

Tissue-level Consequences of Segmental Abnormalities in *Hirudo verbana*: A Morphological and Histological Approach

Hirudo verbana'da Segmental Anormalliklerin Doku Düzeyindeki Sonuçları: Morfolojik ve Histolojik Bir Yaklaşım

Halime Tozak Yıldız¹, Ertan Katırcı¹, Alican Bilden², Merve Kahraman², Nebahat Çağlayan¹, Muttalip Çiçek²

¹Kırşehir Ahi Evran University Faculty of Medicine, Department of Histology and Embryology, Kırşehir, Türkiye

²Kırşehir Ahi Evran University Faculty of Medicine, Department of Medical Parasitology, Kırşehir, Türkiye

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ABSTRACT

Objective: This study aimed to investigate the histological and morphological differences between healthy and deformed specimens of the medicinal leech *Hirudo verbana*, with particular focus on tissue integrity and cellular organization.

Methods: We conducted comparative histological analysis using haematoxylin and eosin staining on tissue sections obtained from both healthy and deformed leeches. The evaluation included examination of epithelial layer integrity, muscle tissue organization, secretory cell distribution, and pigment accumulation patterns.

Results: Healthy specimens demonstrated well-preserved epithelial layers with regularly arranged circular and longitudinal muscle fibers. The secretory cells (T1, T2A, and T2B types) maintained normal distribution and activity, while melanin pigment showed limited and organized deposition in connective tissues. In contrast, deformed leeches exhibited significant structural abnormalities including disrupted epithelial layers, disorganized muscle architecture (particularly in circular muscles), and reduced T2B secretory cell populations. Notable accumulation of mononuclear immune cells, accompanied by increased melanin deposition, was observed in association with inflammatory foci. Morphological abnormalities included body segmentation defects, tissue atrophy, functional impairment of suckers, and compromised mobility.

Conclusion: Our findings demonstrate that deformation in *Hirudo verbana* leads to substantial histological and morphological alterations affecting epithelial integrity, muscular organization, secretory functions, and pigmentation patterns. These changes may significantly impact the leeches' biological functionality and therapeutic potential. Further investigation using immunohistochemical techniques and molecular analyses are warranted to elucidate the underlying mechanisms of these pathological changes.

Keywords: *Hirudo verbana*, histopathology, deformation, medicinal leech

ÖZ

Amaç: Bu çalışmada, tıbbi sülük türü *Hirudo verbana*'nın sağlıklı ve deforme olmuş örnekleri arasındaki histolojik ve morfolojik farklılıkların, özellikle doku bütünlüğü ve hücresel organizasyon üzerindeki etkilerinin araştırılması amaçlanmıştır.

Yöntemler: Hem sağlıklı hem de deforme olmuş sülüklerden elde edilen doku kesitlerinde hematoksilin ve eozin boyama kullanarak karşılaştırmalı histolojik analiz gerçekleştirdik. Değerlendirme, epitel tabaka bütünlüğünün, kas dokusu organizasyonunun, salgı hücresi dağılımının ve pigment birikim modellerinin incelenmesini içeriyordu.

Bulgular: Sağlıklı örnekler, düzenli olarak düzenlenmiş dairesel ve uzunlamasına kas liflerine sahip iyi korunmuş epitel katmanları gösterdi. Salgı hücreleri (T1, T2A ve T2B tipleri) normal dağılım ve aktiviteyi korurken, melanin pigmenti bağ dokularında sınırlı ve organize birikme gösterdi. Buna karşılık, deforme olmuş sülükler, bozulmuş epitel katmanları, düzensiz kas mimarisi (özellikle dairesel kaslarda) ve azalmış T2B salgı hücresi popülasyonları gibi önemli yapısal anormallikler gözlemlendi. Enflamatuvar odaklarla ilişkili olarak, melanin birikiminin artmasıyla birlikte mononükleer bağışıklık hücrelerinin belirgin birikimi gözlemlendi. Morfolojik



Address for Correspondence/Yazar Adresi: Prof. MD., Muttalip Çiçek, Kırşehir Ahi Evran University Faculty of Medicine, Department of Medical Parasitology, Kırşehir, Türkiye

E-mail/E-Posta: muttalipcicek@hotmail.com **ORCID ID:** orcid.org/0000-0003-4807-4482

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anormallikler arasında vücut segmentasyon kusurları, doku atrofisi, vantuzların işlevsel bozukluğu ve hareket kabiliyetinin azalması yer aldı.

