	Polymorphonuclear leukocytes	Fibroblasts	New vessels	Collagen
0	thickening of cut edges	absent	absent	absent	absent-granulation tissue
1	migration of cells (<50%)	mild-surrounding tissue	mild-surrounding tissue	mild-surrounding tissue	minimal-granulation tissue
2	migration of cells (≥50%)	mild-granulation tissue	mild-granulation tissue	mild-granulation tissue	mild-granulation tissue
3	bridging the excision	moderate-granulation tissue	moderate-granulation tissue	moderate-granulation tissue	moderate-granulation tissue
4	keratinization	marked-granulation tissue	marked-granulation tissue	marked-granulation tissue	marked-granulation tissue

Bioethics

The experiments were carried out by the Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447-IV dated 02.21.2006), with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986), as well as by the requirements of the Bioethics Committee of Kharkiv Medical Academy of Postgraduate Education (protocol No. 2 dated 6 September 2022).

Results

The concentrations of PDGF, PIIINP, and IL-8 in the blood serum of animals of various groups are presented in Fig. 1.

Statistically significant differences were found in the expression of PDGF in the blood serum of animals with chronic wounds when using PBM therapy. In experimental group 1, the level of PDGF increased by 2.7 times, and in experimental group 2 — by 1.9 times.

Thus, the use of PBM therapy leads to a significant increase in the degree of aggregation, the time to reach the maximum rate of aggregation, and the rate of aggregation in the first experimental group compared with the corresponding indicators in the control group when using three concentrations of ADP as an aggregation inducer: 2.5, 5 and 10 μmol/L. The degree of aggregation, the time to reach the maximum aggregation rate, and the aggregation rate of the second experimental group did not have significant differences compared to similar indicators in the control group (Table 2). All aggregation diagrams were single-phase reversible curves.

Histological analysis showed that 28 days after surgical modeling of wounds, their complete epithelization was noted in all groups. The central zone of the wound was macroscopically a narrow scar. Upon microscopic examination of wound samples, the histostructure of the newly formed epidermis generally corresponded to the norm, the division into layers was quite clear, and keratinization of the stratum corneum was present.

In most of the wound, the regenerate in animals of the control and experimental groups had signs of the normal dermis: a characteristic arrangement of fairly dense bundles of collagen fibers,

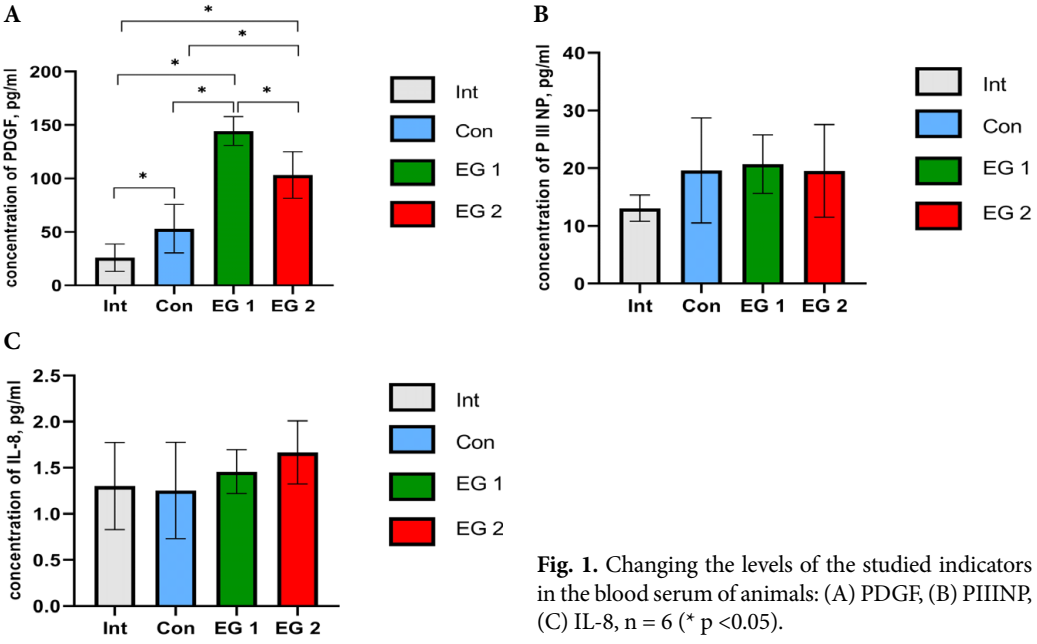

Fig. 1. Changing the levels of the studied indicators in the blood serum of animals: (A) PDGF, (B) PIIINP, (C) IL-8, n = 6 (* p <0.05).

