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Research Article

A study on levels of cytokines and their regulators in the adrenal gland of scorpion-envenomed albino male rats

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Abstract
Scorpion envenomation is a major global health challenge, with the *Leiurus* genus being particularly hazardous. The venom of the newly described species, *Leiurus macroctenus*, remains poorly understood regarding its composition, organ-specific effects, and the ability to induce immune response in affected tissues. This study analyzed the levels of pro- and anti-inflammatory cytokines and their regulators – transcription factors (NF-κB, HIF-1α) and heat shock proteins (HSP-60, HSP-70) – in the albino male rats adrenal gland following intramuscular injection of *L. macroctenus* venom. The results revealed a significant increase in the content of all studied proteins, peaking 24 h after venom administration (except for HSP-60, which peaked at 3 h). Among pro-inflammatory cytokines, IL-8 showed the most prominent elevation, exceeding control value by 79% at 24 h ($p < 0.001$). Among anti-inflammatory cytokines, IL-4 content increased to a greater extent, being 43% above control ($p < 0.001$). Similarly, NF-κB and HSP-70 exhibited the largest increases among their corresponding groups, with levels at 24 h being 31% and 17% higher, respectively, than those in the control ($p < 0.001$). By 72 h, the values of most parameters, except for HSP-60, remained significantly elevated despite a downward trend. Such findings may reflect the ability of *L. macroctenus* venom to induce an inflammation in the rat adrenal gland. These results contrast with data observed in the kidneys and lungs of rats exposed to the same venom, which may be attributed to the unique microenvironment of adrenal gland and the interplay between stress hormones and immune signals within this organ.

Keywords: Adrenal gland, Cytokines, Envenomation, Inflammatory response, *Leiurus macroctenus*.

INTRODUCTION

Currently, scorpion envenomation remains a major global public health challenge, with approximately 1.5 million cases estimated worldwide annually (Mabunda *et al*., 2024). Although the risk of scorpion envenomation is highest in rural areas of tropical and subtropical regions, climate change extends scorpion habitats, facilitating their expansion into developed countries (Pucca *et al*., 2025; Godoy *et al*., 2021; Ahmadi *et al*., 2020; Moradzadeh Roozbehani *et al*., 2023). Thus, the number of scorpion envenomation cases is expected to continue to rise over time.

Scorpions belong to the phylum *Arthropoda*, class *Arachnida*, and order *Scorpiones*. They are currently represented by over 2,600 species, with the *Buthidae* family accounting for about 95% of envenomations (Mabunda *et al*., 2024; Puzari *et al.*, 2025; Bavani *et al*., 2022; Tobassum *et al*., 2020). While most scorpion stings usually result in non-life-threatening local symptoms, approximately 5% of reported cases are severe, exhibiting systemic manifestations and requiring hospitalization; about 0.3% of these severe cases are fatal, resulting in an estimated 3,250 deaths globally each year (Tobassum *et al*., 2020 and Almeida *et al.*, 2025).

