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DOI <https://doi.org/10.32782/2226-2008-2025-2-2>P. T. Muraviov^{1,2} <https://orcid.org/0000-0001-7733-885X>M. H. Bezpalko^{1,2} <https://orcid.org/0009-0006-1338-4236>D. M. Davydov¹ <https://orcid.org/0009-0000-3659-6764>

THE EFFECT OF COLLAGEN HYDROLYZATE ON WOUND HEALING IN THE CONDITIONS OF EXPERIMENTAL MODELING OF THE COURSE OF THE WOUND PROCESS IN RATS

¹ Odesa National Medical University, Odesa, Ukraine² Municipal Non-Commercial Enterprise "Odesa Regional Clinical Medical Center" of the Odesa Regional Council, Odesa, Ukraine

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P. T. Muraviov^{1,2}, M. H. Bezpalko^{1,2}, D. M. Davydov¹

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Despite the existence of multiple trends toward chronic wounds treatment, certain misunderstandings regarding the regeneration of connective tissue determine the need to address this problem.

The aim of the study was to determine the features of post-traumatic regeneration in experimental conditions of collagen hydrolysate complex applying.

Materials and methods. In the rats of the main group (13 (43.3%)), hydrolysate of collagen was applied to the treatment process, in the comparison group (17 (56.7%)), the regeneration processes took place without additional influence.

Results. Significant differences in the formation of the microcirculatory system began to be observed from the 7th day. The differences in capillary density were statistically significant. The same situation in the density of newly formed capillaries on the 14th day of the experiment was detected. In addition, on the 28th day after the injury, the main group of animals demonstrated more expressed collagen formation and more mature connective tissue development. In contrast, the comparison group of animals showed developing granulation tissue with slower maturation and a significantly higher presence of residual inflammatory infiltrate. Newly formed nerve trunks were predominantly observed in the group of animals treated with collagen.

Conclusions. The effectiveness of collagen hydrolysate to improve post-traumatic regeneration processes was demonstrated in an experimental model of induced wound process.

Keywords: rats, wound, regeneration, collagen, wound treatment.

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П. Т. Муравйов^{1,2}, М. Г. Беспалько^{1,2}, Д. М. Давидов¹

ВПЛИВ ГІДРОЛІЗАТУ КОЛАГЕНУ НА ЗАГОЄННЯ РАН В УМОВАХ ЕКСПЕРИМЕНТАЛЬНОГО МОДЕЛЮВАННЯ ПЕРЕБІГУ РАНОВОГО ПРОЦЕСУ В ЩУРІВ

¹ Одеський національний медичний університет, Україна² КНП «Одеський обласний клінічний медичний центр» Одеської обласної ради, Одеса, Україна

У щурів основної групи в лікувальний процес було залучено гідролізат колагену, в групі порівняння процеси регенерації відбувалися без додаткового впливу. Достовірні відмінності у формуванні судинного мікроциркуляторного русла почали спостерігати із 7-ї доби спостереження. Також у щурів основної групи відмічено більш раннє закриття новоутворених судин, швидше дозрівання сполучної тканини з формуванням більш впорядкованого колагенового каркасу. Засвідчено ефективність застосування гідролізату колагену з метою поліпшення процесів післятравматичної регенерації на моделі спричиненого ранового процесу в експерименті.

Ключові слова: щури, рана, регенерація, колаген, лікування ран.

Introduction. The problem of wound healing of various etiologies in surgical patients is one of the most common and debatable issues, considering its medical, social, and economic aspects [1; 2]. The various approaches to surgical wound treatment differ in their diversity and consistently have both supporters and opponents regarding the

components of medicinal and surgical treatment [1–5]. In recent years, there have been research results on the effect of collagen hydrolysate on fibroblast proliferation, restoration of tissue basophil count, organization and maturation of collagen fibers, leading to the formation of dense connective tissue [6]. Other methods aimed at accelerating the healing of soft tissue wounds also deserve attention, including the use of biologically active substances, sorbents, nanoparticles, metal ions, homeopathic remedies, hyperbaric oxygen therapy, ozone therapy, laser therapy, and VAC

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Стаття поширюється на умовах ліцензії



therapy [1–3; 7]. Certain attention is given to a wide range of such methods aimed at the wound surface debridement, as biological, physicochemical, and mechanical [7–9; 10]. However, certain misunderstandings regarding the regeneration of connective tissue in focus of today's realities, concerning mine-explosive and gunshot wounds, determine the necessity of this problem solving [11; 12].