Sonuç: Bulgularımız *Hirudo verbana*'daki deformasyonun epitel bütünlüğünü, kas organizasyonunu, salgılama işlevlerini ve pigmentasyon desenlerini etkileyen önemli histolojik ve morfolojik değişikliklere yol açtığını göstermektedir. Bu değişiklikler sülüklerin biyolojik işlevselliğini ve terapötik potansiyelini önemli ölçüde etkileyebilir. Bu patolojik değişikliklerin altında yatan mekanizmaları açıklamak için immünohistokimyasal teknikler ve moleküler analiz kullanılarak daha fazla araştırma yapılması gerekmektedir.

Anahtar Kelimeler: *Hirudo verbana*, histopatoloji, deformasyon, tıbbi sülük

INTRODUCTION

Freshwater leeches are segmented, hermaphroditic worms that feed on blood. Their bodies are highly segmented, including brain components, with each segment containing distinct organs such as ganglia and testes. Two suckers are responsible for crawling and attachment, while the anterior region contains three jaws equipped with numerous teeth. Leeches typically bite warm areas of the host and extract blood through rhythmic contractions. They use sharp, saw-like teeth in their jaws to make incisions in the host's skin, facilitating blood extraction. Simultaneously, they secrete saliva containing anticoagulants, such as hirudin, to prevent clotting and ensure uninterrupted feeding (1-3). Feeding usually lasts 15-40 minutes, during which a single leech can consume 10-15 mL of blood. Digestion is facilitated by symbiotic microorganisms such as *Aeromonas hydrophila* and *Pseudomonas hirudinia*, along with various digestive enzymes (4-6). Furthermore, it is important to consider the physiological characteristics of leeches after feeding, particularly their ability to store blood in their stomachs for long periods without spoilage, as highlighted in the literature (6).

Two thousand years ago, in ancient Egypt, leeches were first used for medical purposes. In recent years, leech therapy, known as hirudotherapy, has regained popularity (7). In 1884, Haycraft discovered a substance in leech saliva that prevents blood clotting, which was later identified as hirudin, an effective anticoagulant (8). During the 1980s, medical leech therapy became increasingly important in plastic and reconstructive surgery, particularly in reattachment of severed limbs and flap surgeries, as a supportive method to enhance blood circulation. Recognizing its medical significance, the United States Food and Drug Administration approved the sale and use of leeches in plastic and microsurgery in 2004 (9). Currently, bioactive substances obtained from medicinal leeches have shown therapeutic potential in diverse clinical settings, including antihypertensive effects, anticoagulant activity, anti-inflammatory properties, and enhanced wound healing following surgical interventions (10).

With the increasing use of leeches for medical purposes in the treatment of various diseases, uncontrolled harvesting of medicinal leeches from natural habitats has become a growing concern. Several factors negatively impact leech populations, including the decline of wetland habitats, the global reduction of amphibian species (e.g., frogs), the abandonment of traditional grazing practices in favor of stable-based livestock farming, drainage of wetlands for agriculture, and various other agricultural activities (11-13). As a result of these challenges, the decline in leech populations and the rising demand for safe, sterile, and traceable medicinal leeches have made it necessary to establish open or closed-system leech breeding facilities for sustainable production.

Morphological abnormalities in medical leeches may arise due to physical trauma experienced during their growth and feeding process after emerging from the cocoon or during their use for

therapeutic purposes, as well as the inability to fully replicate their natural habitat. These abnormalities result in irreversible segmentation damage, leading to the detachment of the distal part of the body, which subsequently causes infection and ultimately the death of the leech. This pathological condition is highly undesirable for medical leeches, which are both labor-intensive to cultivate and have significant economic value. In this study, we aimed to histologically investigate the underlying causes of these morphological deformities, which are a major concern in the global medical leech farming industry, leading to mortality and economic losses.