Table 2. Indicators of functional activity of platelets in animals of all groups on the 28th day of the experiment when using ADP of various concentrations as an aggregation inducer.

ADP concentration	Indicators of aggregation activity of platelets	Groups of animals			
		Int	Con	EG1	EG2
2.5 µmol/l	Degree of aggregation, %	27.27 ± 2.22	17.15 ± 2.07*	36.25 ± 5.35**	25.33 ± 2.62
	Aggregation time, s	61.17 ± 3.50	52.83 ± 3.19	74.00 ± 6.36*,**	51.33 ± 1.63
	Aggregation rate for 30 s, %/min	56.77 ± 2.11	48.05 ± 2.69	74.75 ± 9.23*,**	61.07 ± 6.04
5 µmol/l	Degree of aggregation, %	63.07 ± 4.80	30.85 ± 3.73*	60.15 ± 6.44**	35.00 ± 5.25*
	Aggregation time, s	121.50 ± 6.42	80.33 ± 6.17*	113.33 ± 8.56**	88.33 ± 4.28*
	Aggregation rate for 30 s, %/min	87.12 ± 8.33	58.50 ± 3.16*	95.75 ± 8.57**	62.27 ± 6.05*
10 µmol/l	Degree of aggregation, %	75.62 ± 5.37	45.45 ± 2.34*	71.28 ± 7.90**	51.23 ± 2.86*
	Aggregation time, s	176.67 ± 8.71	130.00 ± 10.04*	176.50 ± 13.48**	144.67 ± 3.85*
	Aggregation rate for 30 s, %/min	95.78 ± 7.25	67.35 ± 3.23*	88.85 ± 7.50**	74.80 ± 5.19*

* p <0.05 compared to the Int group ** p <0.05 compared to the Con group

a small number of cellular elements, and vessels. The formation of skin appendages (hair follicles and sebaceous glands) was also noted here.

In animals of the control group, in the central zone, filled predominantly with young granulation tissue, areas with signs of fibroblast proliferation, and newly formed capillaries remained. There was a slight diffuse lymphohistiocytic infiltration, and neutrophilic granulocytes were sporadic, likely reflecting mild chronic inflammation. The bundles of collagen fibers here were thin and chaotic (Fig. 2A).