The aim of the study was to determine the features of post-traumatic regeneration in experimental conditions of collagen hydrolysate complex applying.

Materials and methods. The study of the wound healing process involved 30 one-year-old Wistar rats weighing 250–300 grams without signs of chronic or acute diseases. During the experiment, the animals underwent a 10-day quarantine period and were kept in standard vivarium conditions on a full-fledged diet with free access to water. Animal preparation, all interventions, anesthetics, and withdrawal from the experiment were carried out in full compliance with the requirements of the Guidelines of the State Pharmacological Center of the Ministry of Health of Ukraine (Kyiv, 2001), as well as the GLP rules provided by the European Commission for the supervision of laboratory and other studies, by Code of Scientist of Ukraine. The injuries were inflicted in the laboratory, using a Voltran Ekol ES55 pneumatic gun that simulated a gunshot wound. The animals were fixed on a wooden board. The target site of the injury was the hind limbs, the bullet caused damage to the skin, subcutaneous fat, and muscles with minor blood loss, without bone damage. The entrance hole size was (0.3 ± 0.5) mm, the wound edges were relatively even with the surrounding tissue. The length of the wound channel was up to 1 cm. No animals died during the experiment.

The animals were divided into two groups: the main group (13 (43.3%) rats), which received collagen hydrolysate, and the comparison group (17 (56.7%) rats), in which post-traumatic tissue regeneration occurred without additional treatment. Wound healing was assessed visually and by cytological material collecting (smear marks) from the wound surface. The research methods were macroscopic, pathohistological (study of structural changes in tissues and cells, histological features of damage and regeneration in the injured zone), morphometric (study of the

microcirculatory bed), and immunohistochemical (determination of the cellular composition of the tissue regeneration zones and verification of the vascular bed). The results were processed using variational statistical methods of analysis using Microsoft Office Excel 2016 software. Statistical processing of the experimental study results was carried out by the methods of variation analysis using the Student's test. The difference was considered statistically significant at $p < 0.01$.

Results and Discussion. It was found that on the 7th day after the injury, capillaries with predominantly vertical growth relative to the wound lumen were forming in the edges of the wound channel (Fig. 1). Moreover, the number of capillaries in the walls of the wound channel was greater compared to the derma around the inlet. In some of the capillaries stasis were detected.

Besides the qualitative method of the vascular bed verifying in the edges of the experimental wounds in the rats' limbs, a quantitative assessment of the newly formed microcirculatory bed was carried out. The morphometric study of the average diameter of the newly formed vessels and capillary density provided the following results (Table 1).

The dynamics of the vascular bed changes in the edges of the wounds in the experimental animal groups were monitored until the 14th day of the experiment. It allowed to fix a substantial number of vessels in the microcirculatory bed, both in the newly formed granulation tissue (Fig. 2) and in the inlet zone tissues (Fig. 3) in both groups. Capillaries with no particular orientation relative to the course of the wound or the skin surface were predominant, and there were no signs of stasis or thrombosis in the capillary lumen.

To objectify the study of the microcirculatory bed features, a morphometric analysis was performed. The average diameter of vessels from the zone of neoangiogenesis and the density of newly formed capillaries were assessed (Table 2).

After 14 days, in the rats of the main group treated with collagen hydrolysate, the amount of purulent-necrotic exudate on the wound surface significantly reduced, and the consistence of the exudate also changed. It consisted

