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ARTICLE

Morphological characteristics of the testes and epididymides in a rat excisional wound model under the influence of the medicinal leech *Hirudo verbana*

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ABSTRACT A standardized excisional wound model was established in male rats by removing circular full-thickness skin areas with a diameter of 1.5 cm. Animals were divided into two groups (n = 20 per group): a control group, in which wound healing occurred spontaneously without treatment, and an experimental group, in which one medicinal leech (*Hirudo verbana*, body weight 0.5–0.7 g) was applied adjacent to the wound area on days 1, 3, 7, and 14 after injury. On day 30, the testes and epididymides were collected for histological examination. Histological analysis of the testes in control animals revealed a reduced number of spermatocytes, an increased proportion of defective germ cells, and partial arrest of spermatogenesis in some seminiferous tubules compared with the experimental group. Morphometric assessment demonstrated a significant decrease in seminiferous tubule diameter and spermatogenic epithelial height in the control group. In contrast, the experimental group exhibited preservation of testicular architecture with closely packed seminiferous tubules and an intact spermatogenic epithelium. Examination of the epididymis showed that animals treated with *Hirudo verbana* had a significantly larger duct diameter, a wider lumen, and a preserved pseudostratified epithelium compared with controls. In the control group, degenerative changes of the epididymal epithelium and luminal narrowing were more frequently observed. These findings indicate that medicinal leech application during excisional wound healing is associated with improved histological preservation of the testes and epididymides in rats.
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Introduction

According to the World Health Organization, male infertility affects up to 40% of men, posing a potential threat to reproductive health. One of the primary causes of male infertility is the high sensitivity of the male reproductive system to various chemical and physical agents. Among the most vulnerable structures are the generative tissues of the testes, which, at certain stages of spermatogenesis, can be affected even by minor environmental changes (Nebesna et al. 2023; Saad et al. 2020; Stetsuk et al. 2023; Konovalenko 2020). Recent literature includes numerous studies on spermatogenesis under the influence of various environmental factors affecting the testes (Nebesna et al. 2023; Koval et al. 2023; Seliukova et al. 2020). Disorders of spermatogenic and hormonal functions in the testicles can result from acute and chronic circulatory disturbances in the scrotal organs, mechanical damage to the testes-epididymides complex, and general body intoxication (Davoodi et al. 2021; Koval et al. 2023). Testicular and epididymal lesions may result from both direct and indirect effects, including stress from excisional wounds and their healing process (Davoodi et al. 2021; Seliukova et al. 2022; Mostafavi Yazdi et al. 2022; Jiao et al. 2024; Peña et al. 2024; Virtanen et al. 2023; Raziyeva et al. 2021; Volovar et al. 2023). Disruptions in wound healing can lead to the development of various diseases, particularly in the reproductive system, which is highly sensitive to even minor fluctuations. Therefore, researchers are continuously searching for therapeutic agents that can act locally on the affected area and restore overall homeostasis.

One such therapeutic group is immunomodulators, which can indirectly influence various pathologies through the immune system. The medicinal leech (ML), known for its broad therapeutic effects due to the presence of over 100 biologically active substances in its body (Aminov et al. 2017; Aminov et al. 2021; Aminov et al. 2022; Aminov et al. 2023; Bruno et al. 2019; Mostafavi Yazdi et al. 2022; Davoodi et al. 2020; Dudhrejiya et al. 2023; Xia et al. 2020; Jacobsen et al. 2020; Ünal et al. 2023;

Ghods et al. 2019; Irsal-E-Alaq et al. 2021; Balasooriya et al. 2021; Sharma et al. 2020), is a prime example. As demonstrated in our previous studies, ML increases the total number of active spermatozoa in the ejaculate (Bruno et al. 2019). Additionally, it has been shown that ML species such as *Hirudo orientalis* and *Hirudo medicinalis* enhance regenerative and reparative processes during wound healing (Irsal-E-Alaq et al. 2021; Seliukova et al. 2022). Given the broad spectrum of ML's effects, it became pertinent to investigate the influence of the medicinal leech species *Hirudo verbana* on the morphological structure of the testes and epididymides during the healing of an excisional cutaneous wound.

