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Morphometric changes of hair follicles in rat excisional wounds treated by *Hirudo verbana* extract

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Ethical approval:
Prior to the commencement of this study, the Bioethics Committee of Zaporizhzhia National University reviewed and approved the study protocols as well as procedures related to animal housing and their use in the experiments (protocol no. 1 from 30 October 2025).

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The study investigated the regenerative potential of a water-salt extract of the medicinal leech *Hirudo verbana* in excisional skin wounds of rats, focusing on its influence on hair follicle restoration. Sixty male rats were divided into control and experimental groups, the latter receiving topical applications of the extract during wound healing. Histological and morphometric analyses were performed on days 3, 7, and 30 after injury. Treated animals showed earlier transition to the proliferative phase, improved skin structure, and increased density and diameter of hair follicles compared with the control group. By day 30, the number of follicles exceeded that of intact skin, indicating activation of follicular neogenesis and maturation. The therapeutic effect is associated with the bioactive compounds of the leech extract, which exhibit anti-inflammatory, anticoagulant, and regenerative activities, improving local circulation and tissue oxygenation. These findings demonstrate that *Hirudo verbana* extract effectively promotes hair follicle regeneration and accelerates the overall healing of excisional wounds. Due to its natural origin, safety, and multifunctional biological properties, it may serve as a promising biotechnological agent for skin repair and wound management.

Key words: *Hirudo verbana*, water-salt extract, excisional wound, hair follicle, wound healing, leech therapy, tissue regeneration

Introduction

Excisional wound healing involves complex tissue remodeling processes to restore skin integrity, encompassing distinct but overlapping phases of inflammation, proliferation, and tissue remodeling. Among the regenerative elements, hair follicles (HFs) serve as a vital reservoir of epithelial progenitor cells contributing to wound re-epithelialization and neogenesis [15]. The stages of hair follicle cycling — including anagen (growth), catagen (regression), and telogen (rest) — play critical roles in modulating the speed and quality of wound healing. Recent research has underscored that active hair follicle cycling, especially during the anagen stage, significantly accelerates reparative healing of cutaneous defects, likely through enhanced cellular proliferation and growth factor secretion [5].

Traditional and modern medicine have long sought natural compounds that could potentiate wound healing, highlighting the pharmacological promise of medicinal leeches (*Hirudo medicinalis*). The water-salt extract derived from medicinal leeches is rich in biologically active substances such as hirudin, calin, and various growth-promoting peptides, which collectively contribute to enhanced tissue regeneration. Experimental models have demonstrated that this extract positively influences multiple facets of wound healing — accelerating inflammation resolution, promoting angiogenesis, modulating immune responses, and markedly increasing hair follicle neogenesis in excisional wounds [2, 18].

The aim of this study was to evaluate the therapeutic efficacy of a water-salt extract of the medicinal leech *Hirudo verbana* on the morphological properties of hair follicles during the healing process.

Materials and Methods

Experimental animals and housing conditions

The study was conducted on 60 adult male white laboratory rats weighing 245–260 g. Animals were maintained under standard vivarium conditions (temperature 20–25 °C, relative humidity ≤55 %, natural light/dark cycle) and provided with balanced pelleted feed and water *ad libitum*. Following wound induction, each animal was housed individually in aseptic cages with weekly bedding changes. No signs of bacterial contamination were observed in any group throughout the experiment.

All experimental manipulations complied with the International Guidelines for Biomedical Research Involving Animals, the national Joint Ethical Principles for Animal Experiments (Ukraine, 2001), and Directive 2010/63/EU of the European Parliament and of the Council (22 September 2010). The protocol was approved by the Bioethics Commission of the Faculty of Biology, Zaporizhzhia National University (protocol no. 1 from 30 October 2025).

Medicinal leeches (*Hirudo verbana*) were maintained using the modern jar method at the Educational and Research Laboratory of Cellular and Organismal Biotechnology, Zaporizhzhia National University, in accordance with TU U 05.0-02125243-002:2009 “Medicinal Leech” and the sanitary-epidemiological approval of the Ministry of Health of Ukraine (no. 05.03.02-06/49982, dated 12.08.2009).

