Basic Research Article

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# Efficiency of medical leech on experimentally induced incisional wound healing in rats

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**Abstract**

**Objectives:** This study was conducted in response to the increasing interest in understanding the effects of both modern and traditional complementary medicine on incisional wound healing. Herein, it was aimed to investigate the wound healing effects of medicinal leech therapy and leech saliva in an experimentally created incisional skin wound model.

**Methods:** Fifteen rats underwent full-thickness incisions on their dorsal regions and were randomly assigned to five equal groups, as the Leech Saliva (LS) group, where wounds were treated topically with leech saliva once daily; Leech Therapy-1 (LT-1) group, where leech therapy was administered once at the beginning of the experiment; Leech Therapy-2 (LT-2) group, where leech therapy was applied twice, on days 0 and 3; Positive Control (PC) group, where wounds were treated daily with Phyto cream containing *Triticum vulgare*; and Negative Control (NC) group, where no treatment was given.

**Results:** Wound healing was assessed daily, and the experiment continued until complete healing was observed. At the conclusion, the wound size, appearance, and histological features were analyzed to compare healing progress across the groups.

**Conclusions:** Medicinal leech therapy was observed to have a positive wound healing effect in the rat model.

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**Keywords:** medicinal leech therapy; incisional wound; *in vivo*

## Introduction

The skin, a multi-layered organ, serves as a protective barrier against pathogens, aids in preventing dehydration, and is crucial for thermoregulation [1]. Various external factors like burns, trauma, and infections can affect its integrity [2]. Wounds are classified into partial and full thickness, with the latter involving loss of deeper skin layers and fat, disrupting blood vessels and often resulting in scarring upon healing. Wound care encompasses diverse treatments such as debridement, offloading pressure wounds, hyperbaric oxygen therapy, whirlpool therapy, compression therapy, magnetic therapy antibiotics, ultrasound treatment, and electrical stimulation. Additionally, numerous wound-care products like hydrogels, alginates, and antimicrobials are available [3]. Despite stringent protocols, inappropriate wound healing processes may occasionally occur. Successful wound healing requires all phases to proceed in a proper sequence and timeframe [4].

The search for new therapeutic agents for wound healing has recently increased. There is increasing interest in the potential positive effects on both wound healing and wound care through the use of traditional complementary and alternative medicine methods such as plant extracts, larval therapy, and leech therapy [5, 6].

Leech therapy has been endorsed as a dependable and efficient treatment for various painful and inflammatory disorders [7, 8]. Leeches have been used since 200 BC, but their popularity rose significantly during the medieval era and continued as a widely practiced method of bloodletting into the 19th century [9].

Numerous studies have identified various bioactive molecules in leech saliva [8, 10–15], such as anti-inflammatory agents, anti-coagulants, anti-bacterials, vasodilators, anti-ischemic compounds, anesthetics and analgesics [13, 16–18]. Many proteins in leech saliva have unknown functions, but several well-known proteins, such as Eglin C, bdellins, hyaluronidase [19], acetylcholine, saratin [13, 19], hirudin [20], factor Xa inhibitor, chloromycetin [13, 15], platelet-activating factor antagonist [21], and etheromacin, are thought to possess wound healing properties [22]. These proteins are a combination of substances that are delivered from the salivary glands of the medicinal leech to the lesion site and have important biological and pharmacological effects that support the healing process locally.

An examination of the literature revealed that few investigations have been published in peer-reviewed journals on leech treatment in *in vivo* models [23–26]. However, no studies were found investigating the effects of leech saliva in incisional wound healing models and wound contraction. Hence, the current study aimed to investigate the wound healing effects of medicinal leech therapy, particularly focusing on leech saliva in an experimentally created incisional skin wound model.

# Methods

## Study design

A total of 15 rats were randomly and equally distributed into five experimental groups, comprising the Leech Saliva (LS) group, Leech Therapy-1 (LT-1) group, Leech Therapy-2 (LT-2) group, Positive Control (PC) group, and Negative Control (NC) group. The LS group received a topical application of leech saliva once daily throughout the experimental period, as detailed in the Leeches and Saliva Collection section. At the beginning of the experiment, one leech treatment was applied once for each rat in the LT-1 group. The LT-2 group underwent leech therapy at two intervals, on days 0 and 3. Scoring of the healing in the wound model after the first treatment application was performed on days 1, 3, and 7. The NC group did not receive any treatment or therapy. The PC group was treated with Phyto cream, containing the active ingredient *Triticum vulgare*, once daily throughout the experimental period.