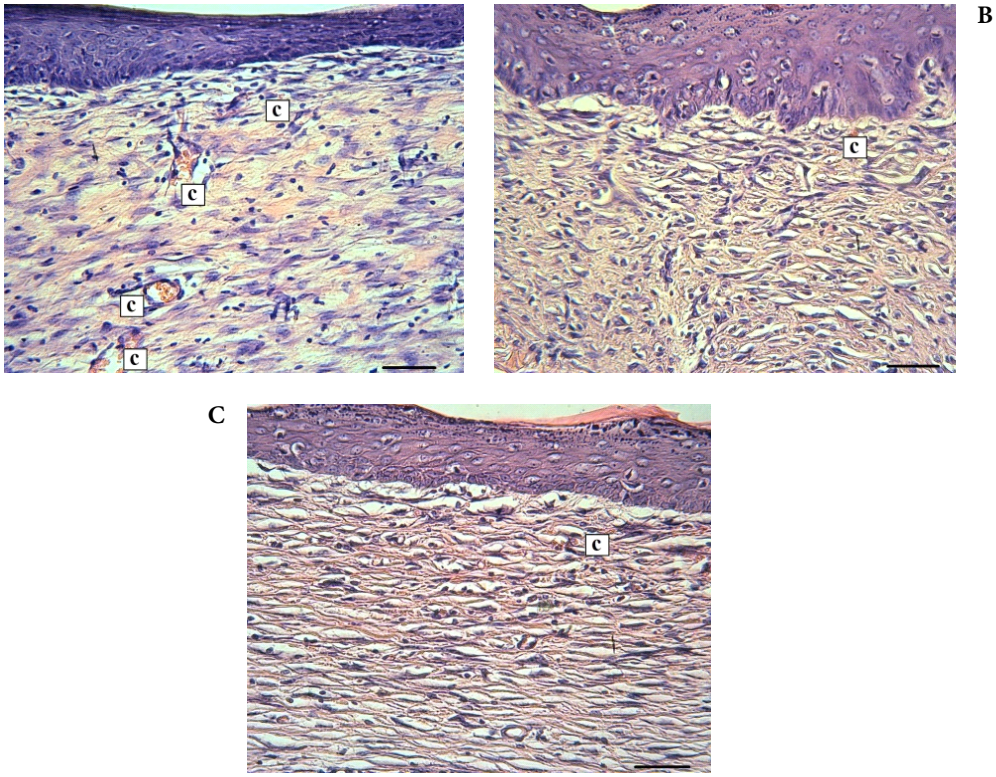

Fig. 2. The central area of the wound after 28 days in an animal of: (A) control group — young granulation tissue with a significant number of fibroblasts and capillaries, a small diffuse inflammatory infiltration; (B) group EG 1 — maturing connective tissue with a large number of fibroblasts and single capillaries; (C) group EG 2 — maturing connective tissue with a small number of fibroblasts and fibrocytes, single capillaries; c — newly formed capillaries; hematoxylin-eosin; scale bar 50 μm.

In the rat samples of the first experimental group, the central part of the defect was filled with maturing connective tissue with a small number of newly formed vessels, and collagen fibers were combined into bundles of sufficient thickness. A significant number of fibroblasts with large brightly colored nuclei and processes were noted, which reflects their functional activity. Single inflammatory elements were represented by macrophages and lymphocytes (Fig. 2B).

In the rat samples of the second experimental group, the central area consisted of maturing connective tissue with a small number of fibroblasts and fibrocytes, bundles of collagen fibers were

of sufficient thickness and located parallel to the surface of the wound, newly formed vessels were single (Fig. 2C).

Histological indicators of wound healing in animals of three groups were calculated in all areas of the regenerate and are shown in Fig. 3.

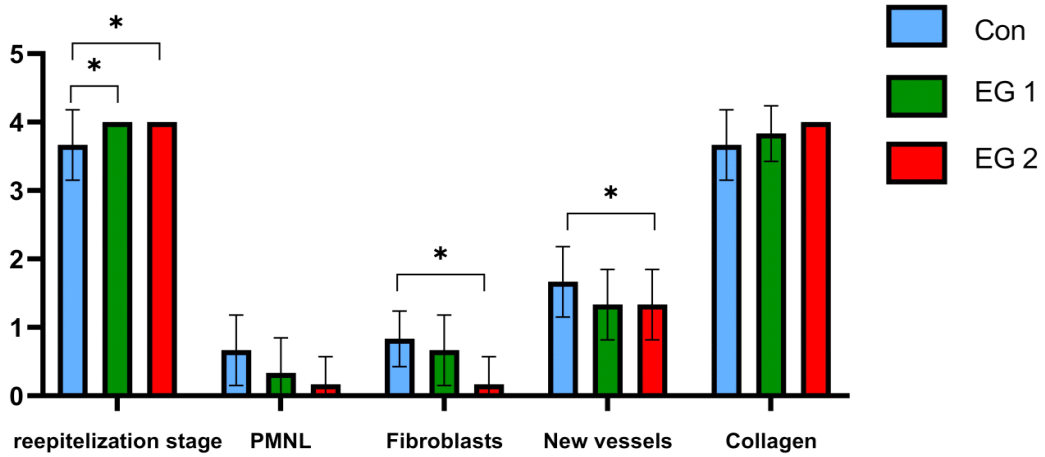

Fig. 3. Data from semi-quantitative analysis of wound healing indicators in animals of three groups for 28 days, n = 6 (* $p < 0.05$).

Histological examination data may indicate a positive effect of PBM therapy on the wound healing process, which was more pronounced in the second experimental group. Animals in this group showed the most rapid maturation of granulation tissue, which persists at the remodeling stage in the central part of the wound, with no signs of inflammation.

Discussion

It is known that there are four major overlapping phases of wound healing, namely hemostasis, inflammation, proliferation, and remodeling. Understanding the differences in wound healing phases can help identify effective treatments to speed healing and prevent infections and complications [14]. Researchers have mainly focused on studying the initial phases of wound healing. We looked at the remodeling stage, the final phase of wound healing, which involves the maturation and organization of newly synthesized tissue.

