RESEARCH PAPER

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Neuroprotective effects of hirudotherapy in cerebral ischemia/reperfusion injury: an experimental rat model

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This study evaluated the *in vivo* effects of hirudotherapy (medicinal leech therapy) on microcirculation and inflammation following global ischemia-induced cerebral ischemia/reperfusion (I/R) in rats. Rats were divided into three groups of eight (control, I/R, and hirudotherapy) for this study. Global ischemia was created in the I/R and treatment groups by occluding both common carotid arteries. According to the I/R procedure, two *Hirudo verbana* leeches were applied bilaterally to the mastoid regions. Serum and tissue samples were analyzed for biochemical parameters including superoxide dismutase (SOD), interleukin-6 (IL-6), nitric oxide (NO), calcitonin gene-related peptide (CGRP), glutathione peroxidase (GPx), catalase (CAT), endothelin-1, tumor necrosis factor alpha (TNF-α), ischemia modified albumin (IMA), and hypoxia-inducible factor-1 alpha (HIF-1α). Histopathological methods were used to examine edema, degeneration, necrosis, and hemorrhagic changes in brain tissue samples. The I/R group showed significantly increased tissue TNF-α levels compared to controls. Tissue IMA and HIF-1α levels were significantly higher in the treatment group. Treatment significantly reduced degeneration, necrosis, edema, and hemorrhage in brain tissues, as shown by histopathology. Histopathological analysis shows significantly less tissue damage in the hirudotherapy group compared to the I/R group, suggesting that hirudotherapy reduces brain damage after cerebral ischemia and reperfusion.

Key words: cerebral ischemia; reperfusion injury; hirudotherapy; neuroprotection; oxidative stress

INTRODUCTION

Hirudotherapy, also known as medicinal leech therapy, is an ancient therapeutic method that continues to be used in modern medicine (Whitaker et al., 2004). Used in traditional medicine and modern science, this treatment's history dates back to antiquity (Whitaker et al., 2004; Adams et al., 1988). Cited often by early physicians for its effectiveness, this treatment remains a focus of modern attention because of its biological makeup and chemical interactions (Adams et al., 1988; Haycraft, 1884).

Sushruta Samhita, written by the famous Indian physician Sushruta, contains the first known written reference to hirudotherapy (Amr & Tbakhi, 2007). This paper describes the use of medical and non-medical leeches as an alternative to surgical interventions. The dissemination and progress of hirudotherapy were significantly influenced by renowned physicians like Hippocrates, Galen, and Ibn Sina, who used and taught this treatment to their students (Whitaker et al., 2004; Haycraft, 1884).

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Scientific and technological progress led to the 1884 discovery of *Hirudin,* an enzyme secreted by leeches, by English scientist John Berry Haycraft (Haycraft, 1884). The chemical structure and effects of about 105 substances from the secretion have been examined, with thorough analysis of roughly 30. Studies show that hirudin, calin, eglin, destabilase, and hyaluronidase enhance blood circulation, inflammation reduction, edema resolution, and healing acceleration (Adams et al., 1988).

Hirudotherapy, using leeches from special farms, is a legal practice in various European and Asian nations, including Turkey, with physicians administering treatment (Kübranur et al., 2025). The applications of hirudotherapy are diverse, spanning fields like plastic surgery and orthopedics. From neurological disorders and skin conditions to circulatory and musculoskeletal problems, it yields promising results in many fields (Ünal et al., 2023). However, given the potential risks of this treatment in cases of pain, anemia, or anticoagulant or antiaggregant drug usage, thorough evaluation and preventative measures are essential to minimize side effects and complications.

A decrease or absence of blood flow to a tissue defines ischemia. Reduced oxygen and nutrient delivery due to ischemia impairs tissue function by disrupting cellular metabolism. Interrupted blood flow is restored by reperfusion. Though helpful for tissue healing, reperfusion unfortunately also causes oxidative stress, inflammation, and tissue damage through several mechanisms. This condition, known as ischemia/reperfusion (I/R) injury, is characterized by a paradoxical exacerbation of cellular dysfunction and death upon restoration of blood flow to previously ischemic tissue, driven by oxidative stress, mitochondrial dysfunction, inflammatory cytokine release, and activation of cell-death pathways (Akhtar et al., 2025).

Medical leech saliva contains noteworthy bioactive enzymes with anticoagulant, antithrombotic, anti-aggregant, anti-inflammatory, and neurotrophic effects (Becanim et al., 2022). The anti-inflammatory properties are mediated in part through suppression of pro-inflammatory cytokines such as TNF-α and IL-1β, as well as inhibition of NF-κB signalling pathways (Becanim et al., 2022).

Because of these effects, hirudotherapy speeds up healing for circulatory disorders, joint diseases, and nervous system issues. Specifically, enzymes including hirudin, calin, eglin, destabilase, and hyaluronidase improve tissue blood circulation, decrease inflammation, and offer benefits for I/R injury related to circulatory disorders. Hirudin inhibits thrombin, reducing micro-thrombus formation; calin inhibits collagen-dependent platelet aggregation, preventing re-occlusion; eglins suppress serine protease-mediated inflammatory cascades; and hyaluronidase reduces oedema by improving interstitial fluid dynamics (Becanim et al., 2022; Schenker et al., 2006).

Despite a lack of specific research, clinical observations hint at potential benefits of hirudotherapy for neuronal damage caused by I/R (Schenker et al., 2006). Biochemically and histologically studying hirudotherapy is crucial within this context. For cerebrovascular diseases, migraines, and transient ischemic attacks, this therapeutic approach offers hope.