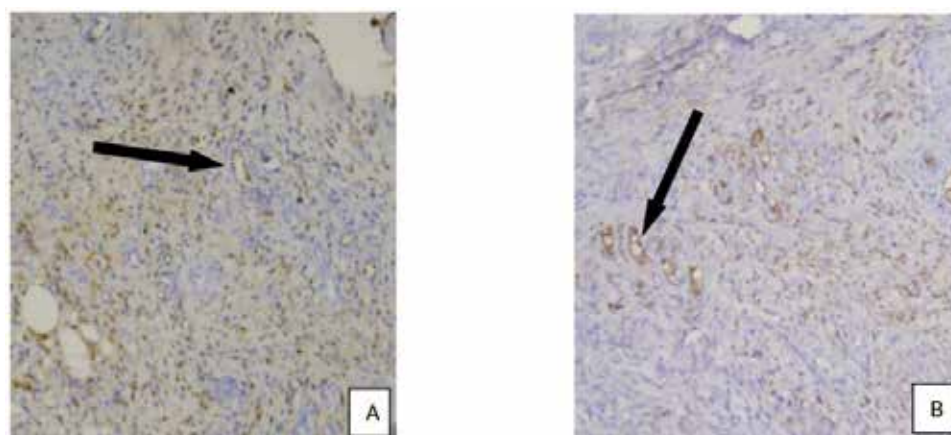


Fig. 1. Vessel density and diameter in the edges of the wounds in rats from the main group (A) and the comparison group (B) on the 7th day of the experiment. IHC reaction with CD34, H&E stain, magnification 100x

Table 1

The rats' limbs wounds wall microcirculatory bed features

Group	The average diameter of the newly formed vessels, μm	The capillary density, %
The Main group (n = 13)	15.4 ± 2.1	$18.7 \pm 1.2^*$
The comparison group (n = 17)	12.1 ± 2.4	$14.6 \pm 1.4^*$

Note: * – Differences are statistically significant ($p < 0.001$).

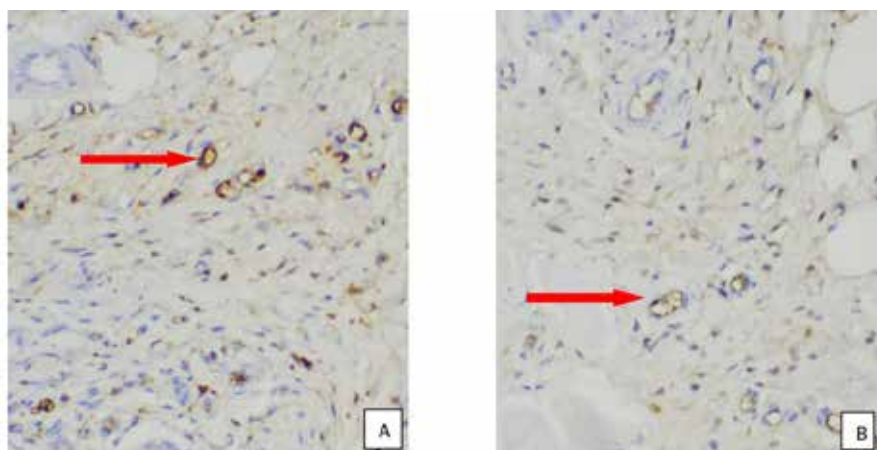


Fig. 2. Location and course of vessels in the wound wall of rats from the main group (A) and the comparison group (B) on the 14th day of the experiment. IHC reaction with CD34, magnification 100x

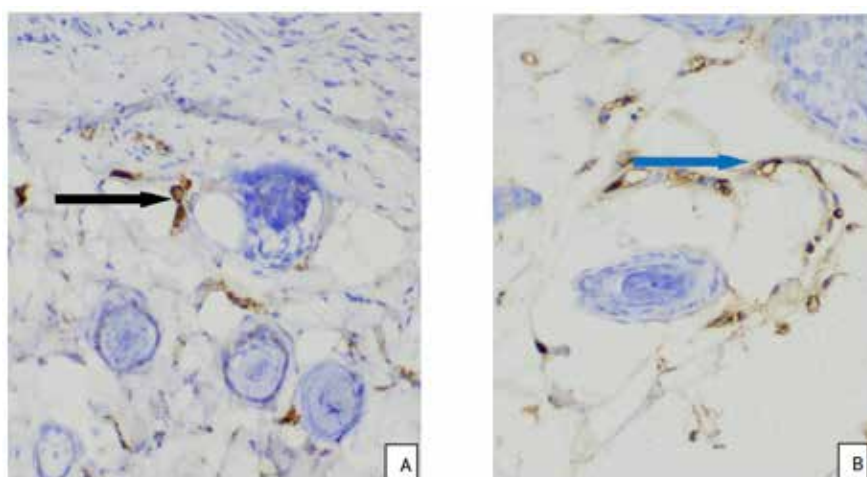


Fig. 3. Capillaries in the inlet zone derma of rats from the main group (A) and the comparison group (B) on the 14th day of the experiment. IHC reaction with CD34, magnification 100x