Material and methods

Experimental animals

The study was conducted on 40 adult male white laboratory rats weighing 250–270 g. Animals were housed under standard sanitary and hygienic conditions in a certified vivarium. During the experiment, rats were kept individually in plastic cages at a temperature of 20-25 °C, relative humidity ≤55%, and under a natural 12 h light/dark cycle. Animals received a standard balanced laboratory diet and had free access to water. After creation of the excisional wound, animals were housed individually with weekly bedding replacement under aseptic conditions. Throughout the observation period, no clinical signs of wound infection or systemic bacterial complications were observed in any experimental group. All experimental procedures were performed in accordance with the International Guidelines for Medical and Biological Research Using Animals, the national Joint Ethical Principles of Animal Experiments (Ukraine 2001), and Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes, as well as the protocol from the bioethics commission at the Faculty of Biology of Zaporizhzhia National University for the planned research (protocol No. 1, 15.01.2025).

Study design and excisional wound model

Animals were randomly assigned into two experimental groups (n = 20 per group):

1. Control group – excisional wound healing without any treatment.
2. Experimental group – excisional wound healing with application of *Hirudo verbana*.

Under ketamine anesthesia (40 mg/kg body weight, intraperitoneally), the dorsal interscapular region was shaved and disinfected. A full-thickness circular excisional skin wound with a diameter of 1.5 cm was created using surgical scissors according to a standardized template, ensuring uniform wound size in all animals. All procedures were performed under aseptic conditions.

Treatment in the medicinal leech (*H. verbana*) group started 24 h after wound creation. One medicinal leech (*H. verbana*), weighing 0.5-0.7 g, was applied adjacent to the wound area on days 1, 3, 7, and 14. After completion of feeding, the leech was removed and disposed of according to biosafety procedures. Control animals received no intervention.

Medicinal leeches (*H. verbana*) were maintained using the modern jar method and were bred at the educational-scientific-research laboratory of cellular and organismal biotechnology at Zaporizhzhia National University (TU U 05.0-02125243-002:2009 "Medicinal Leech," sanitary-epidemiological opinion of the Ministry of Health of Ukraine No. 05.03.02-06/49982, dated 12.08.2009).

Histopathological examination

On day 30 of the experiment, animals were euthanized, and the testes and epididymis were collected. To ensure reproducibility and comparability of results, tissue sampling was performed in an anatomically standardized

Table 1. Morphological changes of the testes and epididymides in rats on the model of an excisional wound (n = 20 per group).

Group animals/day	Testes		
	Seminiferous tubule diameter (μm)	Tubular lumen diameter (μm)	Epithelial height (μm)
30 day/control (n=20)	98.96±2.44	68.13±2.11	21.70±0.24
30 day/experimental (n=20)	122.37±2.65*	71.85±1.98	24.89±0.45*
Group animals/day	**Epididymides**		
	Duct diameter (μm)	Lumen diameter (μm)	Epithelial height (μm)
30 day/control (n = 20)	60.34±2.09	52.60±2.66	8.76±0.12
30 day/experimental (n=20)	117.32±2.77*	78.98±2.87*	9.03±0.13*

Note: *– $p < 0.05$ vs control. Values are mean ± SEM.

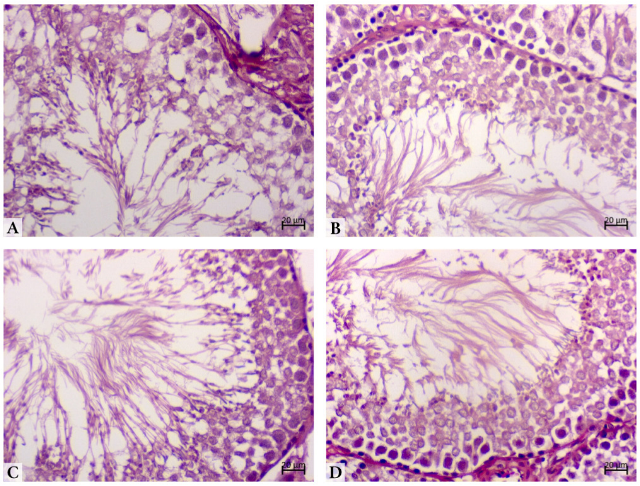

Figure 1. Morphological structure of the seminiferous tubule. A, C – control; B, D – experimental.

manner:

Testes: Entire testes, including the tunica albuginea, were collected for preparation of serial transverse sections.