Our study aimed to examine the *in vivo* effects of hirudotherapy on microcirculation and inflammation after global ischemia in a rat cerebral I/R model. Biochemical analysis using sandwich ELISA (for TNF-α, IMA, HIF-1α, SOD, IL-6, NO, CGRP, GPx, CAT, and EDN1) and histopathological examination using haematoxylin-eosin (HE) staining were used to evaluate the effects of hirudotherapy on I/R injury; these results could inform new treatments for cerebrovascular and circulatory conditions.

METHODS

This study is an experimental animal study and does not involve human subjects or clinical trial design. This study received ethical approval from the Nesa Laboratory Animal Center's Local Ethics Committee (January 5th, 2024, No. 023). The present study is derived from a previously approved experimental protocol conducted during the author's thesis period. Standard experimental animal protocols guided the study. The experiments involved twenty-four male Wistar albino (*Rattus norvegicus*) rats, supplied by the Nesa Laboratory Animal Breeding and Experimental Research Center. Rats in the study were 4–5 months old and had a mean weight of 250±50 grams. Animals were housed in conventional cages under a 12-hour light/dark cycle at 22±2°C, with *ad libitum* access to standard rodent pellet chow and tap water throughout the experimental period (Ünal et al., 2025; Ünal et al., 2026). Ethical standards and animal welfare guidelines were followed throughout the research, aiming to minimize animal stress and ensure reliable results. The number of leeches and the application duration were selected according to previously reported experimental protocols evaluating the effects of hirudotherapy on ischemia-related tissue injury and microcirculation.

Source and Maintenance of Leeches

MS Leeches, a Ministry of Agriculture and Forestry-approved farm, supplied the *Hirudo verbana* leech-

es for this medical study. Leeches were maintained in glass containers of purified, chlorine-free drinking water, under appropriate room conditions for optimal aeration. Every 1-2 days, the water was refreshed.

Grouping

The animals were categorised into three equal groups of eight: Control Group, I/R Group, and Treatment Group.

- Control Group: Control animals did not receive any surgical procedures. They received intraperitoneal ketamine (50–100 mg/kg) and xylazine (5–10 mg/kg) for anaesthesia and were euthanised 30 min after anaesthesia induction by intracardiac blood collection under deep anaesthesia.
- I/R Group: In this group, the common carotid arteries (CCAs) were occluded bilaterally using aneurysm clips for 30 min, followed by 30 min of reperfusion. The animals received intraperitoneal ketamine (50–100 mg/kg) and xylazine (5–10 mg/kg). The animals were sacrificed immediately following the 30-minute reperfusion period (approximately 90 min total duration from anesthesia induction).
- Treatment Group: In this group, cerebral ischemia was induced by bilateral occlusion of the common carotid arteries for 30 min, followed by 30 min of reperfusion, as described in the I/R group. Following reperfusion, two small *Hirudo verbana* leeches (0.5–0.7 g each) were applied bilaterally to the mastoid processes. The selection of two small leeches was based on established protocols to minimize systemic anticoagulant load, as hirudin and calin are potent anticoagulants associated with clinically significant bleeding risks (Lok et al., 2013; Deshmukh et al., 2024). Bilateral application ensured symmetric delivery consistent with the bilateral CCA occlusion model. The mastoid process was selected as the primary application site due to its close anatomical proximity to the internal carotid artery, internal jugular vein, and cerebral vasculature, allowing bioactive compounds to rapidly enter the cerebral circulation *via* the carotid and vertebrobasilar systems (Coudwell & MacDonald, 2007). Leeches detached spontaneously after 30–45 min of feeding. Animals were euthanized under deep anesthesia immediately following leech detachment, yielding a total experimental duration of approximately 120–125 min from anesthesia induction (Mumcuoglu, 2014; Ünal et al., 2026). Representative intraoperative photographs illustrating bilateral common carotid artery exposure, occlusion, and hirudotherapy application are provided in Supplementary Fig. 1.