Table 2

The rat's wound's wall vascular bed features on the 14th day after injury

Group	The average diameter of the newly formed vessels, μm	The capillary density, %
The Main group, (n = 13)	13.1 ± 1.1	$10.5 \pm 1.1^*$
The Comparison group, (n = 17)	12.0 ± 1.4	$8.2 \pm 0.9^*$

Note: * – Differences are statistically significant ($p < 0.001$).

of fibrin and a mixture of keratinocytes, with remnants of inflammatory cells appearing as nuclear fragments, resembling grains and dust. At the same time, there were clear signs of epidermization at the edges of the wounds, both due to the regeneration of basal cell groups of the squa-

mous epithelium (Fig. 4, A) and through migration from the lateral preserved areas of the epidermis (Fig. 4, B). The newly formed multilayered squamous epithelium showed signs of dyskeratosis (uneven hyperkeratosis, parakeratosis) and consisted of 8–14 cell layers.

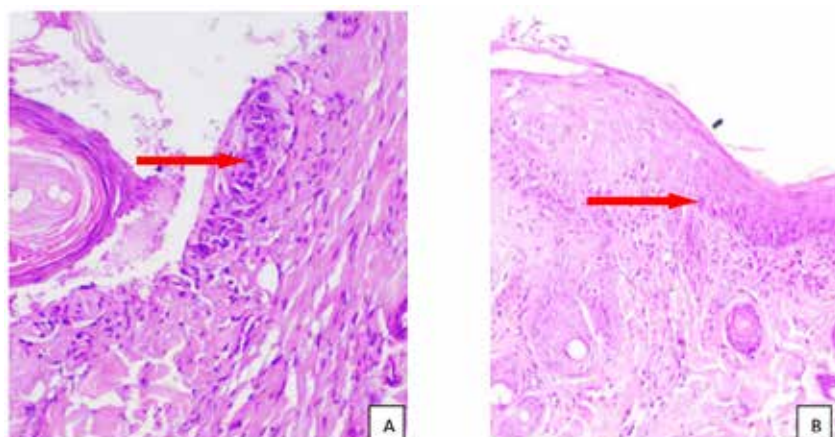


Fig. 4. Edges of the wound inlet in rats from the comparison group, 14th day of the experiment, proliferation of basal cells and formation of the growth zone (A), formation of a new layer of covering multilayered squamous epithelium (B), H&E stain, magnification 100x

The epidermis showed not only signs of dyskeratosis but also shedding of the surface layers (at the level of the granular layer). In the thickness of the epidermis, diffuse, mildly expressed inflammatory infiltration by granulocytes was detected. Distinct inflammation at the level of the dermal papillary layer had a striped pattern, and expressed edema of the derma with collagen fibers separation was also observed. It is important to note that the dermal edema was significantly more expressed than the inflammation.

The skin appendages were represented by hair follicles with uneven regenerative changes, including an expanded proliferation zone involving from 1/3 to 1/2 of the epithelial layer thickness of the hair follicle, with the formation of thin hair shafts and the appearance of several hair shafts within a single follicle.

In the comparison group changes were also documented in the exudate on the surface of wound inlet, where protein masses and fragments of hair shafts predominated, and cellular elements showed signs of breakdown and lysis. Around the wound, there was a reduction of epidermal layers number, with signs of excessive keratinization (Fig. 5). In the epidermis, the number of layers ranged from 6 to 10, but skin appendages showed no particular features.

In both experimental groups, distinct dermal edema with separation of collagen fibers was observed, though it was somewhat more expressed in the comparison group.

In the walls of the wound inlet in both groups, the inflammatory infiltrate had a lower density, but it was an uneven mixed-cellular inflammation. Regarding the vessels, capillaries, arterioles, and venules predominated, vascular dystonia in the microcirculatory bed, accompanied by venule dilation was also detected.