METHODS

Acquisition of Deformed Medicinal Leeches and Experimental Groups

Medicinal leeches (*Hirudo verbana*) that developed morphological and neurological deformities in their body segments due to various factors during cultivation at the Kırşehir University Leech Production and Research Unit were included in the study. Seven doctor-sized and medium-sized leeches exhibiting body constrictions were assigned to the "Group 2-deformed leeches" group. All leeches had been fed last one month prior to the experiment.

According to the Regulation on the Working Procedures and Principles of Animal Experiments Ethics Committees, an Ethics Committee Approval Certificate is required for vertebrate animals. Since medicinal leeches are invertebrates, they are not included in the definition of "experimental animals." Therefore, no ethics committee approval was required. Informed consent could not be obtained because the study did not involve human participants, patient-derived samples, or any direct or indirect intervention in human subjects.

Group 1 "C" (Control): Morphologically normal doctor-sized healthy leeches (DSHL) and medium-SHL (MSHL) (n=8).

Group 2 "Deformed Leeches": Doctor-sized leeches with body deformities (DSDL) and medium-sized leeches with body deformities (MSDL) (n=7) (Figure 1).

Their larger size allows them to consume more blood (approximately 10-15 mL), making them suitable for long-term treatments.

Doctor-sized leeches (8-15 cm in length, 4-6 g in weight) and medium-sized leeches (6-10 cm in length, 2-4 g in weight) were used in this study. The DSDL are capable of consuming approximately 10-15 mL of blood, whereas medium-sized leeches can consume 5-10 mL. Both groups possess anticoagulant and bioactive secretory functions, which are important for therapeutic applications (14).

Anesthesia and Fixation of Medicinal Leeches

The anesthesia process was initiated by placing the medicinal leeches in a 10% ethanol solution. The ethanol concentration

was gradually increased until the leeches ceased movement and response. Once relaxed in 70% alcohol, the leeches were fully anesthetized within approximately 15-30 minutes. Following anesthesia, the leeches were gently passed between the fingers to remove excess mucus and blood residues (15). Finally, in order to preserve tissue integrity and prepare the samples for subsequent analyses, all leech specimens were fixed in 10% formalin for 1 hour and then anatomically divided into anterior, middle, and posterior segments.

Histopathological Studies

The body parts of leeches from each experimental group were fixed in 10% formalin for 72 hours. Following fixation, the samples were washed under running tap water, passed through a graded ethanol series (50%, 70%, 80%, 96%, and 100%), subsequently, the tissue samples were embedded in paraffin blocks and labeled accordingly. Serial sections of 5 µm thickness were taken from the paraffin blocks of healthy leeches, matching the regional numbers of the deformation areas identified in the deformed specimens.

The sections were incubated at 56 °C overnight, then deparaffinized using xylene and rehydrated through a graded ethanol series before being washed in water. To examine the general histological structure, the sections were stained with hematoxylin and eosin. After staining, they were dehydrated again through an increasing ethanol series, cleared in xylene, and mounted with Entellan before being covered with a coverslip. Finally, the prepared slides were analyzed under a light microscope (Nikon Eclipse Si, Tokyo, Japan). The histopathological evaluation was primarily performed by the researcher. To ensure methodological reliability, all slides were independently re-evaluated in a blinded manner by a veterinary pathologist, with the results showing consistent agreement between both evaluators.

Statistical Analysis

No statistical analysis was required in the study.

RESULTS

Comparative analysis between healthy and deformed leeches across both size groups revealed pronounced morphological and physiological abnormalities in the deformed specimens. Irregular body segmentation, tissue loss in the distal region, atrophy, and in some cases, body ruptures were detected (Figure 1). Certain regions exhibited hardening, irregular muscle contractions, and uncoordinated crawling movements, along with reduced reflex responses. Due to movement restrictions, a decrease in body flexibility and locomotor capacity was noted. Additionally, loss of tissue pigmentation, weakening of the sucker function, and mucus accumulation in the segmented areas were observed. Furthermore, tissue damage, vascularization disorders, and lesion formation were identified in the affected regions.