# Animals

Fifteen rats (male *Wistar-Rattus norvegicus*), locally sourced and aged 9–11 weeks, were individually housed in hard-bottomed plastic cages, each with albino bedding. The selected rats weighed approximately 350 ± 10 g and were kept at a constant temperature, in an air-conditioned room, with a 12:12 h light/dark cycle. The rats were given standardized pelleted diet and tap water *ad libitum*. The water temperature was kept at 21 ± 0.5 °C and the relative humidity was between 25 and 30 %. Animal procedures and care were carried out in accordance with the guidelines of the Experimental Animal Ethics Committee of Kırşehir Ahi Evran University [27].

## Experimental wound model

Combined anesthesia consisting of 0.7 mL/kg of phosphate buffered saline (PBS), 15 mg/kg of xylazine, and 70 mg/kg of ketamine was prepared, and then the rats were anesthetized by intraperitoneal injection. After anesthesia, the hair on the back of the rats was shaved and disinfected with 70 % ethyl alcohol. Then, a linear incisional wound of 20 mm in length and 0.5 mm in depth was created 2 cm from the vertebral column.

## Leeches and saliva collection

Medicinal leeches were obtained from a local supplier in Kırşehir Province, Türkiye, and collected from freshwater. The leeches were placed in ventilated plastic containers filled with non-chlorinated tap water, which were kept in a separate room at room temperature, and the water in the containers was renewed every two days.

Before starting the experiment, the leeches were fasted for a while. A phagostimulatory solution consisting of 0.15 M of sodium chloride and 0.001 M of arginine was prepared, heated to 37 °C, and then fed to the medical leeches using a glass funnel wrapped with parafilm. After feeding, the leeches were allowed to cling to the parafilm-covered funnel until they detached spontaneously (Figure 1). The leeches were then removed from the funnel and transferred to a closed plastic test tube immersed in a container with ice, where they remained for 15–20 min, and became completely

**Figure 1:** Feeding leeches with a phagostimulatory solution through a paraffin membrane and the LS obtained as a result of regurgitation.

immobile. The consumed solution was released and expelled from their bodies, and any remaining solution was expelled by gently squeezing the leeches from the posterior to the front. They were then revived by immersing them in warm water (37 °C) for 15–30 min.

Only colorless liquids were collected and passed through a 0.45-μm membrane filter. The filtered liquids were centrifuged at 2,000 rpm for 15 min, and the resulting liquid was termed leech saliva (LS) and used fresh in the experimental procedures [16].

## Leech therapy (LT-1 and LT-2)

Leeches were placed near the wound, specifically 5–6 mm away from it, rather than directly on the wound itself. Leech therapy in humans typically lasts around 30 min. However, due to the smaller size of rats, the leeches were applied for just 5 min.

## The measurement of the wound area and healing process

The wound healing process was evaluated using the planimetric method [28]. The wound dimensions were measured with a ruler on days 0, 3, and 7 of the experiment and photographed with a digital camera. The wound areas were evaluated with a specialized image processing software designed for scientific analysis. The following formula was used to calculate the percentage of wound closure:

$$\text{Wound healing (\%)} = (A_0 - A_t)/A_0 \times 100$$

Here, $A_0$ is the initial wound area and $A_t$ is the wound area at the moment of imaging.

## Histopathological analysis

At the end of the seven-day experiment, the rats were euthanized via an overdose of combined xylazine, PBS, and ketamine. Wound samples were collected at the specified time points along with the surrounding normal skin. The tissues were fixed with 10 % neutral buffered formalin and embedded in paraffin. Then, 5–6 μm sections were taken, deparaffinized, and stained with Masson's trichrome and hematoxylin & eosin (H&E), according to standard histological staining methods. The sections were then examined under a light microscope [29, 30].

## Statistical analysis

The Kolmogorov–Smirnov and Shapiro–Wilk tests were used to evaluate normally distributed data, and the non-parametric Kruskal–Wallis test was used for non-normally distributed data. Data were expressed as the mean ± standard deviation (SD), median, and min–max. $p<0.05$ was accepted as statistically significant.

# Results

The pharmacological properties of the bioactive substances in the leeches were studied to assess the efficacy of medicine leech in wound healing, using a rat model. During the experiment, the rats were in good general health, did not lose weight and showed no signs of inflammation, allergic reactions, or bleeding.

## Wound healing

By day 7, the wounds in the NC group remained partially open, while those in the LS, LT1, LT2, and PC groups were completely closed. In addition, when the LS group was compared with the other groups, the growth rate of the hair around and above the wound was higher than the other groups (Figure 2).

## Wound healing rates in the groups

The mean dimensions of the wound areas in all the groups were taken [31], and the wound healing rates on days 3 and 7 were recorded, respectively (Figures 3 and 4). In the statistical comparisons made between the groups, the wound healing rate in the NC group was low, while the healing rates in the LS, LT-1, and LT-2 groups were high ($p<0.05$) (Table 1). The fastest healing was observed in the LS group.