During the wound healing process, dysregulation of cell signaling can lead to decreased cell function and the development of chronic wounds [15]. PBM therapy was used in our work as a regulator of the remodeling processes of chronic wounds. It is known that laser exposure has a dose-dependent effect [16]. In this regard, the effect of PBM was studied at a wavelength of 660 nm and an energy density of 1 J/cm^2, with experimental groups differing in laser radiation power.

Our work demonstrated that platelet aggregation activity during wound healing at the remodeling stage depended on the parameters of PBM therapy used. Work studying the effect of PBM on

platelets is limited. According to the literature, exposure to PBM therapy significantly increased the number of megakaryocytes in the bone marrow of mice with thrombocytopenia and, accordingly, the number of platelets increased [17]. In a study by Rola *et al.*, low-energy laser irradiation reduced whole blood platelet aggregation in an in vitro model [18]. Our previous studies showed that when using FBM therapy on the seventh day of wound healing, aggregation activity depended on the laser radiation parameters used [12, 19]. Previously demonstrated results obtained when studying the transition stage of inflammation to the proliferation stage are consistent with the data of this study at the remodeling stage.

We also previously showed that PBM therapy is a regulator of reparative processes at the level of intercellular mediators [20]. In this case, an important aspect is the optimization of the parameters of the therapy used. In our work, exposure to PBM therapy led to an increase in PDGF in two experimental groups compared to the same indicator in animals in the control group. PDGF is known to help regulate many cellular activities associated with the wound healing process, including fibroblast mitogenesis and multicellular mitogenesis, angiogenesis, and chemotaxis [21]. In tissue remodeling, PDGF helps break down old collagen by upregulating matrix metalloproteinases [22]. The histological analysis performed in our study confirms a reduction in inflammation, a decrease in newly formed vessels, and a better organization of collagen fibers in groups of animals whose wound defects were exposed to PBM.

The results obtained in our work demonstrate that using PBM does not affect the expression of PIIINP in the remodeling phase. PIIINP is used as a biomarker for increased collagen III synthesis [23]. By day 28 post-wound simulation, PIIINP concentrations remained elevated in experimental animals compared with those in intact animals but were lower than the PIIINP levels at day 3 post-wound simulation shown in our previous study [24]. According to the literature, an increase in PIIINP levels after thermal injury indicates a fibrogenic component of wound healing [25]. The absence of significant differences in the levels of PIIINP in the blood serum of the studied animals of the control and experimental groups apparently indicates a minimal risk of developing fibrotic reactions.

When studying the effect of PBM therapy of various parameters on the levels of IL-8, it was shown that no statistically significant differences in the concentrations of this cytokine were found in comparison with the same indicator in animals of the control group at the remodeling stage. IL-8 has antihypoxic, recruitment of cell homing, and angiogenesis-promoting functions [26]. There are limited studies in the literature on cytokines in long-term wound healing. The use of laser radiation in combination with a photosensitizer as an adjunct to therapy led to a decrease in the level of IL-8 in the gingival fluid of patients with periodontitis [27].

Conclusions

A model of chronic hypoxic wounds demonstrated the positive effect of PBM therapy on healing.

When analyzing platelet aggregation at the stage of remodeling during the healing of chronic wounds, a dose-dependent effect of PBM was noted. The use of PBM therapy led to an increase in PDGF levels in the blood serum of experimental animals.

Histological examination data indicate a positive effect of PBM therapy on the wound healing process. There was a reduction in inflammation, a decrease in the number of newly formed vessels, and better organization of collagen fibers in groups of animals whose wound defects were exposed to PBM.

PBM therapy is most effective when choosing optimal application parameters, and in this case, it can be a reliable method of treating wounds.

Further work is needed to study the cellular and molecular mechanisms of damage repair processes, as well as to find the optimal parameters of PBM therapy for chronic wounds treatment. This will expand the experimental base and improve the clinical application of this method in chronic wounds treatment.

Author contributions

All authors contributed to the study conception, design, or analysis. Conceptualization: S.P.; Methodology: N.B.; Preparation of the material, data collection, and analysis: M.K., O.L.; Writing — original draft preparation: N.B.; Writing — review and editing: M.K., O.L.; Supervision: S.P. All authors read and approved the final manuscript.

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Conflict of interest

None declared.

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