Basic Research Article

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Investigation of the therapeutic effects of medicinal leech treatment on lower extremity ischemia–reperfusion injury

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Abstract

Objectives: This study aims to investigate the therapeutic effects of medical leech treatment (MLT) on ischemia/reperfusion (I/R) injuries in the lower extremities of rats.
Methods: A total of 18 Wistar Albino rats were used and randomly assigned to three groups: Sham (n=6), I/R (n=6), and MLT (n=6). The I/R group rats underwent I/R induction in the femoral artery using an atraumatic vascular clamp. The same was done for the MLT group, after which MLT was carried out on the gastrocnemius muscle region of each rat. Histopathological analysis of the tissue and biochemical analysis was performed on the serum and tissue samples of the rats of each group.
Results: According to the histopathological analysis, the scores of edema, degeneration, necrosis and inflammation of the MLT group were significantly lower than the I/R group ($p<0.001$). In the tissue and serum biochemical analysis, no statistically significant differences were detected among groups for Interleukin-6 (IL-6), Superoxide Dismutase (SOD), Calcitonin Gene-Related Peptide (CGRP), Nitric Oxide (NO), Tumor Necrosis Factor-α (TNF-α), Hypoxia-Inducible Factor-1α (HIF-1α), Ischemia-Modified Albumin (IMA), Glutathione Peroxidase (GPx), Catalase (CAT) and Endothelin-1 (EDN-1) levels.
Conclusions: Although biochemical analysis results showed no significant differences among the groups, the histopathological analysis suggests notable clinical therapeutic effects of MLT in I/R models. Further research is warranted to substantiate these findings.

Keywords: hirudotherapy; leech treatment; ischemia; reperfusion; necrosis; inflammation

Introduction

Ischemia is the inadequate delivery of oxygen and nutrients to organs and tissues in the body because of restricted blood supply. This phenomenon results in hypoxia, acidosis, inflammation, and endothelial dysfunction, among many other cellular and biochemical cascades [1]. Without interventions, critical limb ischemia develops in the affected areas, especially in the lower extremities, which can cause pain, muscle degeneration and gangrene [2, 3]. To prevent this, the conventional approach is to perform procedures like surgical bypass, angioplasty, stenting and thrombolytic therapies [4, 5]. The main goal of these procedures is to re-establish blood and oxygen delivery before permanent damage to the tissue is done. While these methods can be lifesaving, they could also have significant drawbacks like diffuse microvascular obstruction, inflammation, and poor angiogenic recovery, which may hinder tissue healing and long-term limb salvage [6]. These limitations potentially create a need for therapies that are minimally invasive and also enhance local perfusion, modulate inflammation and support tissue regeneration [7].

A promising therapy known as medicinal leech therapy (MLT) dates back thousands of years and was first documented in ancient Egyptian hieroglyphs. In MLT, leeches feed on the patient's blood, and in return, they deliver various therapeutic bioactive substances from their saliva into the host's bloodstream [8]. Currently, MLT is being explored for its extensive use in the management of chronic

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wounds, venous congestion, and ischemic complications in reconstructive or vascular surgery [9]. Leech saliva contains natural anticoagulants, thrombolytic agents, vasodilators, and anti-inflammatory properties. The therapeutic effects of leech saliva are mainly due to the various pharmacologically significant compounds present in leech saliva, including hirudin, bdellins, eglins, calins, and destabilase. These substances have been found to improve local perfusion and promote survival of ischemic areas [10].

Until now, the literature on MLT for ischemic limb salvage remains limited, with most existing data derived from observational studies rather than high-impact randomized controlled trials. Moreover, to integrate MLT into modern medicine, there is a need to establish a specific treatment protocol through rigorous evaluation under standardized conditions [11]. According to studies, acute limb ischemia has an annual incidence of approximately 1.5 per 10,000 people and is significantly associated with mortality and morbidity [12]. Therefore, there is a great need to explore more effective alternative interventions like MLT when conventional treatments for acute ischemic limbs are inadequate.

This *in vivo* trial investigates the therapeutic effects of MLT on ischemia/reperfusion (I/R) injuries in the lower extremities of rats. By quantifying the results of the serum and tissue analysis by biochemical methods and histopathological findings, we evaluate the impact of MLT on I/R injuries.