In the DSHL, histological examinations revealed that the epithelial layer maintained regular outer contours with preserved tissue integrity. Both the circular muscle (CM) and longitudinal muscle (LM) layers exhibited organized structures, while blood vessels (BVs) were clearly defined and structurally intact. Evaluation of secretory cells indicated that T1, T2A, and T2B cells responsible for active secretion were healthy and equally distributed. Additionally, pigment (P) accumulation was observed in an organized manner beneath the muscle layers. In contrast, the DSDL exhibited epithelial layer irregularities, with disrupted outer contours and epithelial folding due to connective tissue proliferation. There was a loss of structural integrity and disorganization in the CM and LM layers. BV appeared deformed, with mononuclear cell accumulation detected. Regarding secretory cells, a notable decrease in T2B cells was observed, and in certain areas, melanin pigment deposits (melanin Ps) replaced secretory cells. Furthermore, distinct inflammatory cell clusters were detected beneath the epithelium, indicating infection sites (Figure 2).

In the MSHL group, the epithelial layer was found to be intact and well-structured. The CM and LM layers were well-organized, and



Figure 1. Macroscopic images of deformed leeches. (Left) Doctor-sized deformed leech, (right) medium-sized deformed leech. Scale bar: Left=2 cm; right=1.5 cm

the muscle structures maintained their integrity. BV exhibited normal morphology. Secretory cells with active secretion functions (T1, T2A, and T2B) were equally distributed and appeared healthy. P accumulation followed a normal distribution. In the MSDL group, the epithelial layer was disrupted and irregular. The CM and LM layers showed disorganization and loss of structural integrity. BV displayed morphological irregularities and deformation. A notable reduction in T2B secretory cells was observed, with some areas showing replacement of these cells by P accumulation. Additionally, inflammatory cell clusters were identified in the subepithelial tissue regions, indicating tissue damage and immune response activation (Figure 3).

DISCUSSION

This study provides a comparison of the morphological and histological characteristics of healthy and deformed leeches belonging to the species *Hirudo verbana*. Through microscopic evaluations, notable structural differences were identified between the groups, particularly in the epithelial organization, muscular architecture, vascular morphology, secretory cell distribution, and P accumulation. The presence of inflammatory foci and marked tissue disruption in deformed specimens further highlights the pathological consequences of deformation. These findings suggest that physical deformities significantly compromise the biological integrity of medicinal leeches, potentially impairing their physiological functions and therapeutic efficacy. Evaluating these alterations is essential not only for understanding the

mechanisms of deformation but also for improving cultivation conditions and ensuring the quality and safety of leeches used in medical applications.

Medicinal leeches are primarily hematophagous parasites that feed on the blood of their hosts. In healthy leeches, botryoidal cells are arranged in clusters, while in malformed leeches, botryoidal tissue transforms from a clustered arrangement into a typical hollow (tubular) structure. In excessively fed leeches, cracks can occur on the intestinal surface, and tissue destruction in these regions triggers an immunological response. These findings provide a possible explanation for the development of post-feeding morphological deformities and highlight the physiological mechanisms that may underlie tissue damage in leech farming systems (7). In this study, epithelial disruptions were markedly more pronounced in the deformed leech groups, indicating a loss of structural integrity in the outermost tissue barrier. This impairment is particularly critical, as the epithelium plays a vital role in protecting internal tissues from external insults, including microbial pathogens. Hildebrandt and Lemke (16) emphasized that epithelial damage in *Hirudo medicinalis* could compromise the barrier function, predisposing the organism to infections and triggering localized immune responses. Consistent with this, our study observed the presence of inflammatory cell clusters and infection foci beneath the damaged epithelial regions in deformed leeches, supporting the notion of immune activation and tissue defense mechanisms. A different study showed that exposure to various concentrations of cadmium caused altered epithelial cell boundaries, vacuolar degeneration in the epidermis, and