Wound model and experimental design

Full-thickness circular skin wounds (1.5 cm in diameter) were created in the interscapular region under ketamine anesthesia (40 mg/kg body weight) after shaving and aseptic preparation of the skin surface [7, 14]. Rats were randomly assigned to control and experimental groups of 30 animals each. In the control group, wounds healed spontaneously without treatment. In the experimental group, compresses soaked in a water-salt extract of *Hirudo verbana* were applied to the wound area on days 1, 2, 3, 7, 10, and 14.

Preparation of the water-salt extract

The water-salt extract was prepared by the method of Aminov [3]. Thirty-five medicinal leeches (0.8–1.1 g each) were blotted dry on sterile filter paper, fragmented, and homogenized in physiological saline under sterile conditions. The homogenate was filtered, washed, re-dried, and re-ground. The material was resuspended in saline (1:10 w/v), extracted at 4 °C, centrifuged, and filtered through Millipore bacteriological filters. Protein concentration in the supernatant was determined colorimetrically using pyrogallol red-molybdate reagent on a *Beckman Coulter AU480* biochemical analyzer. The final protein concentration of the extract was 0.022 mg/mL [20].

Tissue collection and histological analysis

Samples from the wound bed and adjacent intact skin (within 1 cm of the wound edge) were collected immediately after injury and on days 3, 7, and 30. Tissues were fixed in 10 % neutral buffered formalin for 72 h, embedded in paraffin, sectioned at 5 μm thickness (*Thermo Scientific HM 325*, USA), and stained with hematoxylin and eosin. Micrographs were obtained using a *ZEISS PrimoStar* iLED microscope equipped with an *AxioCam ERc5s* camera. Images were analyzed using *Quantitative Pathology and Bioimage Analysis (Qupath)* software (v 0.4.4, Edinburgh, UK) to calculate the amount of hair follicles and their diameters in periwound.

Statistical analysis

Comparisons were performed between the intact group and each treatment group at corresponding time points, and between control and experimental groups on each day of healing. Data distribution normality was verified using the one-sample Kolmogorov-Smirnov test. Parametric analyses were conducted using Student’s *t*-test with Bonferroni correction in *IBM SPSS Statistics 26.0* (USA). Results are presented as mean ± standard deviation (mean±SD). Differences were considered statistically significant at $P<0.05$.

Results and Discussion

The water-salt extract of *Hirudo verbana* represents a complex mixture of biologically active proteins with considerable therapeutic potential. Its principal constituents, including hirudin, bdellins, eglins, and destabilase, act synergistically to regulate inflammation, coagulation, and tissue repair [2, 3, 10]. Hirudin, a potent natural thrombin inhibitor, not only prevents clot formation but also exhibits pronounced anti-inflammatory effects by suppressing the release of tumor necrosis factor-α (TNF-α) and modulating inflammatory signaling pathways, thereby promoting the resolution of inflammation and improving local tissue viability [9, 19, 24]. Bdellins and eglins, as serine protease inhibitors, attenuate inflammation by inhibiting neutrophil elastase and other proteases, limiting tissue degradation and oxidative damage during the early wound phase [17, 22]. Destabilase possesses thrombolytic, muramidase, and isopeptidase activities that facilitate the dissolution of fibrin clots, improve microcirculation, and support tissue remodeling by enhancing oxygen and nutrient delivery to regenerating tissues [16].

The use of a physiological (water-salt) extract ensures sterility and biocompatibility while preserving the structure and bioactivity of native proteins. Following the method of Aminov [3], the extract was prepared by controlled homogenization, cold extraction, centrifugation,

and sterile filtration, resulting in a standardized protein concentration of 0.022 mg/mL [1, 4, 20]. This procedure maintains protein functionality and ensures the extract's safety and efficacy, enabling modulation of inflammatory, angiogenic, and regenerative processes critical for wound healing. Consequently, the accelerated hair follicle regeneration observed is attributed to improved nourishment and oxygenation of the follicular environment, mediated through a combination of anticoagulant, anti-inflammatory, protease-inhibitory, and thrombolytic mechanisms by increased local blood circulation stimulated by these bioactive compounds [4, 20].