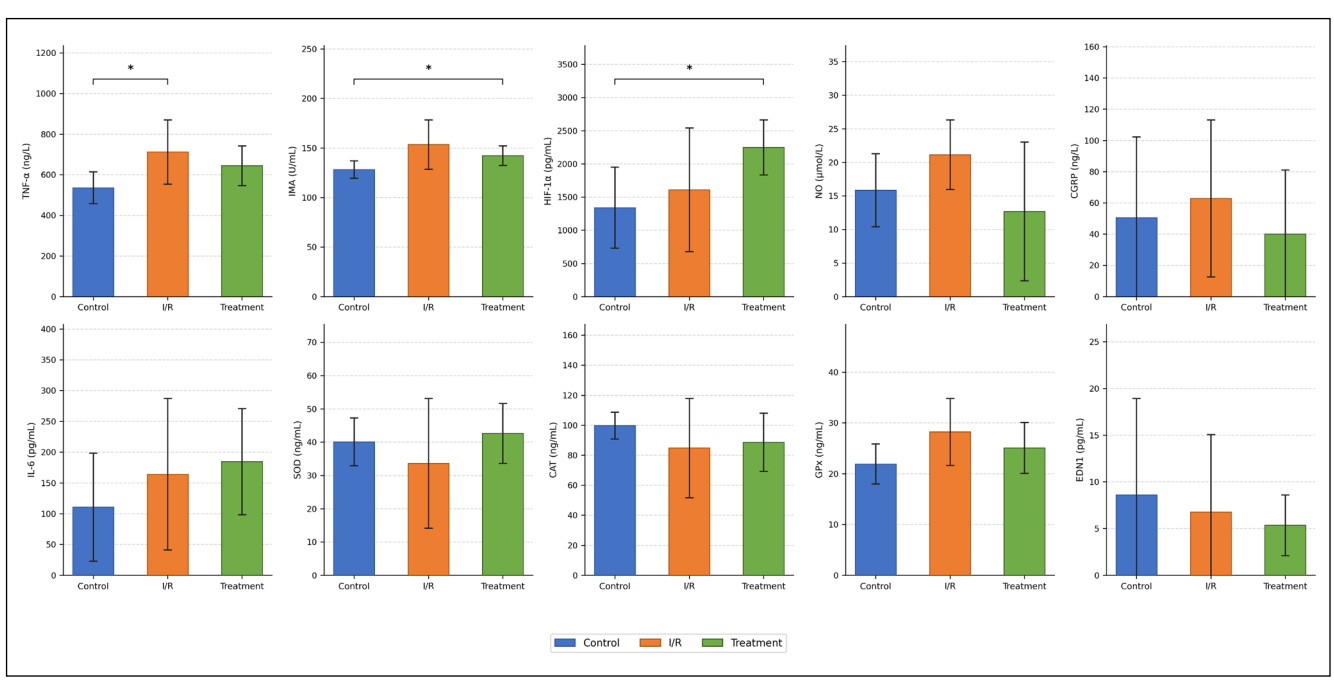

Fig. 1. Distribution of biochemical results of tissue samples among experimental groups. Data presented as mean±SD (n=8 per group). *p<0.05 *vs.* Control (*post-hoc* Tukey/Tamhane test). Y-axes start at zero. TNF-α: tumour necrosis factor-α; IMA: ischemia modified albumin (U/mL: absorbance units per millilitre, manufacturer-defined unit, Sunred ELISA kit); HIF-1α: hypoxia-inducible factor-1α; NO: nitric oxide; CGRP: calcitonin gene-related peptide; IL-6: interleukin-6 (IL-6); SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; EDN1: endothelin-1.

Sample Collection and Analysis

Following euthanasia, intracardiac blood samples and whole brain tissue were collected. Blood samples were centrifuged at 3,000 rpm for 15 min and serum aliquots stored at −80°C for biochemical analysis. The whole brain was removed and divided along the mid-sagittal plane. Tissue designated for histopathological examination was immersion-fixed in 10% neutral buffered formalin (pH 7.4) for 72 hours at room temperature, then dehydrated through a graded ethanol series (50%, 75%, 96%, and 100%), cleared in xylene, and embedded in paraffin. Sections of 5 µm thickness were cut using a Leica RM 2125 RT rotary microtome and stained with hematoxylin-eosin (HE). Tissue designated for biochemical analysis (cerebral cortex) was dissected, snap-frozen in liquid nitrogen, and stored at −80°C until homogenization.

A high-resolution light microscope (Olympus DP-73 camera, Olympus BX53-DIC microscope) was used to meticulously examine all sections. A semi-quantitative assessment of the histology was conducted. Tissue changes (edema, degeneration, necrosis, and hemorrhage) were all recorded during microscopic examination. Histopathological scoring of tissue samples assessed edema, degeneration, necrosis, and hemorrhage on a scale of 0 = none, 1 = mild, 2 = moderate, and 3 = severe. The scoring system was based on Cao et al. (2018).

Approximately 100 mg of cerebral cortex tissue was homogenised in 900 µL ice-cold phosphate-buffered saline (PBS, pH 7.4) on ice, then centrifuged at 3,000 rpm for 15 min at 4°C. Supernatants were promptly aliquoted and stored at −80°C until analysis. Tissue homogenates were prepared at a standardised ratio relative to tissue wet weight, which was precisely recorded for each sample prior to homogenisation, consistent with the protocol used in our group's concurrent studies (Ünal et al., 2025; 2026). ELISA results from tissue homogenates are expressed per unit volume of homogenate (pg/mL, ng/mL, or U/mL as appropriate for each analyte).

Sandwich ELISA analysis of tissue homogenate and serum supernatants determined the levels of Superoxide Dismutase (SOD), interleukin-6 (IL-6), Nitric Oxide (NO), Calcitonin Gene-Related Peptide (CGRP), Glutathione Peroxidase (GPx), Catalase (CAT), Endothelin-1 (EDN1), Tumor Necrosis Factor Alpha (TNF-α), Ischemia Modified Albumin (IMA), and Hypoxia-Inducible Factor-1 Alpha (HIF-1α). The analyses employed Rat ELISA Research Kits supplied by Sunred (Shanghai Sunred Biological Technology Co. Ltd, China).

Haematoxylin-eosin (HE) staining was performed on paraffin-embedded 5 µm sections prepared using a graded ethanol dehydration series (50%, 75%, 96%, and 100%) and xylene clearing prior to embedding.

Statistical Analysis

Data statistical analysis was conducted with Statistical Package for Social Sciences (SPSS) 25.0 software. This study used descriptive statistics as well as comparative statistics. Means and standard deviations (SD) were reported for the numerical data. One-way ANOVA (homogeneous distributions) and Welch's test (non-homogeneous distributions) compared numerical variable distributions across independent groups. *Post-hoc* tests were performed to pinpoint group differences when ANOVA yielded significant results. In instances of equal variances, Tukey's test was implemented; in instances of unequal variances, Tamhane's test was employed. Data distributions and group comparisons were visualized using graphical methods such as box plots and bar graphs. All statistical analyses used a significance level of $p<0.05$.