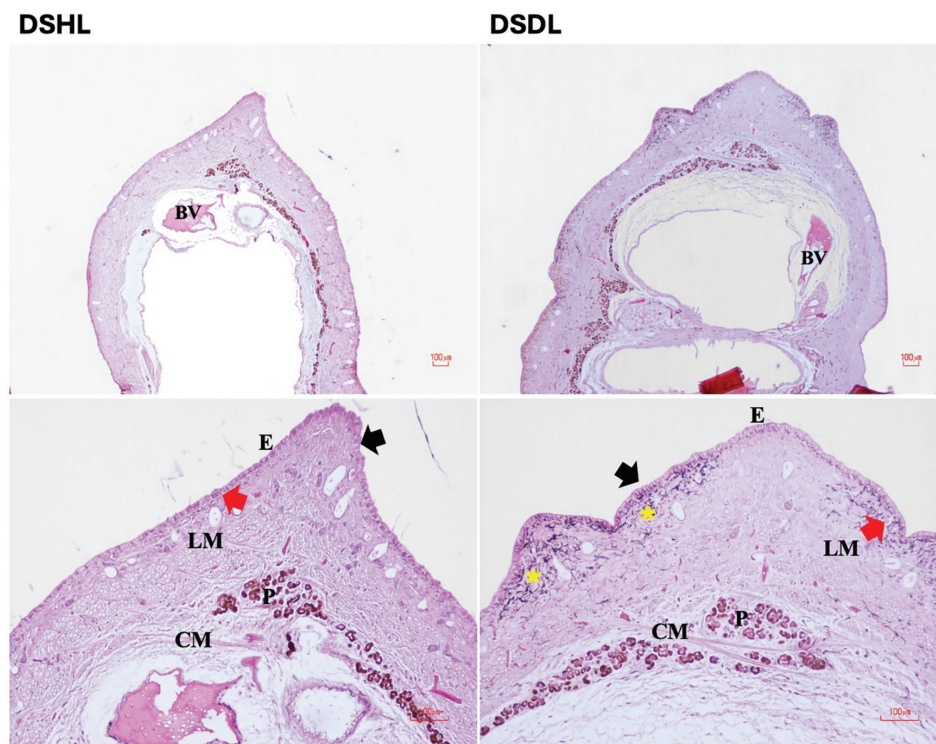


Figure 2. Histological sections of doctor-sized healthy (DSHL) and deformed (DSDL) leeches stained with hematoxylin and eosin. Black arrow: Cuticle, E: Epithelium, CM: Circular muscle, LM: Longitudinal muscle, BV: Blood vessel. Secretory cells with active secretion function are identified as T1 (red arrow). T2A and T2B secretory cells are distributed throughout the subepithelial connective tissue. P: Melanin pigment deposits in the submucosal layer. Infection sites characterized by inflammatory cell infiltration and increased melanin pigment accumulation in the subepithelial region; Yellow stars: Infection sites characterized by inflammatory cell infiltration and increased melanin pigment accumulation in the subepithelial region. Scale bar: 100 µm

structural damage in the cuticle in a freshwater leech species (17). In our study, the increased P accumulation observed beneath the damaged epithelial regions was primarily associated with melanin-like structures. Such pigmentation has previously been linked to stress responses and innate immune activity in invertebrates, where melanin synthesis plays a role in defense mechanisms, particularly under inflammatory conditions (18), which are known to be associated with oxidative stress-induced tissue damage in leeches (17). Importantly, melanin deposition in invertebrates has also been implicated in innate immune defense. It has been demonstrated that phenoloxidase-mediated melanogenesis plays a critical role in encapsulating and neutralizing pathogens, suggesting a functional link between P accumulation and immune activation. As proposed by Salzet (19), melanin and melanin-like compounds may serve not only as by-products of immune activation but also as active agents within antimicrobial defense systems. In this context, the increased P accumulation observed in deformed leeches may reflect a compensatory response aimed at limiting tissue damage and microbial invasion through localized immune activation. These findings reinforce the hypothesis that morphological deformation in medicinal leeches affects not only structural integrity but also elicits physiological stress responses that could potentially compromise their therapeutic reliability.