The partial adipocytes necrosis without a distinct cellular reaction, along with uneven, expressed edema of the intercellular substance in the wound inlet edges was also observed (Fig. 6).

Skin appendages were characterized by an increased growth zone in the hair follicles and the formation of hair shafts with a thinned hair matrix and its uneven density. Sebaceous glands were located in the peripheral zone of the wound, single in number, without signs of hyperfunction.

Collagen fibers in the derma of both experimental groups showed signs of predominantly horizontal orientation relative to the skin surface, partially circular around the skin appendages, and had a disordered structure in close proximity to the wound surface (Fig. 7).

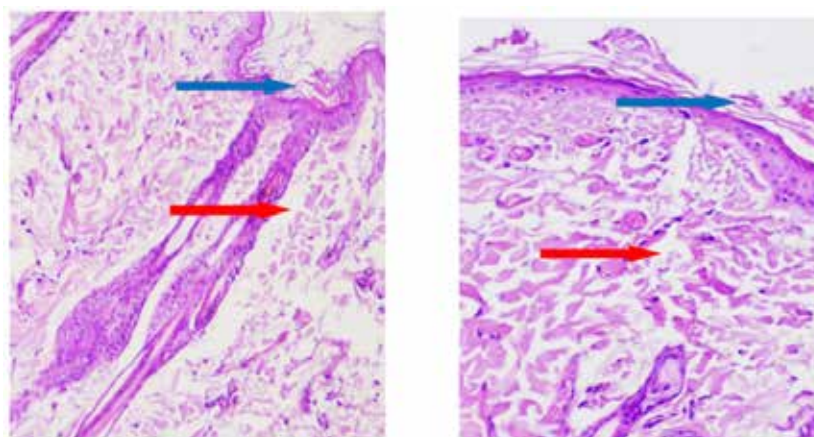


Fig. 5. Keratin scales (blue arrows) on the surface of the epidermis and expressed dermal edema (red arrows) in the edges of the wound in the control group rats, H&E stain, magnification 100x

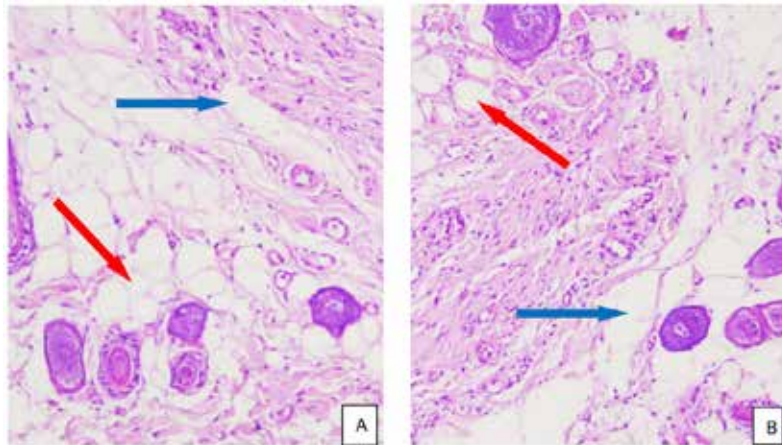


Fig. 6. Wall of the wound channel in the comparison group rat (A) and the main group rat (B), showing expressed edema of the intercellular matrix (blue arrow), focus of adipocyte necrosis (red arrow), H&E stain, magnification 100x