Materials and methods

Animals experiments

This study is an experimental *in vivo* study. 18 female Wistar Albino rats were supplied by NESA Experimental Animals Laboratory, a company that has obtained a license from the Ministry of Agriculture and Forestry for the production, use and supply of experimental animals. The animal experiments were conducted at this laboratory. Animal procedures were approved by the Gazi University Animal Research Ethics Committee (Approval code: G.Ü.ET-25.017). The experiment was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals. Rats were chosen for this study as they are readily bred under laboratory conditions and are widely used in limb I/R models. Six rats were assigned randomly to each of the three groups.

Surgical method for lower extremity I/R

Surgical procedures and sterile techniques were performed in accordance with standard protocols. General anesthesia was induced via intraperitoneal (i.p.) injection of ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). The right groin of each rat was shaved and disinfected with povidone-iodine. The rats were secured in the supine position. After removing the overlying fat pad, a 2-cm oblique incision was made along the inguinal fold. Afterwards, the skin and subcutaneous tissues were dissected to expose the femoral neurovascular bundle. The femoral artery was separated from the femoral vein and nerve, and an atraumatic vascular clamp was applied to induce 2 h of ischemia. During ischemia, tissues were periodically moistened with physiological saline to prevent desiccation. At the end of 2 h, the clamp was removed to initiate reperfusion, and the incision was closed with simple sutures (Figure 1). After completion of procedures, the rats were deeply anesthetized with ketamine–xylazine and appropriate euthanasia was performed 24 h later by intracardiac blood collection.

Experimental design

Overall, a total of 18 rats were divided into the following three groups (Figure 2):

Sham group (n=6): In this model, the right groin was prepared, and the femoral bundle was exposed. I/R was not induced, and the incision was closed with simple sutures.

I/R group (n=6): After the same surgical exposure, the femoral artery was clamped with an atraumatic microvascular clamp for 2 h of ischemia. Tissues were kept moist with saline. The clamp was removed, and the skin was sutured, after which there was 24 h of reperfusion.

MLT group (I/R+ Medicinal Leech Treatment, n=6): After the same I/R surgical exposure, the clamp was removed to initiate reperfusion and the skin was closed. A single leech was applied to the gastrocnemius muscle of each rat under anesthesia after I/R and was allowed 24 h of reperfusion.

Application of the medicinal leeches

Hirudo verbana leeches were obtained from a Ministry of Agriculture and Forestry–approved sterile leech farm, which were supplied by NESA Experimental Animals Laboratory. The leeches were identified under a stereo zoom microscope and maintained in chlorine-free, well-aerated water in plastic containers at room temperature. Water was changed every 2–3 days. For each rat, one small sterile leech (~0.5 g) was applied to the gastrocnemius muscle in the MLT group. The leeches detached spontaneously after feeding

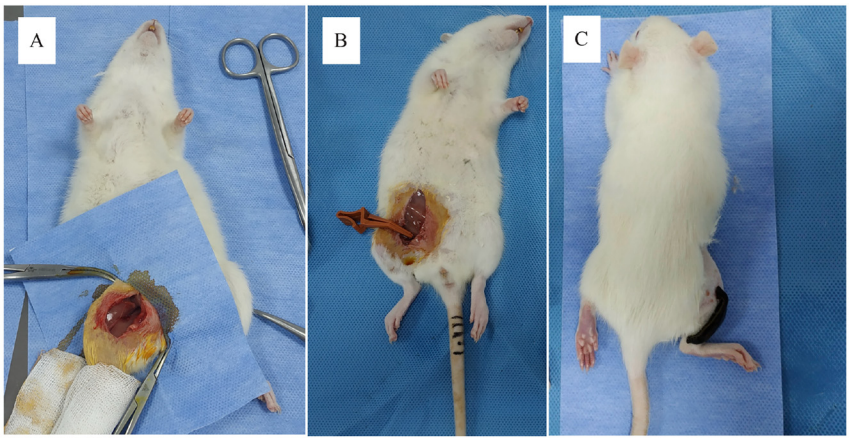


Figure 1: Surgical method for inducing lower extremity I/R and application of MLT. A) Femoral bundle exposed after inguinal incision. B) Femoral artery clamped to induce ischemia. C) MLT performed on gastrocnemius muscle in MLT group.