Epididymides: Morphometric measurements of the caput, corpus, and cauda epididymidis were pooled for analysis; therefore, the results are presented for the epididymis as a whole.

Tissues were fixed in 10% neutral buffered formalin in dark glass containers at room temperature for 72 h. The organs were then processed using standard histological techniques, embedded in paraffin blocks, and serial transverse sections 5 μm thick were cut using a HM 325 microtome (Thermo Scientific, USA). Sections were stained with hematoxylin and eosin (H&E). Microphotographs were obtained using a PrimoStar iLED microscope equipped with an AxioCam ERc5s camera (Zeiss, Germany) and analyzed using ZEN 3.5 software (Blue Edition). This anatomically standardized sampling allows precise comparison of histological and morphometric features of both the testes and the epididymis between experimental groups. Multiple structures were measured per animal, and mean values per animal were used for statistical analysis to ensure independence of observations. At least 10 seminiferous tubules and 10 epididymal ducts were measured per animal.

For morphometric analysis, measurements were performed on **s**tandardized transverse sections. Multiple structures were measured per animal, and mean values per animal were used for statistical analysis to ensure independence of observations.

Statistical analysis

The animal (n = 20 per group) was considered the experimental unit. Data distribution was assessed using the one-sample Kolmogorov–Smirnov test. Between-group comparisons were performed using an independent-samples t test (two-tailed). Homogeneity of variances was checked with Levene's test; when violated, Welch's correction was applied. Statistical analyses were performed in IBM SPSS Statistics v26.0 (IBM Corp., USA). Differences were considered statistically significant at $p < 0.05$. Data are presented as mean ± SEM.

Results

Morphological characteristics of the testes and epididymides in rats subjected to an excisional wound model are presented in Table 1 and Fig. 1-3.

In the experimental group, histological examination revealed a well-preserved testicular architecture. Seminiferous tubules were closely packed, round or oval in shape, and lined with a thick, orderly spermatogenic epithelium (Fig. 1B, D; Fig. 2A, B). Tubular lumens contained germ cells at different stages of differentiation and numerous mature spermatozoa. The mean diameter of seminiferous tubules was 122.37 ± 2.65 μm, and the epithelial height was 24.89 ± 0.45 μm (Table 1). The interstitial tissue contained a moderate number of Leydig cells, mainly localized around small-caliber blood vessels (Fig. 2A, B). In contrast, testes from the control group exhibited structural alterations, including a reduced number of spermatocytes, the presence of defective germ cells, and partial arrest of spermatogenesis in some tubules (Fig. 1A, C; Fig. 2C, D). A significant reduction in tubular diameter and epithelial height was observed compared with the experimental group (Table 1). Vacuolization of

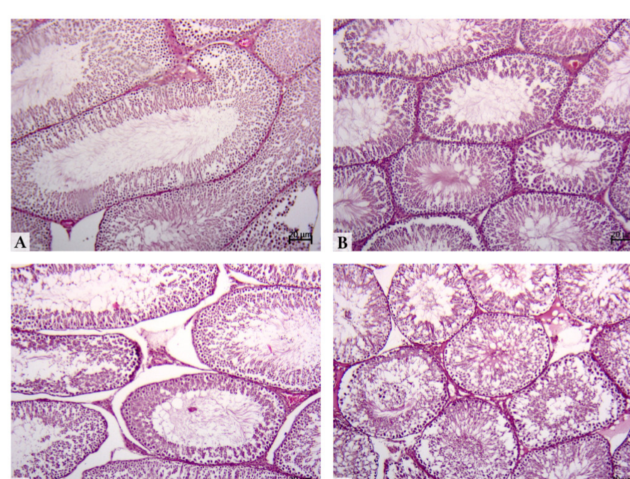

Figure 2. Morphological structure of the seminiferous tubules. A, B – experimental; C, D – control.