RESULTS

Three groups of 8 rats each participated: a healthy control group, an I/R group (global ischemia), and a treatment group (post-ischemia hirudotherapy). The tissue biochemical analysis parameters across the experimental groups are comprehensively detailed in Table 1. Tissue TNF-α levels differed significantly across the three groups (Welch test: p=0.022). *Post-hoc* pairwise analysis revealed a significant increase in the I/R Group (712.44±158.39 ng/L) compared to the healthy Control Group (536.38±78.59 ng/L, p=0.017), indicating that global cerebral ischemia/reperfusion induced a measurable localized pro-inflammatory response. TNF-α levels in the Treatment Group were (644.59±97.61 ng/L), positioned intermediately between the Control and I/R groups, though *post-hoc* pairwise comparisons involving the Treatment Group did not reach statistical significance.

Concurrently, significant alterations were obtained for hypoxic and ischemic biomarkers in brain tissue samples. Tissue hypoxia-inducible factor-1 Alpha (HIF-1α) levels differed significantly among the groups (one-way ANOVA: p=0.042), showing a robust elevation in the Treatment group (2249.51±415.63 pg/ml) compared to the healthy Control group (1339.64±610.73 pg/ml; *post-hoc* p=0.038). Similarly, tissue ischemia-modified albumin (IMA) levels demonstrated statistical significance (Welch test: p=0.013), with a significant variation observed between the Con-

trol (128.31±8.86 U/ml) and Treatment (142.28±9.91U/ml) groups (*post-hoc* p=0.030). No statistically significant differences were observed among the groups for the remaining tissue biomarkers, including SOD, IL-6, NO, CGRP, GPx, CAT, and EDN1 (all p>0.050). Furthermore, serum concentrations for all measured biochemical parameters did not exhibit any statistically significant variations between the groups (all p>0.050) (Table 2). Histopathological findings are summarised in Table 3, and the distribution of histopathological scores among the experimental groups is illustrated in Fig. 2.

Table 1. Distribution of biochemical results of tissue samples among experimental groups.

Tissue Sample	Control (n=8)[a]	I/R (n=8)[b]	Treatment (n=8)[c]	p value	post-hoc
HIF-1α (pg/ml)	1339.64±610.73	1609.63±932.79	2249.51±415.63	0.042**	a-c: 0.038
IMA (U/ml)	128.31±8.86	153.55±24.99	142.28±9.91	0.013*	a-c: 0.030
NO (μmol/L)	15.85±5.43	21.15±5.20	12.68±10.34	0.084*	–
CGRP (ng/L)	50.55±51.66	62.89±50.35	40.03±41.03	0.632*	–
TNF-α (ng/L)	536.38±78.59	712.44±158.39	644.59±97.61	0.022*	a-b: 0.017
IL-6 (pg/ml)	110.56±87.84	164.23±122.98	184.58±86.31	0.333**	–
SOD (ng/ml)	40.10±7.15	33.67±19.47	42.65±9.01	0.517*	–
CAT (ng/ml)	99.84±8.91	84.82±33.04	88.68±19.39	0.405**	–
GPx (ng/ml)	21.91±3.95	28.21±6.60	25.07±5.01	0.083**	–
EDN1 (pg/ml)	8.59±10.34	6.78±8.29	5.36±3.25	0.717**	–

* Welch Test, ** One-way ANOVA Test. Calcitonin gene-related peptide: CGRP, catalase: CAT, endothelin-1: EDN1, glutathione peroxidase: GPx, hypoxia-ınducible factor-1 alpha: HIF-1α, ıschemia modified albumin: IMA U/ml: arbitrary units per millilitre (manufacturer-defined unit for Sunred ELISA series, biologically validated in time-matched rat ischemia-reperfusion models [Ünal et al., 2026]), interleukin-6 (IL-6), L: liter, μmol: micromol, ml: milliliter, ng: nanogram, nitric oxide: NO, pg: picogram, superoxide dismutase: SOD, tumor necrosis factor alpha: TNF-α, U: unit.

Table 2. Distribution of serum results among experimental groups.

Serum Sample	Control (n=8)[a]	I/R (n=8)[b]	Treatment (n=8)[c]	p value
HIF-1α (pg/ml)	784.28±98.57	636.51±259.38	702.39±281.87	0.627**
IMA (U/ml)	82.66±12.65	76.53±7.94	85.38±20.10	0.496**
NO (μmol/L)	8.36±1.61	5.05±3.11	7.11±1.74	0.080**
CGRP (ng/L)	17.38±16.63	20.50±19.78	31.33±9.92	0.251*
TNF-α (ng/L)	215.81±180.93	371.01±36.99	360.03±75.69	0.328**
IL-6 (pg/ml)	54.43±46.26	49.35±23.87	64.17±33.77	0.667**
SOD (ng/ml)	19.37±13.59	18.73±12.18	27.15±2.11	0.194*
CAT (ng/ml)	57.87±13.26	52.29±7.34	60.55±5.94	0.158**
GPx (ng/ml)	11.41±1.91	10.88±1.51	10.86±1.63	0.854**
EDN1 (pg/ml)	30.17±35.82	32.66±22.12	13.66±4.47	0.135*

* Welch Test, ** One-way ANOVA Test. Calcitonin gene-related peptide: CGRP, catalase: CAT, endothelin-1: EDN1, glutathione peroxidase: GPx, hypoxia-ınducible factor-1 alpha: HIF-1α, ıschemia modified albumin: IMA, interleukin-6 (IL-6), L: liter, μmol: micromol, ml: milliliter, ng: nanogram, nitric oxide: NO, pg: picogram, superoxide dismutase: SOD, tumor necrosis factor alpha: TNF-α, U: unit.