## Histopathological results

On day 8, the tissues were fixed in a 10 % formaldehyde solution for 72 h and embedded in paraffin. Then, 5-μm-thick sections were taken, and stained with H&E (Figure 5) to assess wound healing and granulation tissue growth, and Masson's trichrome staining (Figure 6) to assess collagen accumulation. All the samples were analyzed at 100× and 200× magnification under a light microscope, and each

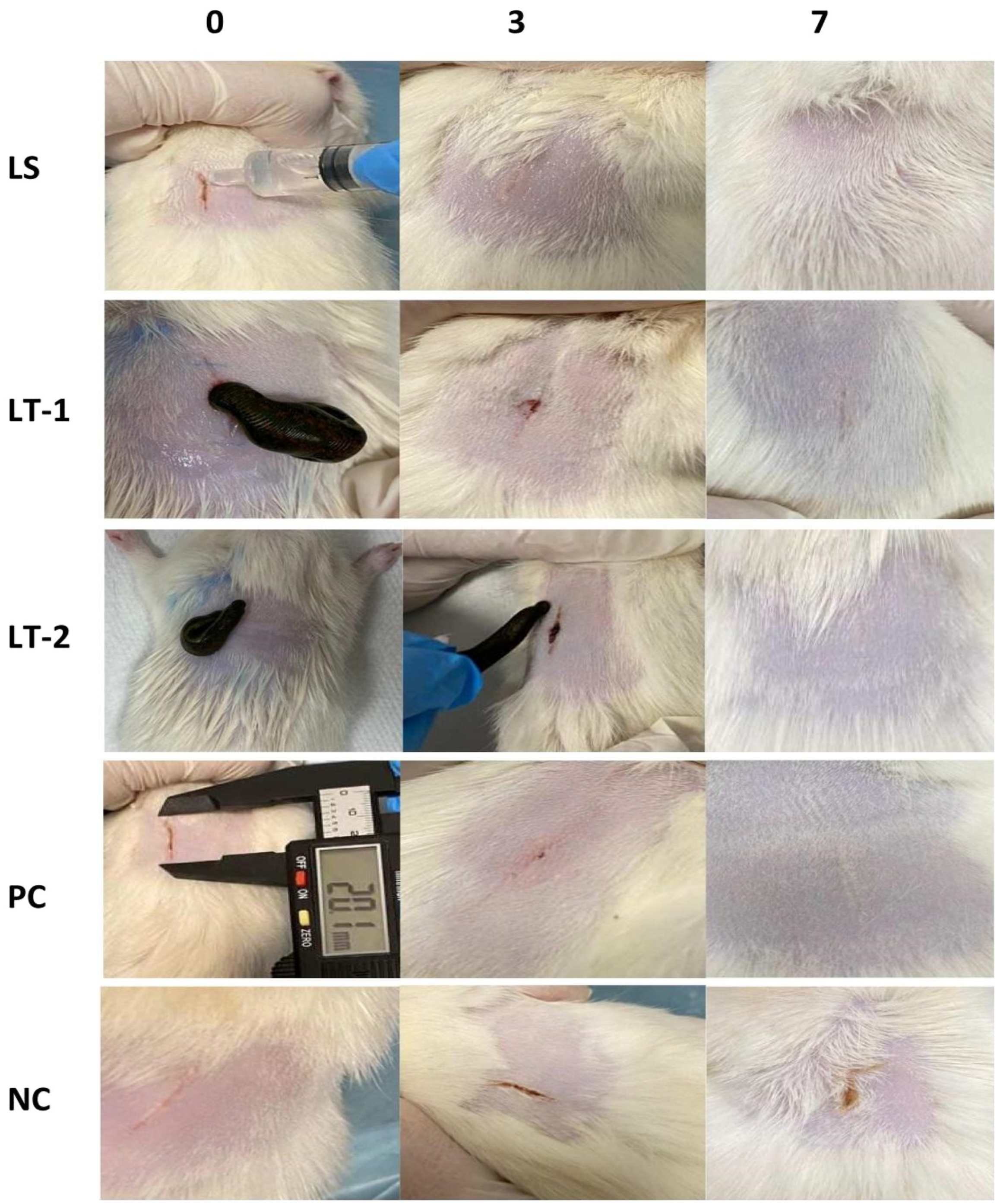


**Figure 2:** Images of the wounds treated in each group on the 3rd and 7th days. Leech Saliva Group (LS); Leech Therapy (LT-1) Group-1; Leech Therapy (LT-2) Group-2; Positive Control (PC) Group; Negative Control (NC) Group.

group was scanned in three image fields. Cellular infiltration was most prominent on the eighth day post-injury, with inflammation predominantly localized to the superficial dermis. Reticular cells and dermal structures were also observed within the hypodermal and muscular layers. Furthermore, in the granulation tissue where neutrophils predominated, numerous blood vessels were evident, along with signs of re-epithelialization. When the experimental groups were evaluated macroscopically, by day 7, the incision wounds in all the groups had healed and were covered