Histological examination of skin samples revealed that the sequence of tissue changes corresponded to the classical stages of wound healing: inflammatory, proliferative, and remodeling. On day 3, during the inflammatory phase, both control and treated groups exhibited a reduced density of hair follicles, reflecting the initial tissue damage and inflammatory response. However, animals treated with the leech extract demonstrated earlier granulation tissue formation and less necrosis, suggesting faster resolution of inflammation and initiation of repair. By day 7, corresponding to the proliferative phase, the experimental group displayed a marked increase in hair follicle number and diameter relative to the control, indicating stimulated epithelial regeneration, enhanced keratinocyte proliferation, and active extracellular matrix remodeling. On day 30, during the remodeling phase, the treated rats exhibited nearly complete restoration of skin structure and even higher follicle density than in intact skin, indicating accelerated dermal regeneration and signs of induction of follicular neogenesis.

Quantitative morphometric analysis confirmed these histological findings. In intact skin (before wounding) about 60–70 % of hair follicles were in anagen, around 20–30 % in telogen, and less than 10 % in catagen (fig.). In the control group, hair follicle density decreased significantly on days 3 and 7 after injury (28.42±1.97 and 32.81±2.71 mm², respectively) (table). On day 3 most follicles in peri-wound were in telogen or early catagen, reflecting a pause in hair growth. By day 7 (proliferative phase), many follicles re-entered anagen. In contrast, the leech extract-treated group showed significantly higher values at the same time points (43.89±3.54 and 48.40±3.73 mm²) and a marked increase by day 30 (54.28±4.21 mm²), exceeding both intact skin (43.28±2.77 mm²) and control values (40.29±2.69 mm²; $P<0.001$). At day 3 all types of hair follicles were noted in approximately same amounts. The presence of numerous anagen follicles supports active proliferation and hair shaft formation, while catagen and telogen follicles indicate phases of follicular remodeling and rest. At day 7 we noticed predominance of catagen and early telogen stages in hair follicles which reflect follicular neogenesis or maturation of new follicles. These data demonstrate enhanced follicular regeneration and suggest activation of resident hair follicle stem cells. Hair follicle diameter, which transiently decreased after wounding, increased significantly in the treated group during the proliferative and remodeling stages (22.19±2.12 μm on day 7 and 23.16±2.78 μm on day 30) compared with the control (21.61±1.74 μm and 19.34±1.75 μm, respectively; $P<0.05$–0.001). This enlargement indicates restoration of follicular maturity and improved structural integrity of the skin under the influence of leech-derived bioactive proteins. In experimental group numerous hair follicles were predominantly in a resting state. This dominance of the telogen phase could reflect later stages in wound healing or normal skin homeostasis, where follicles are resting before regeneration reinitiates.

The mechanistic basis of the therapeutic effect observed in *H. verbana* extract-treated animals likely involves several interconnected molecular pathways. Anti-inflammatory peptides such as hirudin and antistasin-like molecules attenuate oxidative stress and suppress the production of pro-inflammatory cytokines, including TNF-α and IL-1β, thereby protecting tissues from secondary inflammatory damage [10, 13]. Hirudin has also been shown to inhibit inflammasome activation and promote mitophagy, reducing oxidative injury and facilitating tissue recovery [9, 12]. Enzymes such as destabilase contribute to fibrin degradation, improving blood flow and nutrient supply to the regenerating dermis [11]. In addition, certain leech-derived peptides exhibit growth factor — like activity, stimulating keratinocyte proliferation and extracellular matrix synthesis [18, 23]. Proteins such as RNASET2 may participate in collagen deposition and reorganization, ensuring proper structural remodeling [6, 21]. These combined actions result in a microenvironment conducive to tissue repair, angiogenesis, and epithelial regeneration.

The results obtained in this study are consistent with previous reports describing the regenerative properties of leech-derived substances. Aminov et al. demonstrated that extracts of *H. verbana* promote granulation tissue formation, vascularization, and epithelial proliferation, findings that correspond closely with the present histological and morphometric outcomes [4]. A novel contribution of this work is the detailed quantitative evaluation of hair follicle density and diameter across multiple healing phases, providing direct evidence for leech extract-induced follicular neogenesis and maturation.