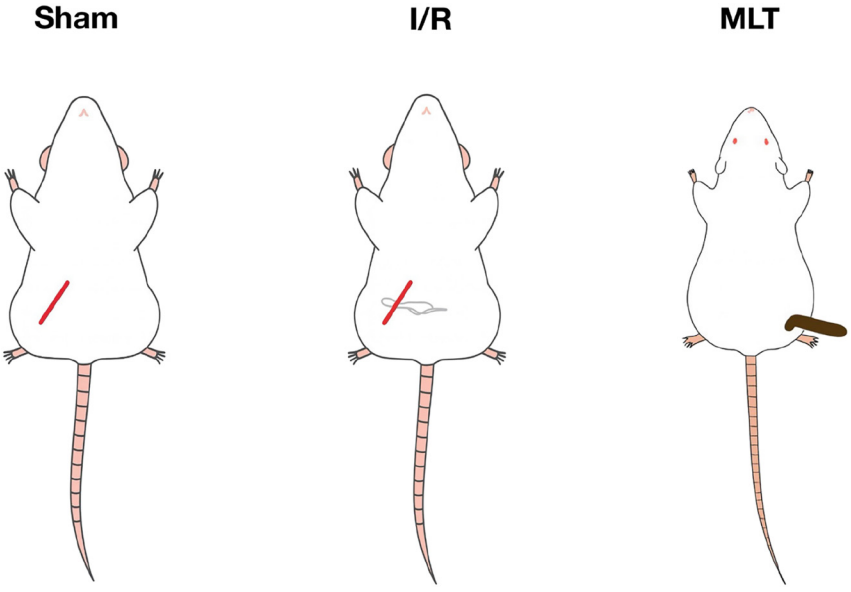


Figure 2: Diagram of the experimental groups: Shams, I/R and MLT group.

(≈0.5 mL blood). Leeches were weighed before and after application. After use, they were disposed of in 90 % ethanol.

Blood and tissue sampling

Blood was transferred to tubes and centrifuged at 3,000 revolutions per minute (rpm) for 15 min (min). Serum samples were aliquoted and stored at −80 °C. Gastrocnemius muscles were excised; a portion was fixed in 10 % formaldehyde for pathology, and the remainder was frozen in liquid nitrogen and stored at −80 °C for biochemical analyses. Muscle tissues were first washed with Phosphate-Buffered Saline (PBS) (pH 7.4). Afterwards, 100 mg of tissue was weighed for each sample. This was followed by the addition of 900 µL PBS to each sample. The mixture was transferred to a tube and homogenized on ice using a bladed homogenizer (Heidolph SilentCrusher). Homogenates were incubated on ice for 15 min, then centrifuged at +4 °C, 3,000 rpm for 20 min. Supernatants were promptly aliquoted and stored until analysis.

Enzyme-linked immunosorbent assay (ELISA)

ELISA analysis was conducted at the Department of Medical Biochemistry Laboratory, Faculty of Medicine, Gazi University. Concentrations of Interleukin-6 (IL-6), Superoxide Dismutase (SOD), Calcitonin Gene-Related Peptide (CGRP), Nitric Oxide (NO), Tumor Necrosis Factor-α (TNF-α), Hypoxia-Inducible Factor-1α (HIF-1α), Ischemia-Modified Albumin (IMA), Glutathione Peroxidase (GPx), Catalase (CAT) were measured in tissue supernatants and serum samples using Rat ELISA Research Kits from the brand Sunred (Shanghai Sunred Biological Technology Co., Ltd., China) were used in this study. Catalog numbers for IL-6,