Table 3. Distribution of histopathological scores of brain tissues among experimental groups.

Brain tissue	Control (n=8)[a]	I/R (n=8)[b]	Treatment (n=8)[c]	p value	*post-hoc*
Degeneration Score	0.00	3.00±0.00	1.25±0.46	<0.001*	a-b: <0.001 a-c: <0.001 b-c: <0.001
Necrosis Score	0.00	3.00±0.00	0.50±0.75	<0.001*	a-b: <0.001 b-c: <0.001
Edema Score	0.00	3.00±0.00	0.75±1.03	<0.001*	a-b: <0.001 b-c: <0.001
Bleeding Score	0.00	2.37±0.51	0.25±0.70	<0.001*	a-b: <0.001 b-c: <0.001

* One-way ANOVA Test.

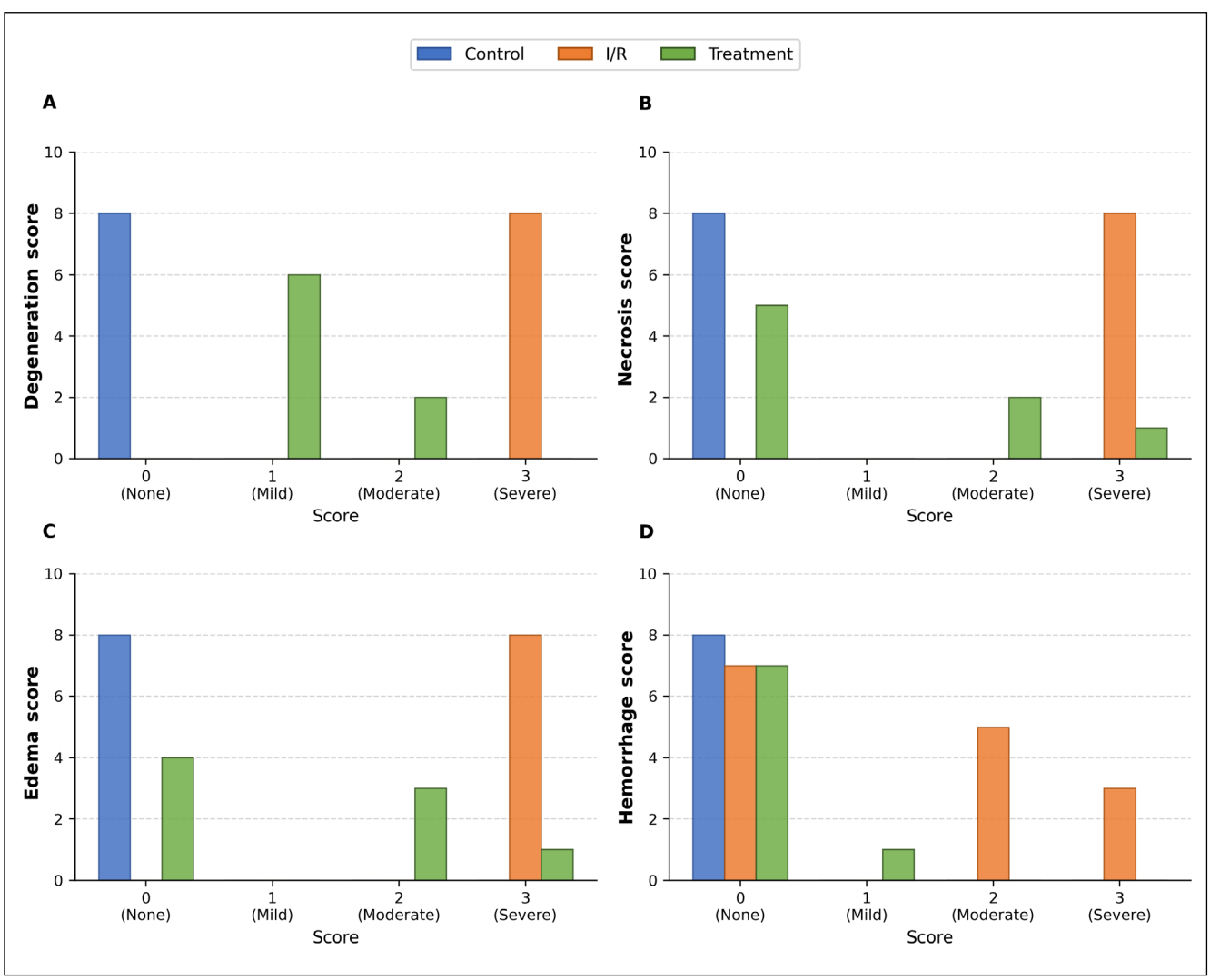

Fig. 2. Distribution of histopathological scores among experimental groups. (A) Distribution of Brain Degeneration Score, (B) Distribution of Brain Necrosis Score, (C) Distribution of Brain Edema Score, (D) Distribution of Brain Hemorrhage Score.