The present results highlight the potential of *Hirudo verbana* extract as a natural biotechnological product for wound-healing applications. Its multifactorial biological activity — which includes anti-inflammatory, anticoagulant, thrombolytic, and tissue-regenerative components — provides a broader spectrum of action compared to many conventional pharmacological agents that are designed to affect specific molecular targets. Moreover, its natural origin, high biocompatibility, and low likelihood of adverse reactions make it an attractive alternative or adjunct in clinical wound management. Further investigations should focus on isolating and characterizing the specific active fractions responsible for these therapeutic effects, determining optimal dosing regimens, and conducting comprehensive preclinical and clinical studies to evaluate efficacy and safety in human subjects.

Table. Morphometric parameters of hair follicles during excisional wound healing in control and after treatment with medicinal leech extract (mean±SD)

Indicator	Intact (day of wounding) (n=60)	Control group (n=30)			Experimental group (n=30)		
		Day 3 (n=10)	Day 7 (n=10)	Day 30 (n=10)	Day 3 (n=10)	Day 7 (n=10)	Day 30 (n=10)
Number of hair follicles, in 1 mm²	43,28±2,77	28,42±1,97 ***	32,81±2,71 *	40,29±2,69 ***	43,89±3,54 ###	48,40±3,73 ** ###	54,28±4,21 *** ###
Diameter of the hair follicle, μm	19,31±1,43	18,44±1,81 *	21,61±1,74 ***	19,34±1,75 **	22,19±2,12 *** ###	23,16±2,78 #	22,89±1,97 ###

Note. * — differences compared to previous time point after wounding within one group (control and experiment) are statistically significant (P≤0.05).
** — differences compared to previous time point after wounding within one group (control and experiment) are statistically significant (P≤0.01).
*** — differences at different times after surgery within one group (control and experiment) are statistically significant (P≤0.001).
# — differences between control and experiment at one time are statistically significant (P≤0.05).
— differences between control and experiment at one time are statistically significant (P≤0.001).

