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The effect of water-salt extract of the medicinal leech *Hirudo verbana* on the regenerative properties of excisional wounds of rat skin

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The process of skin wound healing begins immediately after injury. Wounds remain one of the most pressing scientific and practical problems of modern veterinary medicine. Treatment of skin wounds in our time requires the use of therapy that promotes wound cleansing and facilitates their healing. In recent years, scientists have focused on the study of natural biologically active substances capable of accelerating wound healing and affecting other diseases. Among them a special place is occupied by medicinal leeches, which have a wide range of therapeutic effects due to the presence of over 100 biologically active substances in their body. An important factor in accelerating the healing of skin wounds is perfusion, such an ability is possessed by leech saliva. Given their significant effects, the study of the effect of water-salt extract of *Hirudo verbana* on the regenerative properties of excisional wounds has become relevant. The results of the study indicate that the water-salt extract for topical application accelerates wound cleansing and reparative processes. Moreover, on the 14th day, the wound in animals of the experimental group almost completely healed. On the 30th day, the wounds in the experimental group of rats completely healed, epithelialized and began to actively be covered with hair.

Key words: excisional wounds, skin, medicinal leeches, *Hirudo verbana*, biologically active substances, rats

Introduction

The skin is the largest organ of the organism, playing the role of a barrier that protects the body from adverse environmental factors and pathogenic microorganisms, and is of fundamental importance in maintaining homeostasis [12, 14, 16, 20, 22]. The process of skin wound healing begins immediately after injury. Wounds remain one of the most pressing scientific and practical problems of modern veterinary medicine and medicine [1, 8, 11, 21, 25, 28]. Skin wound healing is accompanied by a phased change in the structure of its structural elements — the epidermis, dermis, and subcutaneous tissue, which depends on the reactivity of tissues and cells in their composition [1, 8, 12, 16]. Treatment of skin wounds in today's world requires the use of therapy that promotes wound cleansing and facilitates their healing. In recent years, scientists have focused on the study of natural biologically active substances (BAS) that can accelerate wound healing and affect other diseases [13, 17, 23]. Among them, medicinal leeches (ML) occupy a special place because of a wide range of therapeutic effects due to the presence of more than 100 BAS in their body [3–6, 9, 18, 24, 26]. An important factor in accelerating the skin wounds healing is perfusion, and the saliva of ML has such ability. ML secrete various anticoagulants like hirudin and factor Xa inhibitors into the wound, preventing scab formation and thereby accelerating the healing process.

Our previous studies and the results by other researchers prove the positive effect of ML *Hirudo verbana* on a rat model on the reparative regeneration of internal organs: thymus, spleen, kidneys, and liver [5, 7, 9, 18, 24].

There are some studies on the effectiveness of ML of two other species, *Hirudo medicinalis* and *Hirudo orientalis*, in healing of incised wounds under primary tension, wound reduction by more than 50 % on day 14 [2, 27]. This accelerated healing may be linked to the presence of peptidases in the ML, which influence the functional activity of various cells, such as endothelial cells, lymphocytes, platelets, and macrophages, thereby promoting granulation tissue formation. Additionally, antihemostatic substances like calins, apyrase, platelet-activating factor antagonists, and hirudin play a role in regulating blood clotting mechanisms. The presence of eglin C also reduces free oxygen radical levels in neutrophils, preventing inflammation and tissue destruction. They have also found use in the treatment of chronic non-healing wounds, such as the diabetic foot ulcers, bedsores, and venous leg ulcers, which was experimentally confirmed in a mouse model, where in animals after the use of leeches, the necrosis decreased and the survival rate of heart valves increased [27]. These effects are likely due to the release of vasodilators, such as histamine-like substances, acetylcholine, and carboxypeptidase-A inhibitors, which increase blood flow to the site of the bite and reduce local swelling. Acetylcholine, in particular, can relax endothelial muscles, dilate blood vessels, and promote microcirculation, delivering fresh oxygenated blood to the affected area. This restores normal blood flow and provides tissues and hair follicles around the wound with the oxygen and nutrients required for regeneration. Also, studies by other researchers indicate the hirudotherapy effectiveness in regeneration of organs in normal conditions and those damaged by traumatic injuries. For example, in the case of diabetic ulcer, in the early stages of healing a human ear wound the use of ML shows increased vascularization and reduced hyperemia of the wound due to the necrotic areas disappearance and rapid wound healing [27]. Given the significant effects of ML, the study of the effect of the *Hirudo verbana* water-salt extract on the regenerative properties of an incised wound has become relevant.