In leeches, muscle fibers are separated from the inner digestive tube by a layer of loose connective tissue (20). The tubular body wall of the leech consists of three distinct muscle layers of varying thickness: circular, longitudinal, and oblique muscle fibers. These three layers, along with dorsoventral muscles, are

primarily responsible for maintaining posture and facilitating various movements such as swimming, crawling, and directional orientation (21). In the literature, disorganization of the muscle layers has been prominently observed in deformed leeches, emphasizing the critical role of muscular architecture in essential biological functions such as locomotion and feeding. Perkins et al. (22) highlighted that structural impairments in the musculature may significantly compromise the leech's physiological performance, including its ability to attach, move, and feed efficiently. Consistent with these findings, this study demonstrated that deformation-related disruptions in the circular and LM layers were evident in the affected specimens, potentially impairing their biological activity.

In this study, histological examination revealed a regular distribution of T1, T2A, and T2B secretory cells in healthy leeches. In contrast, a marked reduction in T2B cells was observed in the deformed groups. According to the literature, T2B cells play an important role in the secretion of biologically active molecules in medicinal leech species such as *Hirudo medicinalis* (10,12,23). Therefore, the decrease in T2B cells may be associated with reduced biological functionality and diminished therapeutic potential. On the other hand, a different study conducted on *Limnatis nilotica* reported that exposure to cadmium resulted in morphological changes in type 1 secretory cells and vacuolar degeneration in the epidermis, while type 2 secretory cells exhibited a numerical increase without morphological alterations. This finding contrasts with our results regarding the reduction in T2B cells, and the discrepancy may be attributed to species-specific