SOD, CGRP, NO, TNF-α, HIF-1α, IMA, GPx, and CAT kits are 201-11-0136, 201-11-0169, 201-11-0348, 201-11-0704, 201-11-0765, 201-11-1371, 201-11-1672, 201-11-1705, and 201-11-5106, respectively. The lower limits of detection for IL-6, SOD, CGRP, NO, TNF-α, HIF-1α, IMA, GPx, and CAT were 1.822 pg/mL, 0.415 ng/mL, 0.906 ng/L, 0.115 µmol/L, 5.127 ng/L, 13.778 pg/mL, 1.284 U/mL, 0.723 ng/mL, and 0.866 ng/mL, respectively. The measurement ranges are 2–600 pg/mL, 0.5–100 ng/mL, 1–300 ng/L, 1–40 µmol/L, 8–1,000 ng/L, 15–4,200 pg/mL, 2–400 U/mL, 0.8–200 ng/mL, and 1–300 ng/mL, respectively. Endothelin-1 (EDN-1) concentrations were measured using a Rat ELISA kit from the brand USCN (Wuhan USCN Business Co., Ltd.; Lot No. L231226791), with a detection limit of 2.71 pg/mL and a measurement range of 6.17–500 pg/mL. During the study, washing steps were performed using a microplate washer (Combiwash Human Diagnostic Worldwide, Germany), and absorbance readings were conducted using a microplate reader (Elx800 UV Universal Microplate Reader, Bio-Tek Instruments, Inc.) at a wavelength of 450 nm.

Histopathological Procedures

Histopathological procedures were conducted at Department of Veterinary Pathology of Harran University. Muscle tissues were fixed in 10 % buffered formaldehyde for 72 h. Then, washed overnight in tap water and processed through graded alcohols (50 , 75, 96, 100 %) and xylene. Afterwards, they were embedded in paraffin, sectioned at 5 µm (Leica RM 2125 RT), and stained with hematoxylin–eosin (H&E). Histomorphological scoring assessed edema, degeneration, necrosis, and inflammation. The scoring was done using numerical values, where 0 indicated “none,” 1 indicated “mild,” 2 indicated “moderate,” and 3 indicated “severe.” [13].

Statistical analysis

Data were analysed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Data distribution was assessed using the Shapiro-Wilk test. Continuous variables were expressed as mean±Standard Error of the Mean (SEM) or Median (interquartile range, IQR). Comparisons between normal distribution of independent groups were performed using one-way ANOVA. Variables that did not show normal distribution were analysed using the Kruskal-Wallis test, and Dunn-Bonferroni post-hoc correction was applied where appropriate. p-value < 0.05 was considered statistically significant.

Results

Histopathological findings

In the histopathological analysis, we observed traces of edema and inflammation, along with degeneration and necrosis, in the I/R group (Figure 3). There was a significant difference in the edema scores among the three groups ($p<0.001$). The sham group and the MLT group had relatively little edema compared to the I/R group. ($p<0.001$; $p=0.043$ respectively). A noteworthy difference was also observed in the tissue degeneration scores among the groups ($p<0.001$). Similar to edema, the sham group and the MLT group had lower scores for degeneration compared to the I/R group ($p<0.001$; $p=0.003$ respectively). Necrosis was also observed among the three groups, and statistically significant differences were noted ($p<0.001$). Pairwise comparisons showed that the sham group and the MLT group exhibited less necrosis compared to the I/R group ($p<0.001$; $p=0.024$ respectively). There was also a significant difference in inflammation among the three groups ($p<0.001$). The sham group and the MLT group had lower inflammation scores compared to the I/R group ($p<0.001$; $p=0.023$ respectively). A detailed comparison of histopathological parameters among the groups is presented in Table 1.

Biochemical findings

In the experimental study, the parameters IL-6, SOD, CGRP, NO, TNF-α, HIF-1-α, IMA, GPx, CAT, and EDN-1 were evaluated in the homogenates prepared from the gastrocnemius muscle tissue of the experimental animals and serums belonging to the sham, I/R, and MLT groups. No statistically significant difference was observed between the groups in terms of tissue and serum biochemical markers. The distribution of the parameters studied in the tissue samples among the experimental groups is presented in Table 2. The distribution of the parameters studied in the serum samples among the experimental groups is presented in Table 3.