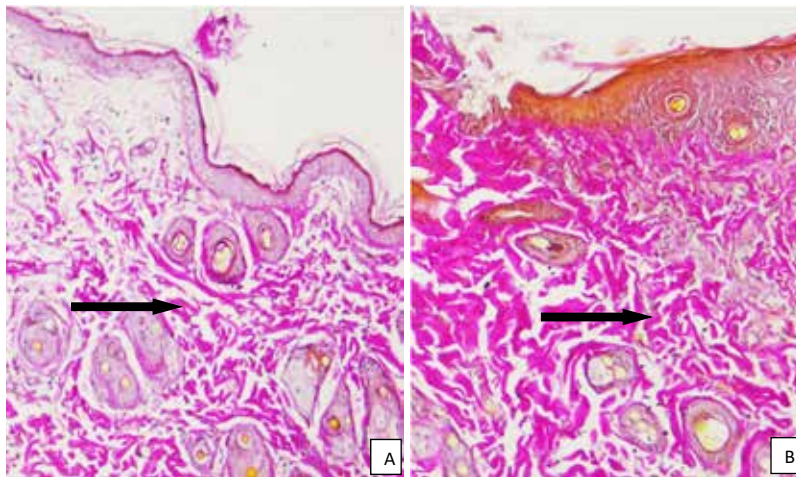


Fig. 7. Arrangement and orientation of collagen fibers in the edges of the wound inlet in the main group (A) and the comparison group (B), Van Hyson stain, magnification 100x

Upon detailed examination of the wound channel, it was evident that the formation of collagen fibers in the animals of the main group occurred through the development of finer fibers with a more organized alignment, showing signs of thickening at the peripheral part of the wound. In the comparison group of animals, collagen fibers were thicker, with a chaotic arrangement, forming thick bands and strips (Fig. 8). Additionally, the formation of a thin collagen capsule around the preserved muscle fibers was observed.

Newly formed nerve trunks were predominantly observed in the group of animals treated with collagen (Fig. 8 (A), arrows), with small-diameter nerve trunks being more common and located primarily next to the newly formed vessels.

It should be emphasized that edema of the intercellular matrix was predominantly observed in the superficial edges of the wounds, in the inlet zone derma of both groups of animals.

On the 28th day of the experiment, the wound inlet was completely closed with a pink scar, without scabbing, and surrounding tissues showed no signs of inflammation.

The peripheral part of the scar was whitish-pink, and microscopically, it was composed of connective tissue (Fig. 9), equally developed in both groups. Among the connective tissue fibers, fibrocytes and isolated histiocytes were present. The wound surface showed signs of epidermization, with a covering squamous epithelium and foci of local hyperkeratosis. The epidermal layers peeled off quite easily from the underlying tissues. The number of cell layers in the epidermis ranged from 4 to 6.

The formed connective tissue in the peripheral part of the scar became denser, with a small number of capillaries. In the edges of the fibrous tissue, skin appendages were observed.

To verify the degree of maturation of the connective tissue, we applied Van Hyson staining. It was found that in the group of rats treated with collagen, mature connective tissue had formed with wide bands of collagen fibers (Fig. 10, A), which exhibited an organized and oriented structure relative to the wound and had a high density of arrangement. In the comparison group of animals, dense connective tissue was also formed with collagen fibers, but they were thinner, and no clear orientation of the fiber

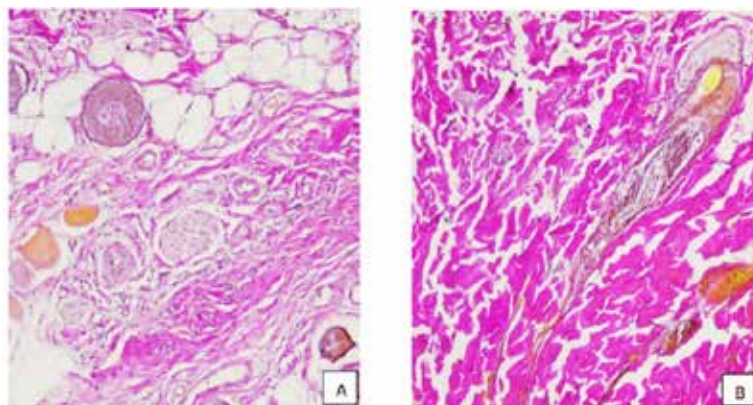


Fig. 8. Peripheral part of the wound in rats from the main group (A) and the comparison group (B), 14th day of the experiment, *Van Hyson* stain, magnification 100x