The toxicity of scorpion venom is determined by its components. The most studied of them are peptides, which constitute about 5% of scorpion venom dry weight. Based on structural features, they are divided into disulfide-bridged peptides (DBPs) and non-disulfide -bridged peptides (NDBPs). DBPs are long- and short-chain neurotoxins targeting voltage-gated Na^+, K^+, Ca^{2+}, and Cl^- channels. The most consequential long-chain Na^+-channel toxins are further categorized into α- and β -toxins based on their mechanisms of action. Toxins affecting channels for other ions belong to the short-chain peptide family (Mabunda *et al*., 2024; Tobassum *et al.*, 2020; Gunas *et al*., 2024a). By acting on ion channels, neurotoxins influence the secretion of neurotransmitters, hormones, and cytokines (Mabunda *et al.*, 2024; Gunas *et al*., 2023a, 2024; Petricevich, 2010). In particular, scorpion α-toxins, through inhibition of Na^+-channel inactivation, contribute to overstimulation of the autonomic nervous system, causing a massive release of catecholamines and acetylcholine, which is manifested by characteristic symptoms of envenomation such as hyperglycemia, hypertension, agitation, tachycardia, restlessness, and hyperthermia (a sympathetic reaction driven by 'catecholamine storm'), as well as diarrhea, hypersalivation, vomiting, lacrimation, miosis, and profuse sweating (a parasympathetic reaction due to cholinergic effects) (Mabunda *et al*., 2024 and Godoy *et al*., 2021). NDBPs, accounting for over a third of all identified peptides in scorpion venom, exhibit antibacterial, antifungal, antiviral, bradykinin-potentiating, immunomodulatory, or other biological activities (Mabunda *et al*., 2024 and Gunas *et al*., 2024a). Scorpion venom also contains proteins, including enzymes such as phospholipases, hyaluronidases, serine- and metalloproteinases, L-amino acid oxidases, as well as enzyme's inhibitors, lipids, nucleotides, glycosaminoglycans, biogenic amines, free amino acids, inorganic salts, and metal (Mabunda *et al*., 2024 and Gunas *et al*., 2024a).

The immune response to scorpion venom typically includes the production of pro- and anti-inflammatory cytokines, chemokines, eicosanoids, and growth factors (Matkivska *et al*., 2024; Adi-Bessalem *et al*., 2013). Upon recognition by pattern recognition receptors (PRRs), some scorpion venom components – venom-associated molecular patterns (VAMPs) – can initiate intracellular signalling pathways culminating in the activation of multiple transcription factors (Reis and Arantes, 2024). Alarmins, such as heat shock proteins (HSPs) and other stress-induced cellular compounds, are damage-associated molecular patterns (DAMPs) that, like VAMPs, can interact with PRRs (Zuliani, 2023; Zininga *et al*., 2018). Nuclear factor kappa B (NF-κB) is a key transcription factor regulating pro-inflammatory cytokine production. However, hypoxia-inducible factor-1 (HIF-1) due to established link between inflammation and hypoxia, and the known hypoxic effects of scorpion venom, may also play a role in modulating the expression of genes encoding inflammatory mediators during scorpion envenomation (Watts and Walmsley, 2019; Ramakrishnan *et al*., 2014 and Salman and Hammad, 2017).

It is known that animal venoms affect the adrenal glands and other organs of the hypothalamic-pituitary-adrenal (HPA) axis, causing adrenal cortical cell hyperplasia, mitochondrial dysfunction, vascular changes, and exacerbated inflammation and oxidative stress (Kobzina-Didukh, 2024; Daachi *et al*., 2020). The adrenal gland contains different cells that produce hormones, cytokines, neurotransmitters, and other substances, creating a unique microenvironment that serves as a key site for bidirectional immune-neuroendocrine interaction mediated by paracrine factors. This crosstalk is pivotal for the regulation of adrenal function and may be especially crucial in the context of the 'catecholamine storm' provoked by scorpion α-toxins, combined with the release of adaptive hormones in response to acute pain (Mabunda *et al*., 2024; Kanczkowski *et al.*, 2016 and Santhosh *et al.,* 2016).

Among the family *Buthidae*, the genus *Leiurus* stands out as one of the most hazardous (Mabunda *et al*., 2024 and Pucca *et al*., 2025). Until recently, this genus was considered monospecific for *L. quinquestriatus*, but after significant taxonomic reclassification, it now includes 22 species, each with a distinct geographic range (Ward *et al*., 2018; Seiter *et al*., 2016; Afifi *et al*.,

2016). *L. macroctenus*, described in 2014 by Lowe *et al*, is distinguished from other *Leiurus* species by a set of morphometric and morphological characteristics; its existence has also been confirmed at the genetic level (Lowe *et al*., 2014 and Alqahtani and Badry, 2020). Although the precise composition of *L. macroctenus* venom is undetermined, venoms from phylogenetically related species are known to contain the aforementioned components (Afifi *et al*., 2016). Nonetheless, the venoms from even closely related species sometimes exhibit substantial quantitative and qualitative variations in their composition (Tobassum *et al*., 2020).