Discussion

This randomized *in vivo* experiment investigates the effects of MLT on I/R injuries in rats. The evaluation was done based on the histopathological evaluation of gastrocnemius muscle assessing edema, degeneration, necrosis, and haemorrhage scores in the samples. According to the analysis, the findings indicate that MLT exerts therapeutic effects on I/R injuries

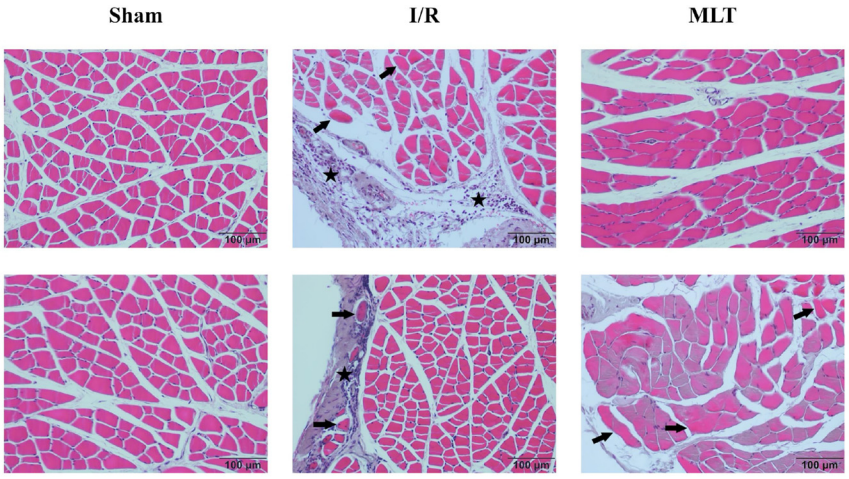


Figure 3: Histopathological evaluation with light-microscopic images of hematoxylin–eosin (H&E)–stained gastrocnemius sections of the Sham, I/R and MLT group. Sections stained with hematoxylin & eosin (H&E) assess edema, inflammation, degeneration and necrosis. Inflammation/edema: ★, degeneration/necrotic muscle cell: ➔.

Table 1: Comparison of histopathological parameters in gastrocnemius tissue among experimental groups.

	Sham	I/R	MLT	p-Value
Edema	0.00 (0.00–0.00)[b]	2.00 (1.75–2.00)[a]	0.50 (0.00–1.00)[b]	<0.001
Degeneration	0.00 (0.00–0.00)[b]	3.00 (3.00–3.00)[a]	1.00 (0.00–1.00)[b]	<0.001
Necrosis	0.00 (0.00–0.00)[b]	2.00 (2.00–2.75)[a]	1.00 (0.00–1.00)[b]	<0.001
Inflammation	0.00 (0.00–0.00)[b]	3.00 (3.00–3.00)[a]	0.50 (0.00–1.00)[b]	<0.001

Data are presented as median (IQR). Different superscript letters (a, b) indicate statistically significant differences between groups. p-Value <0.05 was considered statistically significant.

Table 2: Comparison of biochemical results obtained from tissue samples among experimental groups.

	Sham	I/R	MLT	p-Value
SOD (ng/g tissue)	423.21 ± 40.33	452.43 ± 18.65	415.51 ± 39.62	0.60
TNF-α (µg/g tissue)	0.17 (0.08–7.02)	6.32 (4.66–7.36)	0.33 (0.20–6.64)	0.48
IL-6 (ng/g tissue)	2.73 (2.04–3.05)	0.21 (0.18–1.56)	1.73 (0.19–2.23)	0.51
NO (µmol/g tissue)	167.83 ± 10.78	191.54 ± 8.00	199.97 ± 15.61	0.20
CGRP (ng/g tissue)	778.09 ± 156.76	1,001.12 ± 87.97	601.94 ± 134.91	0.21
GPX (ng/g tissue)	239.00 ± 17.32	259.10 ± 24.52	254.78 ± 22.39	0.79
IMA (U/g tissue)	1,648.33 ± 83.57	1,641.33 ± 76.38	1707.16 ± 78.49	0.85
HIF-1-α (ng/g tissue)	16.19 ± 3.26	18.45 ± 1.07	18.14 ± 0.59	0.69
CAT (µg/g tissue)	1.17 ± 0.05	1.07 ± 0.03	1.00 ± 0.06	0.06
EDN-1 (pg/g tissue)	112.11 ± 32.76	62.28 ± 18.46	96.56 ± 36.06	0.20

Data are presented as mean ± SD or median (IQR), according to data distribution. p-Value <0.05 was considered statistically significant.

Table 3: Comparison of serum biochemical results across experimental groups.