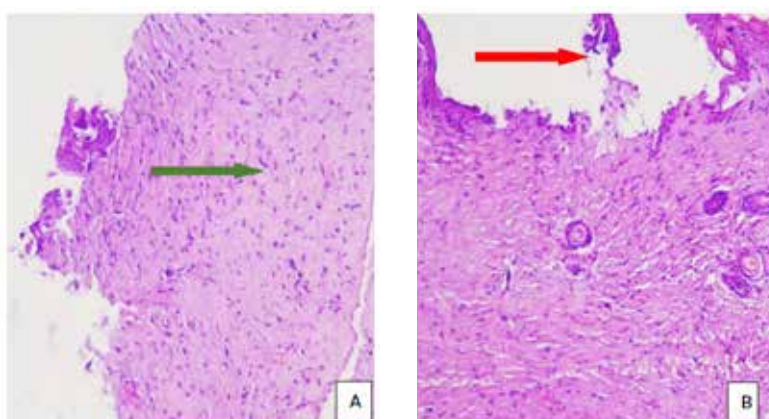


Fig. 9. The surface of the wound in rats (A – the main group, B – the comparison group) on the 28th day of the experiment, formed connective tissue (green arrow), signs of desquamation of the regenerated squamous epithelium (red arrow), *Van Hyson* stain, magnification 100x

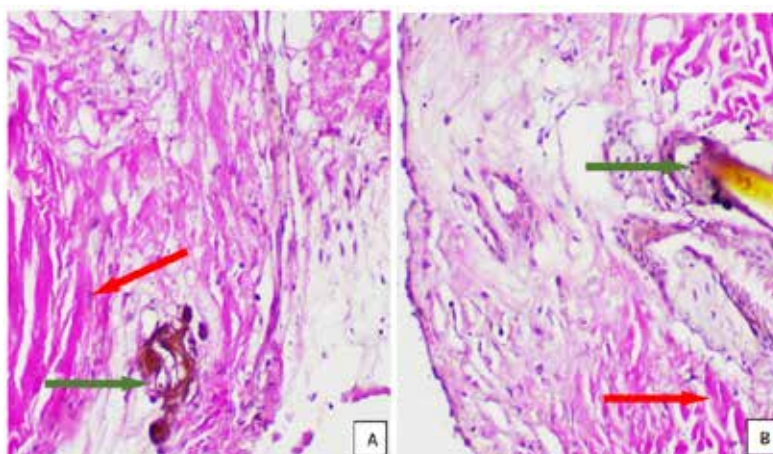


Fig. 10. Formation of collagen fibers (red arrow) in the scar of the injured limb tissue of rats on the 28th day of the experiment (A – the main group, B – the comparison group), foreign material and hair fragments in the scar (green arrow), *Van Hyson* stain, magnification 100x

alignment was observed. It should also be noted that in both groups, the formed connective tissue contained fragments of foreign bodies and damaged hair.

Upon detailed examination of the scar wall in animals from the main group, the formation of mature connective

tissue was observed beneath the epidermis, with a large number of organized collagen fibers and isolated cells (histiocytes, single macrophages). In the group without collagen treatment, the scar on the skin was also formed, but there were fewer collagen fibers, which predominantly

had a disordered alignment. A significant portion of vessels of varying calibers was also noted.

In the deep layers of the wounds in rats from both groups, a noticeable mixed-cell inflammatory infiltrate (lymphocytes, macrophages, histiocytes, plasma cells) was still present, without the presence of neutrophilic granulocytes. On both sides of the zones with inflammatory infiltrates, the formation of collagen fibers was fixed, being thinner in the center of the scar and thicker in the peripheral part (Fig. 11). Additionally, among the formed connective tissue, there were traces of foreign bodies and fragments of damaged hair.

It is evident that there was more expressed collagen formation in the animals of the main group, with more mature connective tissue observed there. In contrast, in the comparison group of animals, a developing granulation tissue was observed with slower maturation, and there was significantly more residual inflammatory infiltrate.

The study of the vascular bed was also conducted both qualitatively – using an immunohistochemical (IHC) reaction with monoclonal antibody CD34, and

quantitatively – through morphometric analysis, counting the density of capillaries and the average diameter of newly formed vessels.

It was found that in the area of the formed scar in the wound site, there were vessels of varying calibers, with a significantly smaller proportion of capillaries. They were distributed evenly in the adipose tissue and were almost absent in the areas of collagen fiber deposition in both animal groups (Fig. 12).