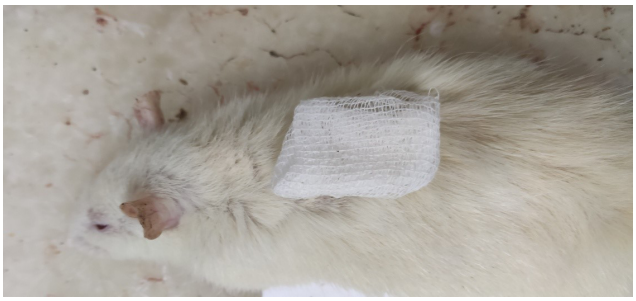

Fig. 1. Compress application moistened with water-salt extract of medical leech

Materials and Methods

The study was conducted on 60 white male laboratory rats weighing 245–260 g. Experimental animals were held in standard sanitary and hygienic conditions. After the experimental cut wounds had been inflicted, the animals were housed in separate cages with bedding changed weekly under aseptic conditions. No signs of contaminant bacterial infection were revealed during the observation period in either group of animals. Throughout the study, the rats were held in a vivarium with temperature 20–25°C, humidity not exceeding 55 %, with a natural light “day-night” cycle, in the individual plastic cages, and received a balanced diet [19].

All the experimental procedures were in accordance with the “International Recommendations for Medical and Biological Research Using Animals” and the national “Joint Ethical Principles of Animal Experiments” (Ukraine, 2001), the Council Regulation 2010/63/EU of the European Parliament and of the Council of September 22, 2010 “On the protection of animals used for scientific purposes”.

The wound model was created as follows: under ketamine anesthesia (40 mg/kg b.w.), after hair removal on the back of the animals, following aseptic and antiseptic procedures. Before the experiment, the animals were randomly divided into 2 groups of 30 animals each. The circular skin areas of 1,5 cm (706,5 mm^2) were excised with surgical scissors using a template [10, 19]. In the control group the wound healing occurred spontaneously without treatment; in the experimental group an aqueous saline extract of medicinal leech was applied to the lesion site in the form of a lotion on days 1, 2, 3, 7, 10, and 14. The concentration of the aqueous saline extract was 0.022 mg/ml (fig. 1).

Treatment began 24 hours after wound creation. The rate of epithelialization and wound edge contraction was calculated based on the decrease in the wound area at specific time intervals.

On days 3, 7, 14, and 30, we measured the wound area in all animal groups, and calculated the healing rate using the formula:

$$V = 100 \times (So - St)/So,$$

where So is the initial wound area, mm^2;
St is the wound area on the day of measurement, mm^2.

The wound healing rate is a relative indicator and it allows characterizing the dynamics of the wound healing process, regardless of the difference in the initial wound area.

Statistical processing of the obtained data was carried out using parametric statistical methods (Student’s *t*-test), after preliminary verification of the samples for normal distribution. The data in the table are presented as mean±SD, using the *IBM SPSS Statistics 21.0* (USA) software packages. Differences were considered significant at a significance level of $P<0.05$.

Table. The dynamics of wound healing in rats (mean±SD)

Day of wound healing	Indicator					
	Control			Experiment		
	Absolute wound area, mm²	Relative wound area, %	V, mm²/day	Absolute wound area, mm²	Relative wound area, %	V, mm²/day
Before excision of the skin flap	176.62±7.66	100.00±6.44	—	176.62±7.66	100.00±6.44	—
Immediately after excision	183.76±8.49	101.96±6.33	—	153.86±9.22	93.33±7.11	—
Day 3	167.33±7.88	97.76±5.97	8.94±1.44	116.84±6.88*	81.33±8.86*	24.06±3.23*
Day 7	136.78±7.12	88.30±8.09	25.56±2.66	78.50±7.11*	66.66±6.79*	48.98±5.89*
Day 14	30.17±2.93	41.65±3.94	83.58±8.77	10.17±1.02*	24.46±2.12*	93.39±8.78
Day 30	6.15±0.99	18.33±2.04	96.65±8.75	1.13±0.21*	8.00±0.78*	99.26±9.11

Note. * — P<0.05 compared to the control group.

Results of the Study

After the wound was inflicted, it was observed that the condition of the wound in all groups was almost identical (fig. 2A): tissues of pink-red color [10, 19].