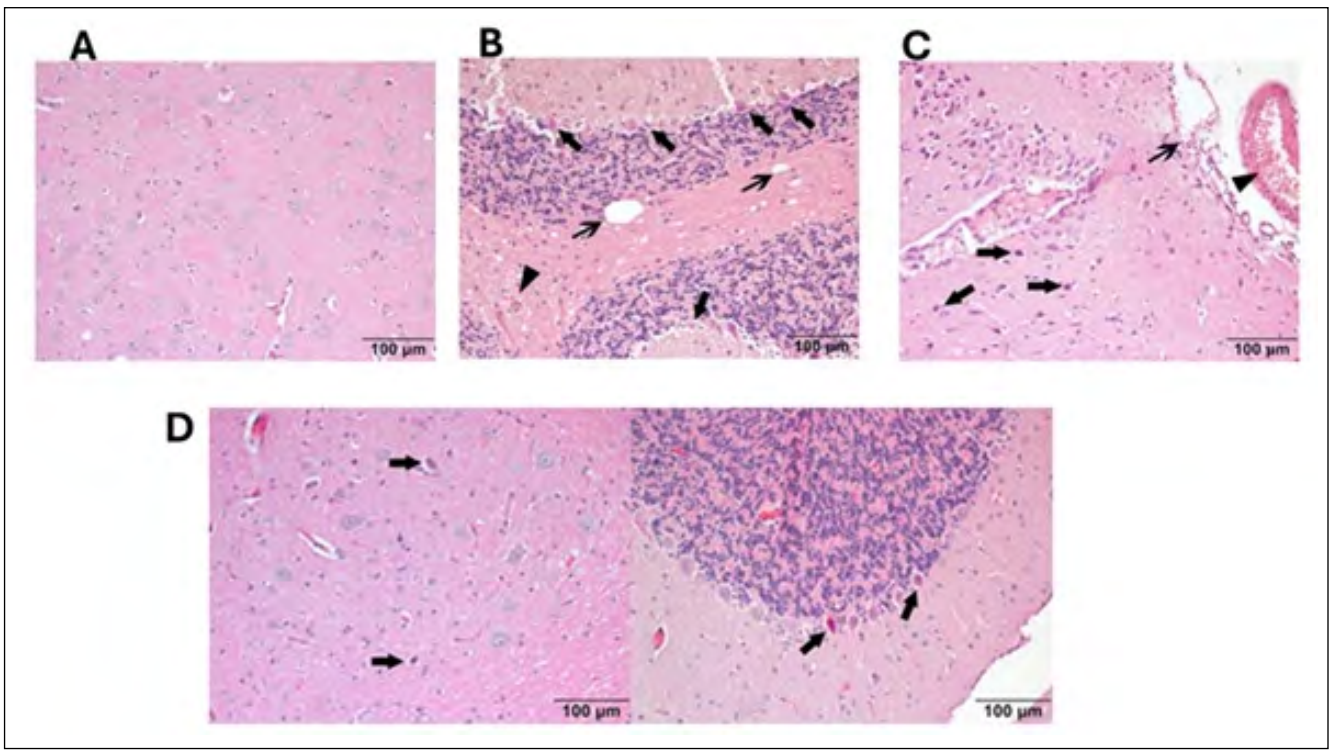

Fig. 3. Histopathological findings in the control, I/R and treatment groups. Haematoxylin-eosin (HE)-stained coronal sections of cerebral cortex. (A) Control Group: Normal cortical tissue architecture with intact neuronal and glial cell morphology identified by morphological criteria on HE staining. No pathological changes observed. (B) I/R Group: Thick arrows: neuronal degeneration and necrosis (eosinophilic cytoplasm, pyknotic nuclei, vacuolisation, lysis); Thin arrow: demyelination; Arrowhead: hyperaemia. (C) I/R Group: Thick arrows: neuronal degeneration and necrosis (eosinophilic cytoplasm, pyknotic nuclei, vacuolisation, lysis); Thin arrow: hemorrhage; Arrowhead: hyperaemia. (D) Treatment Group: Arrows: scattered mildly degenerated cells in representative sections; overall cortical tissue architecture and integrity substantially maintained compared to I/R group.

DISCUSSION

In this study, hirudotherapy reduced oxidative stress and histopathological damage in cerebral ischemia-reperfusion injury. Leech saliva contains biologically active molecules such as hirudin, bdellins, and eglins, which exhibit anticoagulant, anti-inflammatory, and microcirculatory regulatory effects. These properties may play a role in reducing ischemia-reperfusion injury. The cerebral I/R model in this study exhibited significant elevations in TNF-α, IMA, and HIF-1α levels. Hirudotherapy was associated with significant histopathological improvement, accompanied by modulation of these biochemical responses. Compared with the I/R group, the treatment group demonstrated significantly reduced degeneration, necrosis, edema, and hemorrhage. The findings indicate that hirudotherapy offers neuroprotective benefits through anti-inflammatory and microcirculation-enhancing effects.

Hirudin, an anticoagulant in leeches, is key to hirudotherapy's effectiveness against cerebral hypoxia; it prevents blood clots and increases blood flow to the brain (Huan et al., 2023). Increased blood flow enhances clinical outcomes *via* improved oxygen supply to oxygen-deprived tissues (Mousavi et al., 2016). Furthermore, the anti-inflammatory substances in leech saliva could decrease neuronal injury by controlling the neuroinflammatory response to hypoxia (Rowland et al., 2019). Preventing complications of hypoxic brain injury may be helped by controlling inflammation (El Hasnaoui-Saadani et al., 2009). Angiogenesis, stimulated by HIF-1α pathways in response to hypoxia, may be enhanced by hirudotherapy, leading to better oxygenation (Davidson et al., 2015). Clinical applications of hirudotherapy have shown success in venous congestion and wound healing, suggesting potential for cerebral hypoxia treatment (Cheung et al., 2014). Mechanisms like anticoagulation, vasodilation, and reduced tissue pressure in this therapy improve microcirculation, potentially aiding in the healing of hypoxic damage (El Hasnaoui-Saadani et al., 2009; Fan et al., 2013; Cheung et al., 2014; Davidson et al., 2015). Our study showed that hirudotherapy led to significant improvements in both anti-inflammatory parameters (TNF-α, IMA, and HIF-1α) and the results of histopathological

examinations. However, more extensive clinical trials are necessary to determine its effectiveness and safety (Fainberg et al., 2023). It is also important to account for the potential risks of infection and allergic reactions (Fainberg et al., 2023).