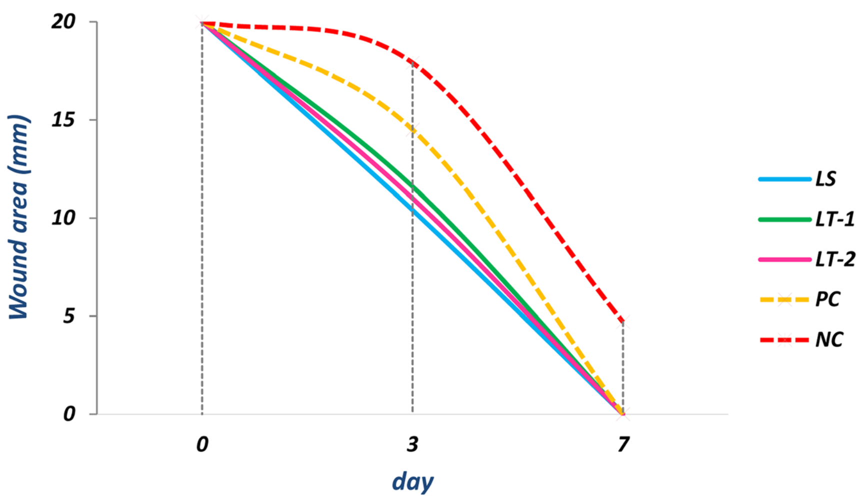


**Figure 3:** The graph of wound area closing rate (mm) over time.

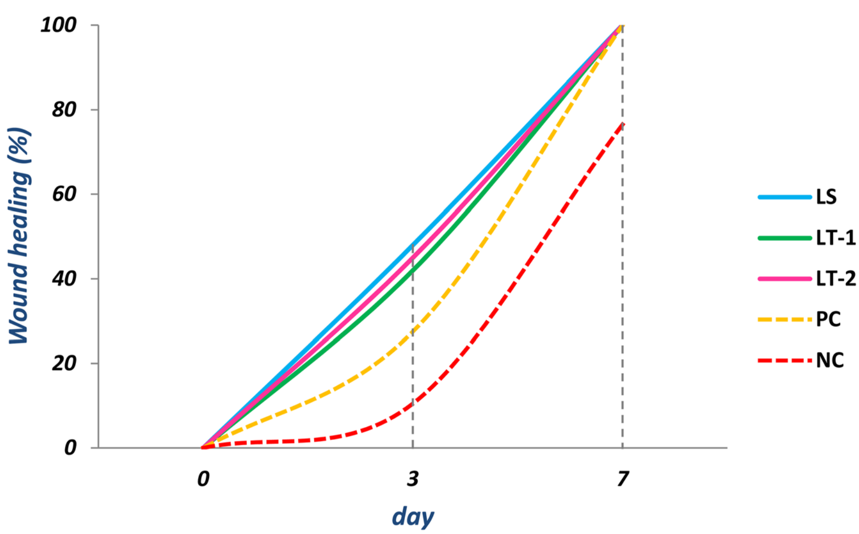


**Figure 4:** The graph of wound area closing rate (%) over time.

**Table 1:** Statistical assessment of wound healing rates across the groups.

| | 0 | 3 | 7 |
|---|---|---|---|
| LS | 20.00 ± 0.00 [20 (20–20)] | 10.43 ± 1.17[c] [10.9 (9.1–11.3)] | 0.00 ± 0.00[b] [0 (0.00–0.00)] |
| LT-1 | 20.00 ± 0.00 [20 (20–20)] | 11.67 ± 1.15[c] [11.7 (10.5–12.8)] | 0.00 ± 0.00[b] [0 (0.00–0.00)] |
| LT-2 | 20.00 ± 0.00 [20 (20–20)] | 11.07 ± 0.91[c] [11.2 (10.1–11.9)] | 0.00 ± 0.00[b] [0 (0.00–0.00)] |
| PC | 20.00 ± 0.00 [20 (20–20)] | 14.50 ± 0.72[b] [14.3 (13.9–15.3)] | 0.00 ± 0.00[b] [0 (0.00–0.00)] |
| NC | 20.00 ± 0.00 [20 (20–20)] | 17.93 ± 0.67[a] [18.1 (17.2–18.5)] | 4.77 ± 0.29[a] [4.6 (4.6–5.1)] |
| Test statistics; p-Value | KW: 0.000; p: 1.000 | KW: 11.333; p=0.023 | KW: 13.846; p: 0.008 |

[a>b>c]Means marked with different letters within the same column indicate statistically significant differences (p<0.05); KW, Kruskal-Wallis test value; p, p-value.

with epidermis, no infection occurred in any of the groups, and hair follicles had begun to form on the skin on the rats' backs.