	Sham	I/R	MLT	p-Value
SOD, ng/ml	24.47 ± 1.94	25.96 ± 2.95	22.01 ± 3.09	0.84
TNF-α, ng/L	311.9 (234.2–439.7)	5.13 (5.13–119.6)	372.3 (232.5–431.9)	0.09
IL-6, pg/ml	88.0 (80.18–96.12)	48.95 (16.39–98.29)	103.5 (61.14–112.2)	0.45
NO, µmol/L	5.28 ± 0.46	6.84 ± 0.53	5.55 ± 1.12	0.38
CGRP, ng/L	27.90 ± 2.13	27.30 ± 2.60	26.37 ± 5.00	0.93
GPX, ng/ml	11.31 ± 1.46	11.46 ± 0.76	10.14 ± 1.48	0.76
IMA, U/ml	89.90 ± 2.63	86.68 ± 4.66	85.70 ± 9.28	0.41
HIF-1-α, pg/ml	649.24 ± 46.89	736.27 ± 28.73	674.58 ± 52.60	0.24
CAT, ng/ml	58.17 ± 4.43	60.85 ± 2.53	64.09 ± 4.53	0.49
EDN-1 (pg/g tissue)	14.49 ± 2.12	9.47 ± 2.22	13.25 ± 2.74	0.49

Data are presented as mean ± SD or median (IQR), according to data distribution. p-Value <0.05 was considered statistically significant.

by attenuating tissue edema, inflammatory responses, and limiting cellular degeneration and necrosis in the affected area. Biochemical analysis of the tissue and serum samples for SOD, IL-6, CGRP, NO, TNF-α, HIF-1α, IMA, GPx, CAT, and EDN-1 levels were also conducted. However no significant differences were noted among the groups in the biochemical analysis.

According to the histopathological analysis, the MLT group showed improved edema, degeneration, necrosis, and inflammatory cell infiltration compared to the I/R group. These findings align with numerous studies on the efficacy of MLT in improving microcirculation by relieving venous congestion, reducing microthrombus formation, inhibiting platelet aggregation, and enhancing tissue oxygenation [9, 14, 15]. Similarly, leech saliva has been shown to reduce necrosis, promote tissue regeneration, and significantly reduce cell inflammation in ischemic flaps and wounds [16, 17]. Similar therapeutic mechanisms of leech saliva are also seen in I/R models. Hirudin, a crucial bioactive substance in leech saliva, has been shown to limit secondary ischemic injuries by enhancing capillary perfusion, inhibiting fibrin deposition, and thereby enhancing microvascular protection [18–20]. These pharmacological properties evidently explain the mechanism by which the MLT group exhibited reduced edema and degeneration scores. Although the biochemical results did not yield a uniform result across all groups, these histopathological improvements provide strong functional evidence, supported by the available literature on the effect of MLT on I/R injuries [21, 22].

In I/R injuries, there is initially insufficient blood flow to a specific part of the body, followed by the sudden restoration of blood flow. This phenomenon triggers a surge of inflammatory mediators in the body, which exacerbates the ischemic injury [23]. Our study found that the I/R, sham and MLT group did not have any significant differences in tissue and serum pro-inflammatory cytokine levels. In most studies, the level of these biomarkers are elevated in the I/R models [24–26]; however, in our research, we found no statistically significant increase in biochemical parameters. At first, these results seem counterintuitive; however, after further research, we discovered that enzymes like SOD, CAT, GPX, and GSH etc. are not uniformly and reliably up regulated in I/R model. In studies involving rat renal I/R models, extensive tubular necrosis and apoptosis were observed, indicating that oxidative damage occurred even without observable up regulation of antioxidant enzymes, possibly due to messenger RNA (mRNA) down regulation, enzyme turnover, or cell loss [27, 28]. Thus giving a potential explanation for the unexpected outcomes in our study.

During the ischemia–reperfusion process, a biphasic immune response is observed. An early pro-inflammatory phase develops, initially characterized by a significant increase in cytokine levels, followed by a late immunosuppressive phase called CARS (Compensatory Anti-Inflammatory Response Syndrome) or immunoparalysis, in which inflammatory markers are suppressed. In this process, while necrosis develops, it is reported that the inflammatory response, which had previously peaked, gradually decreases [29–33]. Therefore, experimental studies with longer durations and different time points will contribute to a clearer understanding of this time-dependent decrease in inflammatory markers. Therefore, another plausible explanation for the lack of significant increases at the biochemical level may be related to the relatively short experimental duration, meaning that cytokines had not yet reached their peak circulating levels. Alternatively, the relevant inflammatory responses may have occurred at the transcriptional level; however, since mRNA expression analyses were not performed, these changes may not have been demonstrated in our study.