Cerebral hypoxia protocols could benefit from hirudotherapy integration, potentially improving patient outcomes through hirudin's anticoagulant and neuroprotective properties (Huang et al., 2021). Hirudin prevents thrombus formation, thus increasing cerebral blood flow and reducing ischemic damage (Huang et al., 2021). In addition, this process can encourage healing in oxygen-starved tissues by supporting angiogenesis and cellular function *via* the HIF-1α pathways (Foster et al., 2009). The combined effects of hirudin and other treatments could be synergistic (Willaims & Pearce, 2006). Innovative treatment strategies combining pharmacological and holistic approaches are enabled by this natural compound.

Bioactive molecules in medicinal leech saliva are particularly responsible for the reduction of degeneration, necrosis, oedema, and hemorrhage observed in this study. Beyond anticoagulation, the thrombolytic and anti-thrombotic dimensions of leech saliva deserve specific attention. Destabilase - a polyfunctional enzyme unique to leech salivary secretion - exerts fibrinolytic activity through isopeptidolysis: the hydrolysis of ε-(γ-Glu)-Lys isopeptide bonds in stabilised fibrin (Baskova & Zavalova, 2001). This mechanism allows dissolution of aged, stabilised clots resistant to plasmin-based lysis - particularly relevant in the context of microvascular occlusion following cerebral I/R injury. Concurrently, eglin-type serine protease inhibitors suppress elastase and cathepsin G activity, dampening the neutrophil-driven inflammatory cascade that amplifies endothelial damage during reperfusion. The combined action of destabilase-mediated microvascular recanalisation and eglin-mediated attenuation of neutrophil proteases likely contributes to the histopathological improvements observed in the Treatment Group.

This study makes significant contributions to the literature, being one of the few to examine hirudotherapy's effects on the brain. Although the effects of hirudotherapy on peripheral circulation have been extensively studied, its impact on cerebral I/R injury remains largely unexplored (Sig et al., 2017; Junren et al., 2021). This situation is highly significant because our study fills a gap in the field. Our findings are similar to those of Mousavian et al. (2022), who showed hirudotherapy's effectiveness in reducing necrosis and improving survival in a peripheral ischemia model. This similarity points to hirudotherapy's possible protective effects across peripheral and central nervous system tissues.

Hirudotherapy may improve microcirculation, increasing oxygen and nutrient delivery to tissues with low oxygen, as evidenced by the reduction in edema, necrosis, and bleeding in the treatment group. Proteins from the leech Hirudo verbana, including hirudin and calin, have anti-inflammatory and anticoagulant effects, possibly increasing cerebral blood flow to reduce edema and cellular degeneration. The histopathological improvements seen in our study align with mechanisms described in other research (Baskova & Zavalova, 2001; Mousavian et al., 2022; Lin et al., 2016; Sig et al., 2017; Junren et al., 2021).

Hirudotherapy effectively modifies inflammatory and oxidative stress responses to cerebral I/R damage, as shown by biochemical and histopathological analyses. The elevation of HIF-1α levels in the Treatment Group, exceeding those of both the Control and I/R Groups, warrants careful interpretation. Rather than representing ongoing ischaemic damage, we propose that this elevation reflects an active adaptive neuroprotective response. Hirudotherapy, by promoting gradual microvascular recanalisation through destabilase-mediated fibrinolysis and hirudin-mediated anticoagulation, likely sustains a controlled sub-lethal hypoxic stimulus sufficient to activate HIF-1α-mediated protective pathways - including angiogenesis, erythropoietin synthesis, and metabolic adaptation - without triggering the oxidative burst associated with sudden reperfusion. This interpretation is supported by the concurrent reduction in TNF-α levels and the significant improvement in histopathological scores in the Treatment Group. It is noteworthy that the reperfusion period was limited to 30 min, which may not have been sufficient to capture the full trajectory of HIF-1α-mediated adaptive responses; extended reperfusion periods in future studies would likely reveal more pronounced neuroprotective effects of hirudotherapy.

Improved histopathology mirroring biochemical changes further supports hirudotherapy's beneficial effects on microcirculation. Hirudotherapy's positive effects on ischemia-induced flap necrosis, leading to significantly higher flap survival rates, were demonstrated by Mousavian et al. (2022). Hirudotherapy shows promise in preserving tissue integrity, as suggested by the study, through better microcirculation and less inflammation. Our findings similarly indicate a direct link between decreased cerebral damage such as degeneration, necrosis, and edema and reduced inflammation and oxidative stress at a biochemical level. Therefore, hirudotherapy mitigates cerebral I/R damage by focusing on inflammation and oxidative stress, based on the results. Improvements in TNF-α, IMA, and HIF-1α suggest hirudotherapy could complement cerebrovascular disease treatments (Baskova & Zavalova,

2001; Mousavian et al., 2022; Mousavi et al., 2016; Zoroddu et al., 2024). A more comprehensive understanding of this effect's scope and mechanisms necessitates larger, longer-term studies.