## Discussion

Since ancient times, leeches have been employed in medical practice with varying degrees of success. These creatures release several anticoagulant substances, such as hirudin, into wounds. These substances prevent the formation of scabs, thereby speeding up the healing process [32]. A study in the literature revealed that leech therapy applied to the epigastric flaps of rats with venous insufficiency can partially reduce flap necrosis caused by venous disruption until surgical venous revascularization is performed [33]. In a study on reconstructive maxillofacial surgery, the effectiveness of leech therapy was examined and it was observed

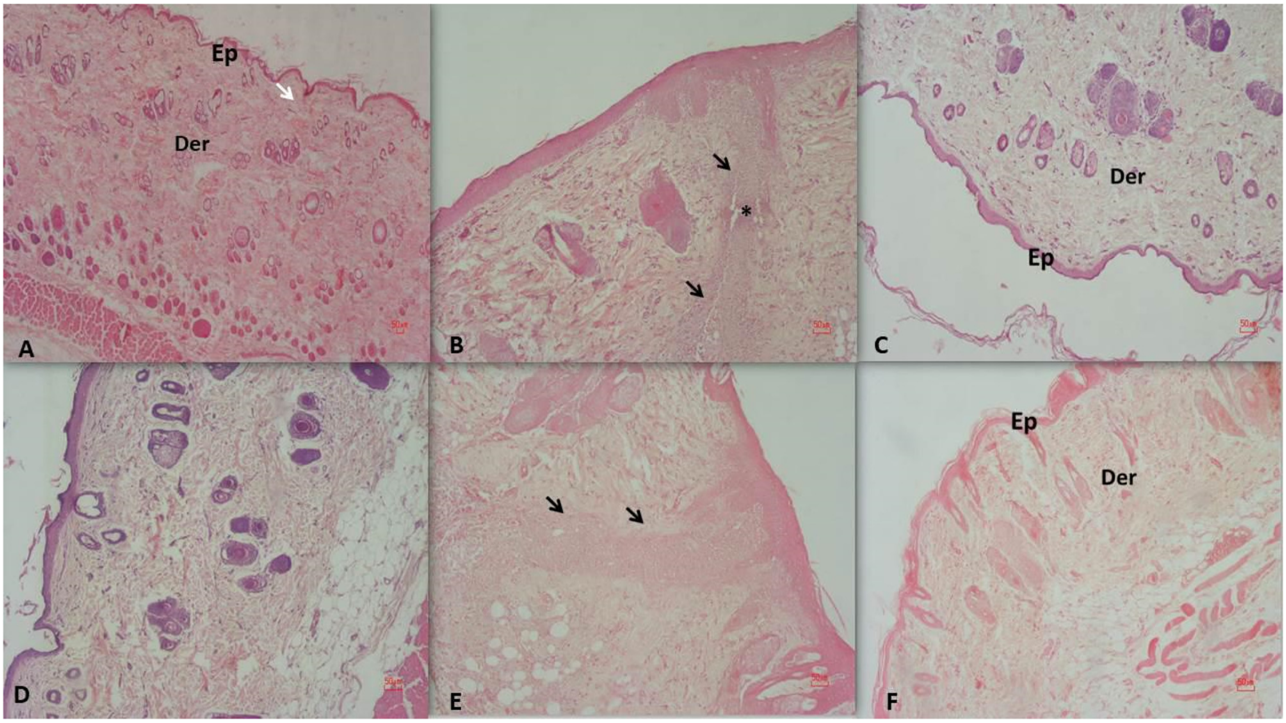


**Figure 5:** H&E staining of wound tissues collected on the 8th day from all groups with skin incisions. (A) Control Group (no incision); (B) Wound Incision Group; (C) Wound Incision + Leech Saliva Group (LS); (D) Wound Incision + LT1; (E) Wound Incision + LT2; (F) Wound Incision + *Triticum vulgare*; black arrow: wound area, *inflammatory cell infiltration; white arrow: normal tissue; Der, dermis; Ep, epidermis.