Conclusions

1. It was shown that the wound healing process in animals from both groups was characterized by expressed inflammation, with a predominance of neutrophilic granulocytes in the initial phase of healing. This was followed by the formation of granulation tissue and subsequent development of scar tissue, with the formation of collagen fibers and closure of most of the newly formed blood vessels.

2. The use of collagen hydrolysate led to a faster maturation of connective tissue, with the formation of a more organized collagen framework, significantly more

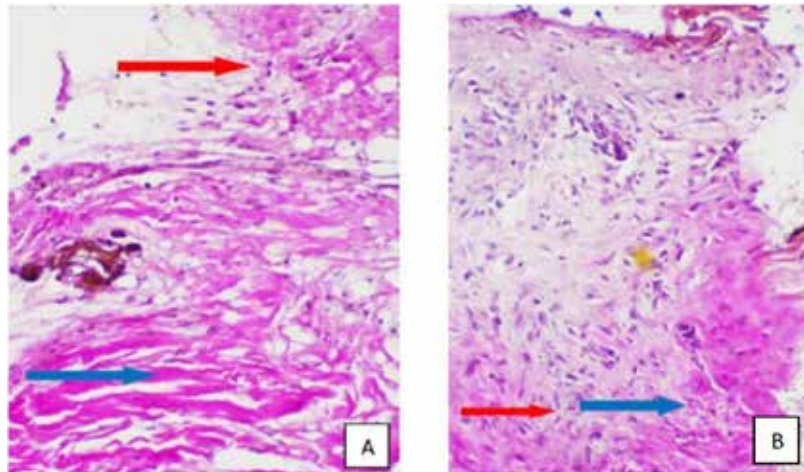


Fig. 11. Deep layer of the wound in rats on the 28th day after the injury (A – the main group, B – the comparison group): focus of inflammation surrounded by collagen fibers (blue arrow); mixed-cell inflammatory infiltrate (red arrow), Van Hysen stain, magnification 100x

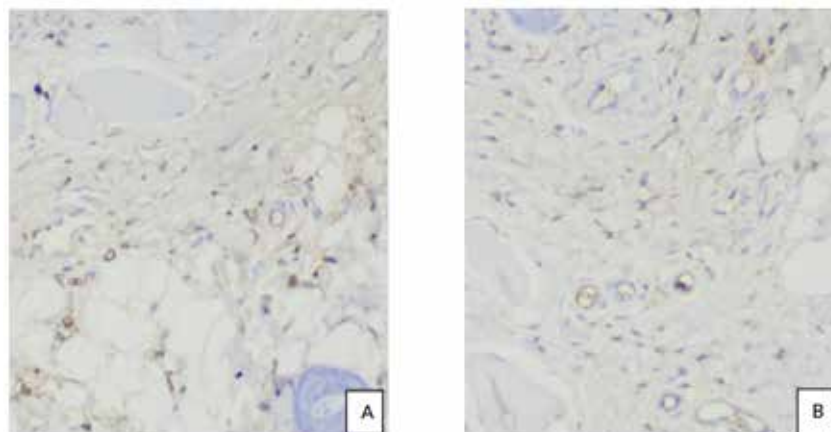


Fig. 12. Vascular bed in the scar tissue of experimental animals from the main group (A) and the comparison group (B), 28th day of the experiment. IHC reaction with CD34, Van Hysen stain, magnification 100x

Table 3

The rat's wound's wall vascular bed features on the 28th day after injury

Group	The average diameter of the newly formed vessels, μm	The capillary density, %
The Main group (n = 13)	12.0 $\pm$ 0.6	5.2 $\pm$ 0.6*
The Comparison group (n = 17)	11.0 $\pm$ 0.5	8.1 $\pm$ 0.7*

Note: * – Differences are statistically significant ($p < 0.001$).

expressed formation of the vascular microcirculatory network, and, as a result, more efficient wound cleaning. There was also earlier closure of newly formed vessels through the formation of mature connective tissue and the development of a high-quality and strong scar.

3. The highlighted pathohistological and morphometric changes indicate the effectiveness of using collagen hydrolysate improving post-traumatic regeneration processes in the experimental model of a wound-induced process.

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Електронна адреса для листування gemostatik@gmail.com