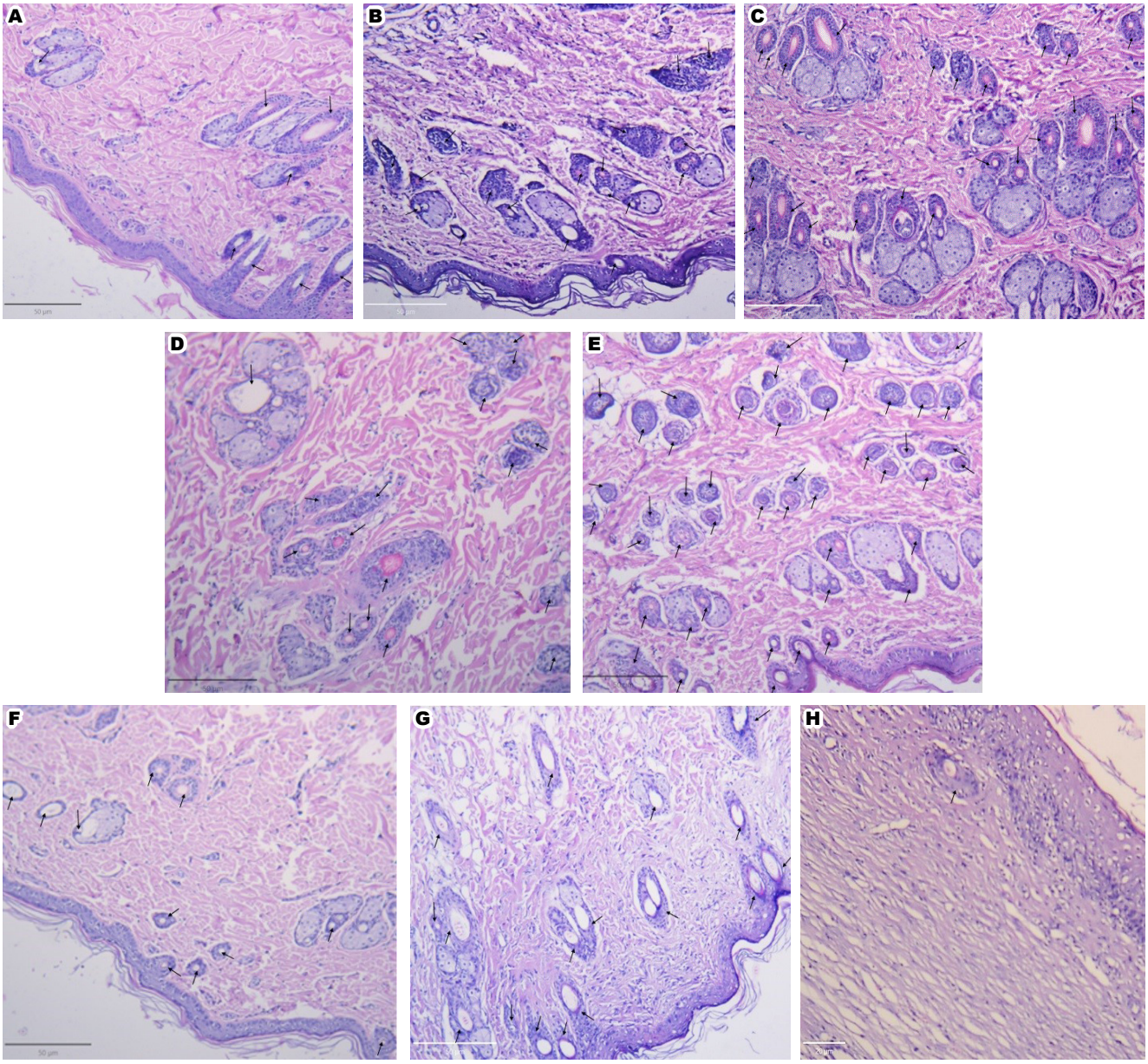

Fig. Microphotographs of a skin flap excised in periwound in control and experimental groups of rats at different periods of healing:
A — Day of wounding, intact; B — Day 3 of wound healing, control group; C — Day 3 of wound healing, experimental group;
D — Day 7 of wound healing, control group; E — Day 7 of wound healing, experimental group;
F — Day 30 of wound healing, control group; G — Day 30 of wound healing, experimental group;
H — Day 30 of wound healing, experimental group. Follicle neogenesis

The water-salt extract of *Hirudo verbana* significantly enhances wound healing by accelerating inflammation resolution, promoting follicular regeneration, and improving skin restoration. Quantitative morphometric data demonstrate increased hair follicle density and diameter, indicating follicle neogenesis and maturation beyond natural recovery. These effects are attributed to the extract's bioactive compounds with anti-inflammatory, angiogenic, and regenerative properties. Given its natural origin and biocompatibility, this extract holds promise as a novel therapeutic agent for wound care. Further molecular research and clinical studies are essential to isolate active components and confirm safety and efficacy in humans.

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Прискорення регенерації волосяних фолікулів під впливом екстракту *Hirudo verbana* у моделі різаної рани у щурів

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У дослідженні вивчено регенераційний потенціал водно-сольового екстракту медичної п'явки *Hirudo verbana* при загоєнні різаних ран шкіри щурів, з особливим акцентом на відновлення волосяних фолікулів. Шістдесят самців щурів поділили на контрольну та дослідну групи; тваринам дослідної групи місцево наносили екстракт під час загоєння ран. Гістологічний та морфометричний аналізи проводили на 3, 7 та 30 добу після нанесення рани. У тварин, що отримували екстракт, спостерігали швидший перехід до проліферативної фази, покращення структури шкіри, а також збільшення кількості й діаметра волосяних фолікулів порівняно з контролем. На 30-ту добу їхня кількість перевищувала показники інтактної шкіри, що свідчить про активацію фолікулярного неогенезу та дозрівання. Терапевтична дія зумовлена біологічно активними сполуками екстракту, які мають протизапальні, антикоагулянтні та регенераційні властивості, покращуючи місцевий кровообіг і оксигенацію тканин. Отримані результати підтверджують, що екстракт *Hirudo verbana* ефективно стимулює регенерацію волосяних фолікулів і прискорює загоєння різаних ран. Завдяки природному походженню, безпечності та комплексній біологічній дії він може бути перспективним біотехнологічним засобом для відновлення шкіри та лікування ран.

Ключові слова: *Hirudo verbana*; водно-сольовий екстракт; різана рана; волосяні фолікули; загоєння ран шкіри; гірудотерапія; регенерація тканин

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