Our study is distinctive in the literature, representing the first instance of using H. verbana leeches for I/R injuries in the lower extremities under *in vivo* conditions. The findings suggest that MLT can be an effective treatment for I/R injuries. Our research makes a significant contribution to the existing literature on Complementary and Integrative Medicine by investigating the *in vivo* application of MLT and examining various biochemical and histopathological parameters in experimental rats.

In this study, the results of the biochemical analysis did not show completely cohesive results. However, the results of the histopathological analysis unanimously indicate that MLT attenuates I/R induced tissue injury to some extent. Lower-extremity I/R injury is a significant clinical problem, particularly in vascular surgery, trauma, and limb reimplantation scenarios [34]. Currently, the treatment protocols focus on revascularization and supportive measures. However, to date, no substantial pharmacological agents have been well established for reperfusion injuries [35]. MLT could be a possible intervention to address these concerns. However, it is essential to be aware of the potential risks associated with MLT. Among them, infections, primarily of the Aeromonas species [36], and anemia should be taken into consideration, and strict measures need to be implemented to reduce complications [37].

While our findings offer promising evidence, several limitations should be taken into consideration. Firstly, the sample size of this study is relatively small, with only three groups. Secondly, the biochemical parameters in this study were collected only at a single time point. The parameters were not investigated at different time points, and long-term outcomes of I/R after 24 h were not recorded. Given that

cytokine and enzyme responses are highly time-dependent following I/R [33], additional sampling points, mRNA expression analyses or extended reperfusion durations may further clarify the temporal dynamics and provide mechanistic insights into the non-uniform results of the biomarkers. Long-term muscle function recovery and contractility were also not investigated. Another limitation of this study is the absence of an additional experimental group in which rats were treated with MLT but did not undergo I/R injury. Inclusion of such a group could have allowed for a clearer demonstration of the independent effects of MLT and a more definitive assessment of whether the observed findings were related to I/R damage or directly to MLT itself.

Conclusions

This study provides compelling experimental evidence that MLT could be a possible therapy for I/R injuries. In the study MLT doesn't make significant modulations of key inflammatory biomarkers, such as SOD, IL-6, CGRP, NO, TNF-α, HIF-1α, IMA, GPx, CAT, and EDN-1 levels which deviate from conventional expectations. However, MLT successfully reduced edema, degeneration, necrosis, and inflammation in tissues. These findings are consistent with the current literature on the pharmacological properties of leech application, particularly its ability to stabilize microcirculation and limit reperfusion-induced tissue destruction [20–22].

Collectively, these results position MLT as a rational treatment for I/R injury, which is both minimally invasive and cost-effective. Overall, this study strengthens the scientific basis for incorporating medicinal leeches into modern strategies for limb salvage and the management of I/R injury in humans. In light of the robust histopathological improvements observed in this study, we encourage further investigation to establish proper treatment protocols. Future studies should incorporate longitudinal designs, dose-response evaluations, and molecular assays to elucidate further the mechanisms underlying the benefits.

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Research ethics: This study was approved by the Gazi University Animal Experiments Local Ethics Committee (Approval no: G.Ü.ET-25.017).

Informed consent: Not applicable.

Author contributions: Conceptualization: K.Ü., B.S.; Data curation: K.Ü., B.S.; Formal analysis: K.Ü., L.İ.; Identification and supply of leeches: K.Ü., B.S.; Investigation: K.Ü., B.S., L.İ.; Methodology: K.Ü., N.Y., L.İ.; I/R procedure: K.Ü. B.S.; Histopathological Procedures: N.Y.; Project administration: K.Ü.; Resources: K.Ü., B.S., N.Y.; Supervision: K.Ü.; Visualization: B.S., A.S.; Writing – original draft: K.Ü., A.S.; Writing – review & editing: K.Ü., A.S.

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Conflict of interest: The authors declared that there is no conflict of interest.

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Data availability: Not applicable.

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