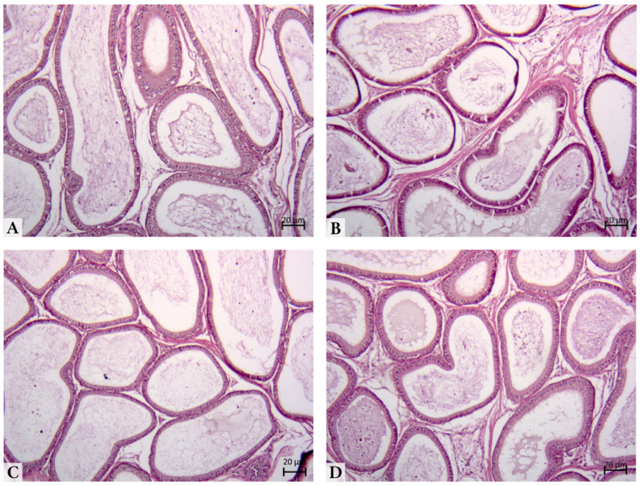

Figure 3. Morphological structure of the epididymis. A, B – control; C, D – experimental.

the epithelium and desquamation of germ cells into the tubular lumen were occasionally detected.

In the experimental group, epididymal ducts exhibited a significantly larger diameter, nearly twofold greater than that of the control group (Table 1). The ducts were surrounded by well-organized stroma and lined with a pseudostratified epithelium, while the lumen was densely filled with germ cells (Fig. 3C, D).

In the control group, epididymal tubules showed partial degenerative changes, including narrowing of the lumen, thinning of the epithelial wall, supranuclear vacuolization of principal cells, and focal disruption of the basement membrane (Fig. 3A, B). Cellular debris and fragments were frequently observed in the lumen.

Discussion

The present study demonstrates that application of the medicinal leech (*H. verbana*) during healing of an excisional wound is associated with preservation of the histological architecture of the testes and epididymides compared with untreated controls. In control animals, a reduction in seminiferous tubule diameter and epithelial height was observed, which may reflect stress-related alterations in spermatogenic processes during wound healing, as reported in previous studies (Konovalenko 2020; Nebesna et al. 2023; Saad et al. 2020; Stetsuk et al. 2023; Virtanen et al. 2023). The presence of an increased number of defective germ cells and epithelial vacuolization further supports this interpretation, suggesting that the wound healing process may indirectly affect testicular morphology through physiological stress responses.

In the epididymides, epithelial vacuolization and elevated numbers of light cells in control animals likely represent degenerative or adaptive changes associated with accumulation of luminal detritus, altered epithelial transport activity, and potential disruption of the epididymal microenvironment (Nebesna et al. 2023; Saad et al. 2020; Koval et al. 2023, Jiao et al. 2024). Such changes have been described as either indirect (e.g., presence of germ cells in the lumen, reduced epithelial height) or direct (structural alterations of the epithelium) manifestations of adverse influences on the male reproductive tract.

While the experimental group exhibited morphological features suggestive of preserved spermatogenic and epididymal integrity, it is important to note that the present study is limited to histological and morphometric assessments. Therefore, definitive conclusions regarding functional fertility outcomes cannot be drawn without additional physiological or reproductive evaluations, such as sperm motility, count, or mating trials.

An increase in the number of spermatozoa has been observed in our previous studies; however, this was assessed through ejaculate analysis, rather than by morphometric evaluation of the testes and epididymides (Aminov et al. 2021) . Importantly, the current study represents the first histological investigation of the testes and epididymides under the influence of the medicinal leech (*H. verbana*) in a rat excisional wound model, providing novel insights into the potential protective effects of leech therapy on male reproductive tissue during wound healing. The dataset presented here has not been published previously and does not overlap with earlier studies by the authors, which focused on ejaculate parameters rather than histological outcomes.

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