Results indicate that hirudotherapy could serve as a supportive addition to cerebrovascular disease treatments. To reduce tissue damage during acute cerebral ischemic events, improved microcirculation and suppressed inflammation are crucial. Anticoagulants and anti-inflammatories within leech saliva regulate vascular tone, resulting in increased oxygen and nutrient flow to ischemic zones and enhancing tissue survival (Baskova & Zavalova, 2001; Junren et al., 2021). Our study, along with Mousavian et al.'s (2022), strengthens the evidence for hirudotherapy's diverse therapeutic effects.

LIMITATIONS

The limitations of this study must be taken into account. The absence of a sham-operated control group represents an important limitation; future studies should include a sham surgery group to isolate the contribution of surgical trauma and anaesthesia from ischaemia-induced injury. Additionally, Bradford protein quantification was not performed for tissue homogenates, which we acknowledge as a limitation for future studies. The short post-reperfusion follow-up period (30 min) prevented assessment of longer-term effects. Oxidative enzyme responses (SOD, CAT, GPx) are highly time-dependent; the acute experimental window may have been insufficient to capture full antioxidant dynamics - consistent with findings from our group's concurrent acute I/R studies (Ünal et al., 2025; Ünal et al., 2026) and supported by Sarikan & Savas (2024), who demonstrated significant antioxidant balance improvements in chronic hirudotherapy. Finally, the small sample size (n=8 per group) warrants cautious interpretation of the results.

CONCLUSION

Cerebral I/R injury was associated with significant alterations in TNF-α, IMA, and HIF-1α levels. Hirudotherapy was associated with significant histopathological improvement and modulation of inflammatory and hypoxia-related biochemical responses. Treatment resulted in significant improvements in histopathological parameters, including reduced degeneration, necrosis, edema, and hemorrhage. These findings demonstrate the beneficial effects of hirudotherapy for cerebral I/R damage, offering a valuable contribution to the literature. Hirudotherapy demonstrated potential neuroprotective effects in this experimental model, as reflected by histopathological improvement and modulation of selected biochemical parameters, suggesting that it may have value as a complementary therapeutic approach for cerebrovascular diseases. Further clinical trials are necessary to verify the effectiveness and safety of this treatment.

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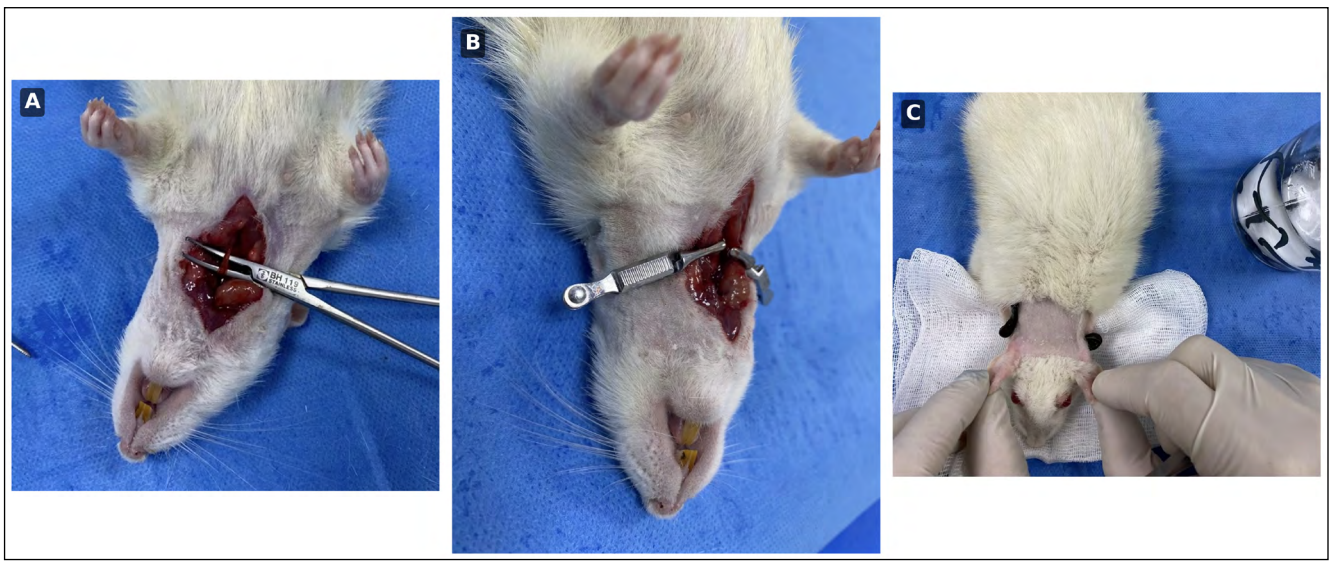
SUPPLEMENTARY MATERIALS



Supplementary Fig. 1. Intraoperative documentation of the experimental procedures. (A) Midline cervical incision with surgical exposure of bilateral common carotid arteries under general anaesthesia (ketamine/xylazine). (B) Bilateral CCA occlusion induced using atraumatic vascular clamps to establish global cerebral ischaemia. (C) Hirudotherapy – bilateral application of small Hirudo verbana leeches (0.5-0.7 g each) to the mastoid processes (processus mastoideus) following reperfusion. Leeches detached spontaneously after 30-45 minutes.