On the 3rd day of observation, the control group was dominated by a post-traumatic inflammatory process, the edges of the wound were roller-shaped, swollen, the wound was covered with thick brown crusts, the bottom was hyperemic, the wound took on a purulent character, with a large amount of purulent content (fig. 2B), which corresponds to the physiological norm and corresponds to the studies conducted by other scientists [10, 11, 15, 16, 19, 28]. In the experimental group, the wounds decreased somewhat in size, actively began to cleanse and become covered with a scab (fig. 2C). This observed effect may be attributed to the presence of bdelins, ellins, and the multifunctional protein destabilase-lysozyme, which not only has destabilase activity but also exhibits lysozyme and antimicrobial properties. As an antibiotic, destabilase inhibits the growth of many bacteria, fungi, and archaea. Moreover, substances such as chloromycetin, theromycin, thermisin, and destabilase in leech saliva demonstrate strong antimicrobial activity by destroying bacterial cell components.

In subsequent observation periods, the significant changes in the wound condition and the healing course were recorded (table). On the 7th day of observation, the macroscopic picture changed even more significantly (fig. 2D, 2I). As can be seen from fig. 2D, the wound in the control group animal practically did not decrease in size, is of a purulent-necrotic nature. In the experimental groups of animals, the wound was completely cleansed (fig. 2I), has small areas, is covered with a scab and actively epithelizes, the wound edges fit tightly to its bottom, on which granulation tissues begin to develop. This accelerated healing may be linked to the presence of peptidases in the ML, which influence the functional activity of various cells, thereby promoting granulation tissue formation.

Further wound healing in all groups was characterized by the development of granulation tissue, covered with epithelium from the edges. The dominance of these processes was significantly stronger in rats of the experimental group, which received an aqueous-saline extract in the form of a compress. On the 14th day of observation, the wound in the control group animals did not heal completely (fig. 2F, 2G). A defect remained, covered with a scab. In the experimental group, the wound almost healed, a small part of it remained in the process of epithelization. Interestingly, in the same animals, the hairline began to actively recover (fig. 2G). The faster regrowth of new hair may be attributed to improved blood supply to hair follicles due to enhanced circulation in the wound area an effect of ML. These effects are likely due to the release of vasodilators, such as histamine-like substances, acetylcholine, and carboxypeptidase-A inhibitors, which increase blood flow to the site of the bite and reduce local swelling. This restores normal blood flow and provides tissues and hair follicles around the wound with the oxygen and nutrients required for regeneration.

On the 14th day, the further process of wound epithelization from the edges and, accordingly, a decrease in the wound area was observed. The pronounced positive changes in the animals of the experimental group receiving an aqueous-saline extract consisted in the formation of the small, clean, oval-stretched wound in the middle of the area of the primary injury [10]. On the 30th day, at the end of the observation, in the control group the wound almost completely healed, a small area covered with a dense scab was observed (fig. 2H). In the experimental group, no wound is visible, since the hairline has recovered, which covered the site of the previous injury (fig. 2I). A significant decrease in the wound size and the rapid restoration of the hairline at the final stages of healing indicated the quickness of regenerative processes. Similar results were obtained by other scientists when analyzing the effect of *Hirudo medicinalis* and *H. orientalis* on the healing of wounds by primary tension [2, 27].