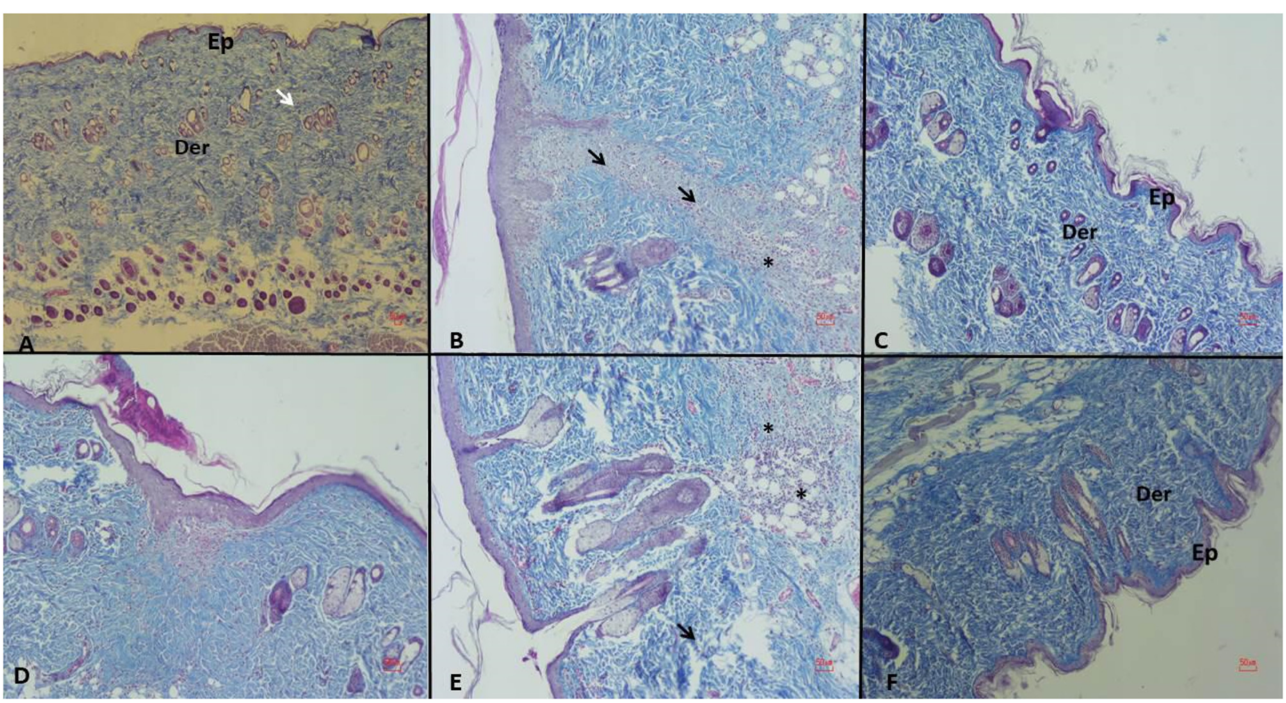


**Figure 6:** Masson's trichrome staining of wound tissues collected on the 8th day from all groups with skin incisions. (A) Negative Control Group (NC); (B) Positive Control Group (PC); (C) Wound incision + Leech Saliva Group (LS); (D) Wound incision + LT-1; (E) Wound incision + LT-2; (F) Wound incision + *Triticum vulgare*; black arrow: wound area, *inflammatory cell infiltration; white arrow: normal tissue; Der, dermis; Ep, epidermis.

that leech therapy was quite therapeutic in cases of venous congestion, both in locally and microsurgically anastomosed flaps [34]. Different studies in the literature have also reported that leech therapy is effective in the treatment of venous occlusion in flaps [35]. Blood circulation in some organs supports thrombolytic, anti-inflammatory, and immune-stimulating activities, strengthens tissue immunity, and improves tissue nutrition [33]. Hence, leeches promote healing by sucking excess blood, reducing tissue swelling, and enhancing blood circulation, while their saliva, rich in over 100 proteins and peptides, including the enzyme hyaluronidase, breaks down the intercellular matrix, encouraging cell motility and division, leading to rapid, effective, and long-lasting pain relief [8, 17].

In the current study, incisional wounds were created in rats in the LT-1, LT-2, LS, and PC groups and their comparative effects on wound healing were investigated. In the first stage of the wound model, active and healthy rats were selected for three experimental groups. By day 7, in the LS group (Figure 2), new hair growth was observed in the created wound area and the skin had healed with almost imperceptible scars. These findings showed the positive effect of LS on hair formation and wound healing. The hair growth observed in the LS group was thought to be due to wound healing, possibly due to increased blood circulation over the wound and nutrition of the hair follicles. Based on the percentage calculations derived from these results, wound healing exceeded 50 % in the LS group, slightly surpassed 20 % in the PC group, and reached 10 % in the NC group by day 3 (Figure 4). When the graph in Figure 3 is analyzed, it is seen that the change accelerated, and the steep slope shows that the observed improvement was mostly realized within the first three days. In the LT-1 and LT-2 groups, the results were similar to those in the LS group. According to the statistical data of these results, changes in wound healing rates over time and significant differences between the groups were observed. Notably, the results from days 3 and 7 indicate statistically significant differences between the treatment and control groups (Table 1). However, skin tissue regeneration was only observed by day 7. These findings demonstrated that leech salivary secretions could confirm the vasodilation, perfusion, subcutaneous capillary enrichment, and anti-inflammatory effects.