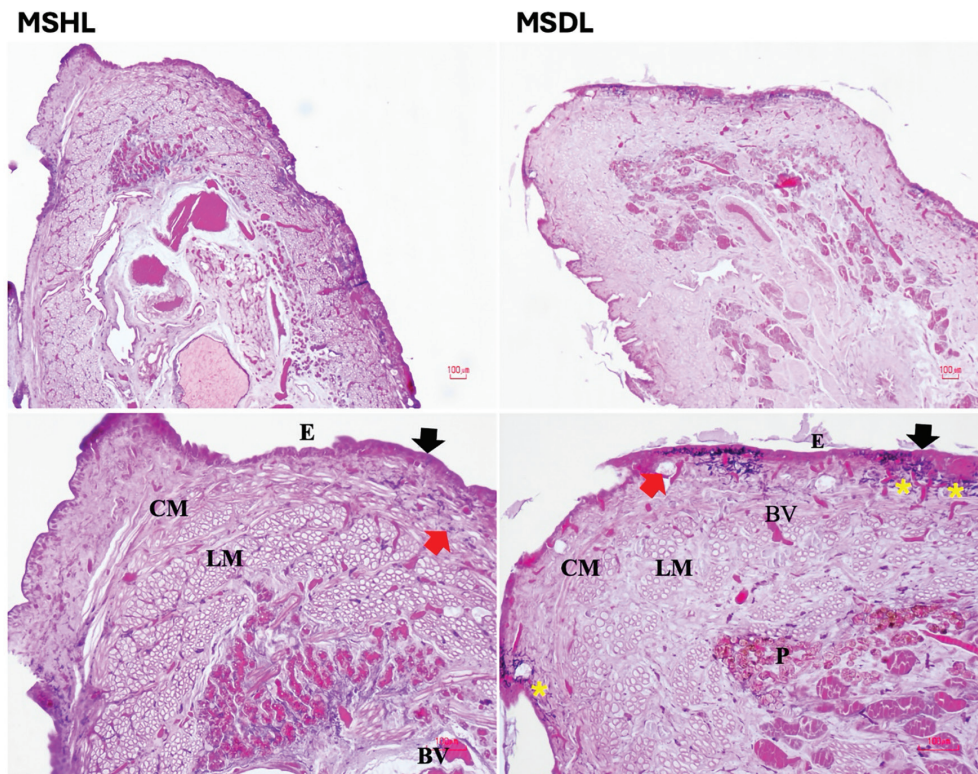


Figure 3. Hematoxylin-eosin stained tissue sections from the medium-sized healthy leech (MSHL) and medium-sized deformed leech (MSDL) groups. Black arrow: Cuticle, E: Epithelium, CM: Circular muscle, LM: Longitudinal muscle, BV: Blood vessel, secretory cells with active secretory function are designated as T1 (red arrow); T2A and T2B secretory cells are distributed throughout the subepithelial connective tissue. P: Melanin pigment deposits in the submucosal layer. Yellow stars: Infection sites characterized by inflammatory cell infiltration and increased melanin pigment accumulation in the subepithelial region. Scale bar: 100 μ m

differences and the distinct nature of the stressors involved. In our study, the deformation is likely linked to biomechanical stress or infection rather than a chemical agent such as cadmium. These observations suggest that various stress factors may elicit cell-type-specific responses depending on the species and tissue type involved (17).

In the deformed groups, infectious foci were also found to be more prevalent. The accumulation of mononuclear cells beneath the epithelium, along with the observed decrease in T2B secretory cells and replacement of these cells by melanin Ps, suggests the activation of a local immune response. Additionally, the presence of distinct inflammatory cell clusters beneath the epithelium indicates localized tissue damage and immune reaction in the affected areas. A more comprehensive investigation of these infections can be achieved through techniques such as immunohistochemistry, which would allow for the characterization of specific cell types and the cytokines they secrete in the infected regions thereby providing deeper insight into the extent and nature of the inflammatory response (24). The inflammatory responses and cellular alterations accompanying tissue damage observed in the study by de Eguileor et al. (25) are consistent with previously described wound healing processes in annelids. In the literature, it has been reported that leeches utilize macrophage-like cells, natural killer-like cells, and granulocytes located in the connective tissue and vascular system to mediate tissue repair. However, this cellular defense mechanism appears to be less rapid and efficient compared to other annelid groups such as oligochaetes, polychaetes, and sipunculids. This difference has been attributed to the reduced coelomic cavity and the limited number of coelomocytes in leeches (26,27). While oligochaetes and polychaetes are capable of initiating relatively rapid tissue repair through the migration of immune cells from the coelomic fluid, leeches exhibit reduced regenerative capacity and increased vulnerability to extensive tissue damage. These findings are particularly significant in explaining the persistence of inflammatory foci and irreversible tissue deformities observed in our study.

CONCLUSION

This study demonstrated that morphological deformities observed in *Hirudo verbana* medicinal leeches can affect several histological parameters including epithelial integrity, muscle organization, secretory cell distribution, and immune responses potentially impairing their physiological functions. The observed reduction in T2B secretory cells, along with the accumulation of melanin Ps in certain regions, suggests a disruption in normal secretory activity. Additionally, increased inflammatory foci and the accumulation of mononuclear immune cells beneath the epithelium indicate a localized immune response, linking deformation to tissue damage and the activation of defense mechanisms. These findings imply that deformation may negatively impact the therapeutic effectiveness of leeches. Further molecular-level studies are needed to better understand the underlying mechanisms. In this context, optimizing leech breeding conditions and improving quality control processes remain essential for ensuring their reliability in medical applications.

*Ethics

Ethics Committee Approval: According to the Regulation on the Working Procedures and Principles of Animal Experiments

Ethics Committees, an Ethics Committee Approval Certificate is required for vertebrate animals. Since medicinal leeches are invertebrates, ethics committee approval was not required according to national regulations.

Informed Consent: Informed consent could not be obtained because the study did not involve human participants, patient-derived samples, or any direct or indirect intervention in human subjects.

Footnotes

*Authorship Contributions

Concept: H.T.Y., E.K., A.B., M.Ç., Design: H.T.Y., E.K., A.B., M.Ç., Data Collection or Processing: H.T.Y., E.K., M.K., N.Ç., Analysis or Interpretation: A.B., Literature Search: H.T.Y., E.K., M.K., N.Ç., Writing: H.T.Y., E.K., M.Ç.

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