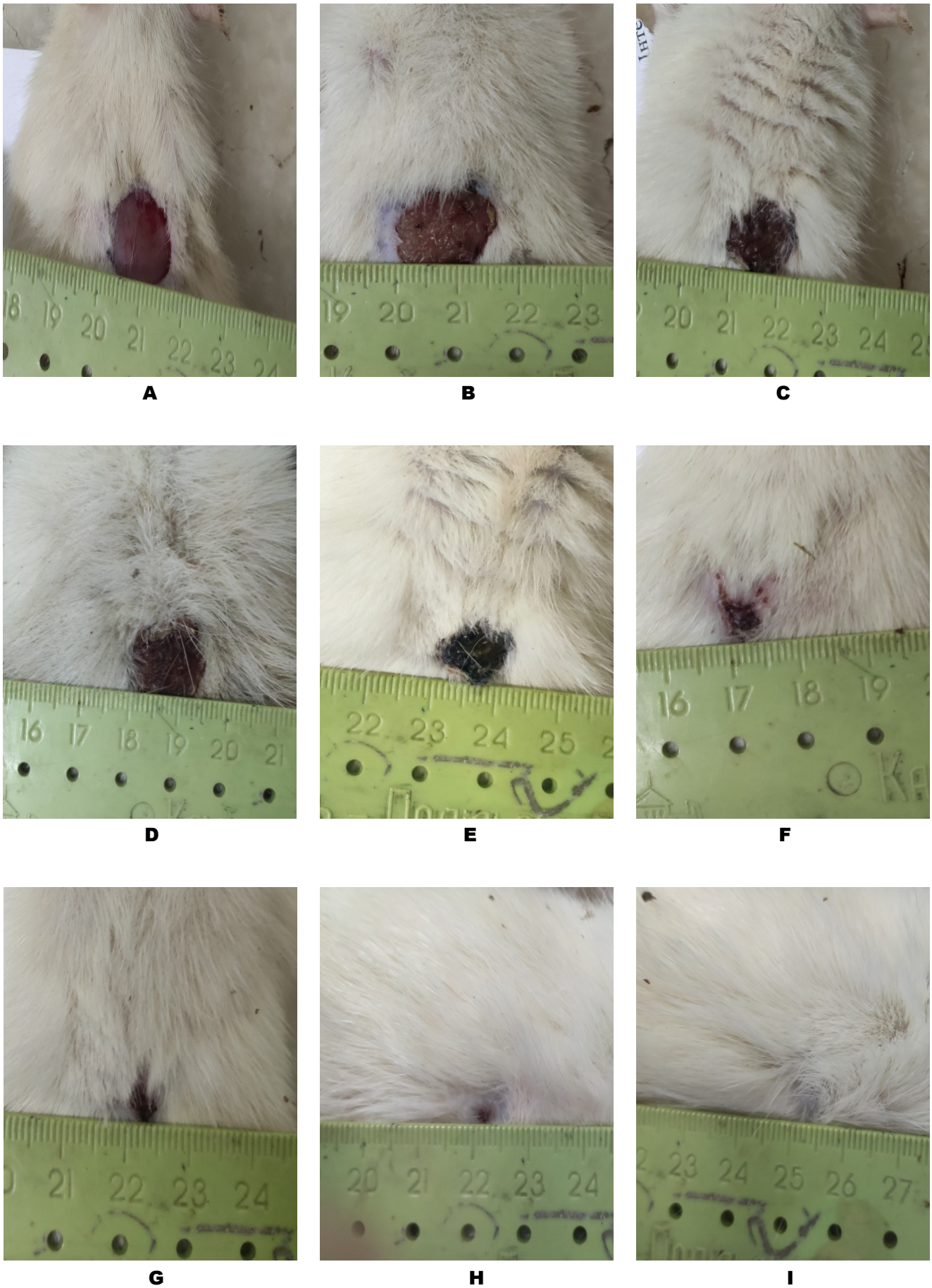

Fig. 2. Wound appearance during healing at different times. A — intact, B — day 3 control, C — day 3 experiment, D — day 7 control, E — day 7 experiment, F — day 14 control, G — day 14 experiment, H — day 30 control, I — day 30 experiment

Thus, according to visual assessments, we can conclude that the water-salt extract with local use accelerates the cleansing of wounds and reparative processes compared with the control. Moreover, on the 14th day, the wound in the animals of the experimental group almost completely healed. On the 30th day, the wounds in the rats of the experimental group completely healed, epithelialized and actively began to be covered with a hairline.

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Вплив водно-сольового екстракту медичної п'явки *Hirudo verbana* на регенеративні властивості різаної рани шкіри щурів

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Процес загоєння шкірної рани починається відразу після травми. Рани залишаються однією з найактуальніших наукових і практичних проблем сучасної ветеринарії та медицини. Лікування ран шкіри в наш час вимагає застосування терапії, яка сприяє очищенню ран і полегшує їх загоєння. Останніми роками дослідники зосередилися на вивченні природних біологічно активних речовин, здатних прискорювати загоєння ран і впливати на інші захворювання. Серед них особливе місце посідають медичні п'явки, які мають широкий спектр лікувальної дії завдяки наявності в їхньому організмі понад ста біологічно активних речовин. Важливим фактором прискорення загоєння шкірних ран є перфузія, і такою здатністю володіє слина п'явки. Враховуючи суттєвий її ефект, актуальним стало вивчення впливу водно-сольового екстракту *Hirudo verbana* на регенеративні властивості різаної рани. Результати дослідження показують, що водно-сольовий екстракт при місцевому застосуванні прискорює очищення ран і репаративні процеси. Причому на 14-ту добу рана у тварин дослідної групи майже повністю загоюється. На 30-ту добу рани в щурів дослідної групи цілком загоїлися, епітелізувалися та почали активно покриватися волосяним покривом.

Ключові слова: різані рани, шкіра, медичні п'явки, *Hirudo verbana*, біологічно активні речовини, щури

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