Previous histopathological studies support the wound-area assessment findings of the present study [36, 37], in which cellular infiltration was most prominent eight days after injury, with inflammation primarily observed in the superficial dermis. Additionally, reticular cells and dermal structures were also noted in the hypodermal and muscular layers. Moreover, in the granulation tissue where neutrophils were predominant, numerous blood vessels were observed, and re-epithelialization was detected (Figures 5 and 6). Acanthosis and papillomatosis were prominent at this stage. In the NC group of the wound model, acanthosis, mild fibrosis, tissue inflammation, fibroblast proliferation and hypergranulosis occurred, and this effect was shown to be in the early stage of skin wound healing. In the PC group, moderate fibrosis and fibroblast proliferation were observed. This finding suggests that wound healing was in the intermediate stage. Moreover, in the LS, LT-1, and LT-2 groups, individual non-monetary incisional wounds were found to be completely healed and no wounds were seen in the histological sections [24, 38] (Figures 5 and 6). In a study on the effect of LT used in the treatment of incisional skin wounds in *in vivo* models, higher success was achieved in the treatment of incisional wound models with medicinal leech therapy [27]. In the current study, when the groups were examined microscopically with H&E and Masson's trichrome staining, it was seen that epithelialization was better in the LS group on day 8 and the lesions regressed with more effective restructuring in the dermis. It was also observed that hair follicles were denser in the LS group. This finding supports the therapeutic properties of the LS in the study.

It was concluded that enzymes in medicinal leech salivary secretions significantly affect wounded tissue by increasing angiogenesis, which improves tissue perfusion. In addition, vasodilators in leech salivary secretions help prevent venous congestion. Given the positive and promising results of medicinal leech therapy on wounds, more detailed research is needed to elucidate its molecular mechanisms.

**Research ethics:** This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Kırşehir Ahi Evran University Animal Experiments Ethics Committee (approval number 2024-08/8).
**Informed consent:** Informed consent was obtained from all individuals included in this study.
**Author contributions:** Alican Bilden: Concept, Design, Analysis or Interpretation, Literature Search, Writing. Özlem Kara: Data Collection or Processing, Analysis or Interpretation. Merve Kahraman: Data Collection or Processing, Writing. Nebahat Çağlayan: Data Collection or Processing. Muttalip Çiçek: Concept, Design. All authors approved the submitted manuscript.
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**Data availability:** The data supporting the findings of this study are available from the corresponding author upon reasonable request.

## References

1. Pereira RF, Barrias CC, Granja PL, Bartolo PJ. Advanced biofabrication strategies for skin regeneration and repair. Nanomedicine 2013;8:603–21.
2. Clark RAF, editor. The molecular and cellular biology of wound repair. New York: Springer; 2013.
3. Sussman C, Bates-Jensen BM. Wound care: a collaborative practice manual. Philadelphia: Lippincott Williams & Wilkins; 2011.
4. Guo S, DiPietro LA. Factors affecting wound healing. J Dent Res 2010;89: 219–29.
5. Sun X, Jiang K, Chen J, Wu L, Lu H, Wang A, et al. A systematic review of maggot debridement therapy for chronically infected wounds and ulcers. Int J Infect Dis 2014;25:32–7.
6. Deshmukh PT, Gupta VB. Embelin accelerates cutaneous wound healing in diabetic rats. J Asian Nat Prod Res 2013;15:158–65.
7. Duzgun G, Celik A, Durmaz Akyol A. Medical leeches and hirudotherapy: where is nurse's role? J Educ Res Nurs 2021;18:276–81.
8. Nair HKR, Ahmad NW, Lee HL, Ahmad N, Othamn S, Mokhtar NSHM, et al. Hirudotherapy in wound healing. Int J Low Extrem Wounds 2020; 1–7. https://doi.org/10.1177/1534734620948299.
9. Whitaker IS, Rao J, Izadi D, Butler PE. Historical article: *Hirudo medicinalis*: ancient origins of, and trends in the use of medicinal leeches throughout history. Br J Oral Maxillofac Surg 2004;42:133–7.
10. Lemke S, Vilcinskas A. European medicinal leeches-New roles in modern medicine. Biomedicines 2020;8:1–12.
11. Müller C, Haase M, Lemke S, Hildebrandt JP. Hirudins and hirudin-like factors in Hirudinidae: implications for function and phylogenetic relationships. Parasitol Res 2017;116:313–25.
12. Mumcuoglu KY. Recommendations for the use of leeches in reconstructive plastic surgery. Evid base Compl Alternative Med 2014: 1–7. https://doi.org/10.1155/2014/205929.
13. Das BK. An overview on hirudotherapy/leech therapy. Indian Res J Pharmaceut Sci 2014;1:33–45.
14. Koeppen D, Aurich M, Rampp T. Medicinal leech therapy in pain syndromes: a narrative review. Wien Med Wochenschr 2014;164:95–102.
15. Abdullah S, Dar L, Rashid A, Tewari A. Hirudotherapy/leech therapy: applications and indications in surgery. Arch Clin Exp Surg 2012;1:172.
16. Abdualkader MA, Merzouk A, Ghawi AM, Alaama AM. Some biological activities of malaysian leech saliva extract. IIUM Eng J 2011;12:1–9.
17. Singh AP. Medicinal leech therapy (Hirudotherapy): a brief overview. Compl Ther Clin Pract 2010;16:213–15.
18. Wiwanitkit V. Leech therapy. Ancient Sci Life 2012;31:141.
19. Zaidi SMA, Jameel SS, Zaman F, Jilani S, Sultana A, Khan SA. A systematic overview of the medicinal importance of sanguivorous leeches. Alternative Med Rev 2011;16:59–65.
20. Herlin C, Bertheuil N, Bekara F, Boissiere F, Sinna R, Chaput B. Leech therapy in flap salvage: systematic review and practical recommendations. Ann Chir Plast Esthet 2017;62:1–13.
21. Asutkar S, Varshney S. An interdisciplinary critical review of leech therapy; evolution, indications, salivary gland secretions and mode of action – the scientific way. J Pharm 2019;9:10–18.
22. Tasiemski A. Antimicrobial peptides in annelids. Invertebr Surviv J 2008; 5:75–82.
23. Moosavian HR, Mirghazanfari SM, Moghaddam KG. Effect of ischemia preconditioning and leech therapy on cutaneous pedicle flaps subjected to prolonged ischemia in a mouse model. Aesthetic Plast Surg 2014;38:1024–9.
24. Zakian A, Ahmadi HA, Keleshteri MH, Madani A, Tehrani-Sharif M, Rezaie A, et al. Study on the effect of medicinal leech therapy (*Hirudo medicinalis*) on full-thickness excisional wound healing in the animal model. Res Vet Sci 2022;153:153–68.
25. Verriere B, Sabatier B, Carbonnelle E, Mainardi JL, Prognon P, Whitaker I, et al. Medicinal leech therapy and *Aeromonas* spp. infection. Eur J Clin Microbiol Infect Dis 2016;35:1001–6.
26. Darestani KD, Mirghazanfari SM, Moghaddam KG, Hejazi S. Leech therapy for linear incisional skin-wound healing in rats. J Acupunct Meridian Stud 2014;7:194–201.
27. Institute of Laboratory Animal Resources (US). Committee on care, and use of laboratory animals. Guide for the care and use of laboratory animals. No. 86. Bethesda, MD: US Department of Health and Human Services, Public Health Service, National Institutes of Health; 1986.
28. Gethin G, Cowman S. Wound measurement comparing the use of acetate tracings and Visitrak™ digital planimetry. J Clin Nurs 2006;15: 422–7.
29. de Almeida SM, da Cruz AD, Ferreira RI, Vizioli MR, Bóscolo FN. Effect of low-dose electron radiation on rat skin wound healing. Braz Dent J 2007;18:208–14.
30. Emami RS, Esmaeili DN, Forouzannia S, Amanpour S, Rabani S, Alizadeh M, et al. Effect of bentonite on skin wound healing: experimental study in the rat model. Tehran, Iran: Acta Medica Iranica; 2006:235–40 pp.
31. Ünal K, Tırık N, Erol ME, İbrahimkhanlı L, Elçi MP, Ayhan H. The investigation of effects of medicinal leech saliva extract on the breast fibroblast cell line *in vitro*: an experimental study. J Tradit Med Compl Ther 2023;6:142–51.
32. Mousavian A, Sabzevari S, Parsazad S, Moosavian H. Leech therapy protects free flaps against venous congestion, thrombus formation, and ischemia/reperfusion injury: benefits, complications, and contradictions. Arch Bone Jt Surg 2022;10:252–60.
33. Battin AO, Hobeika N, Zdilla MJ. Systematic review of medicinal leech therapy in urology. Afr J Urol 2023;29:21.
34. Choi JH, Kim EH, Choi N, Beak CH, Chung MK. Leech therapy for the salvage of complicated reconstructive flaps after head and neck cancer surgery. J Clin Otolaryngol Head Neck Surg 2020;31: 51–6.
35. Boissiere F, Gandolfi S, Riot S, Kerfant N, Jenzeri A, Hendriks S, et al. Flap venous congestion and salvage techniques: a systematic literature review. Plast Reconstr Surg Glob Open 2021;9:3327.
36. Sorg H, Sorg CGG. Skin wound healing: of players, patterns, and processes. Eur Surg Res 2023;64:141–57.
37. Wilkinson HN, Hardman MJ. Wound healing: cellular mechanisms and pathological outcomes. Open Biol 2020;10:200223.
38. Peña OA, Martin P. Cellular and molecular mechanisms of skin wound healing. Nat Rev Mol Cell Biol 2024:1–18. https://doi.org/10.1038/s41580-024-00715-1.