Currently, little is known about the role of inflammation in the pathogenesis of *L. macroctenus* envenomation, and data on the levels of pro- and anti-inflammatory cytokines in affected organs under this condition are also limited. Considering the pivotal role of adrenal glands in the physiological stress response system and immune-neuroendocrine interaction, along with the above mentioned findings regarding the ability of various venoms to impact HPA-axis organs, this study aimed to analyze the levels of cytokines and their regulators – transcriptional factors and heat shock proteins – in the rat adrenal gland after intramuscularly injection of *Leiurus macroctenus* venom.

MATERIALS AND METHODS

Scorpion collection and maintenance

Ten mature specimens of *L. macroctenus* were used in this study. All scorpions were previously wild-collected in Oman and morphologically identified by Mark Stockmann according to Lowe *et al*. (2014) before being housed in a private collection in Ibbenbüren, Germany. Scorpions were maintained separately in transparent plastic containers (10´5´5 cm) containing 1 cm of Exo Terra 'Desert Sand' as substrate. Distilled water was provided ad libitum in centrally placed water bowls and refilled weekly. Environmental parameters were maintained at 25–35ºC temperature, 50–60% humidity, and natural light cycles. Proper aeration was ensured through numerous perforations in container. Feeding occurred once per week with a single *Shelfordella lateralis* cockroach; uneaten prey was removed within 2 days. Monthly, containers were cleaned to remove any food debris.

Venom collection

The procedure of venom collection was performed according to Ozkan and Filazi's electrostimulation method, modified by Yaqoob *et al*. (Ozkan and Filazi, 2004 and Yaqoob *et al*., 2016). Each scorpion was fixed, and electrodes were positioned on the cephalothorax and telson. A 24V electric current was then applied for 5 seconds to the base of the telson, while its opposite edge was directed into a sterile phial to collect the venom. The number of electrode-scorpion contacts varied, up to 10, depending on the volume of collected venom. A two-week interval was maintained between milking acts. The collected venom samples were stored at -20° C until further use.

Animal model and experimental design

Laboratory albino male rats were housed in the accredited vivarium in accordance with the 'Standard Rules for Organizing, Equipping and Maintaining Experimental Biological Clinics (vivariums)'. Animals were kept on a standard diet under controlled conditions (20 –24°C, 30–70% humidity, 12-h light/dark cycle).

Rats selected for the experiment, weighing 180±3 g, underwent a veterinary examination before being randomly grouped, weighed, numbered, and marked accordingly. To ensure unbiased distribution, randomization was performed using a computer-generated random number generator (RAND function in Microsoft Excel).

Experimental rats (n=80) were intramuscularly injected with 0.5 ml venom solution (28.8 µg/mL; LD50 = 0.08 mg/kg), previously dissolved in saline solution (0.9%), while control animals (n=10) were administered 0.5 mL saline solution (0.9%) alone. The selected LD50 value was based on findings published by Gunas *et al*. (2024b). Four experimental groups were formed, corresponding to the 1, 3, 24, and 72 h time points after venom administration. A single control group was utilized for comparison across all time points, as the short experimental duration (72 hours) and the developmental stage of the animals precluded significant age-related or physiological drift. All animals were selected from the same general pool and maintained under identical controlled environmental and nutritional conditions throughout the study to rule out potential batch effects. Twenty rats were initially allocated to each experimental group. Following the expected mortality (approx. 50%), ten surviving animals per group were utilized for the final analysis. At the end of each designated time period, the rats were euthanized by CO_2 inhalation.

Ethical approval

All experiments on animals were performed in compliance with the international principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Council of Europe, 1986). The study was approved by the Ethical Committee of Taras Shevchenko National University of Kyiv (protocol No. 2 approved 19.08.2021).

Rat adrenal glands homogenization

Adrenal glands were isolated and homogenized at 4°C immediately after euthanasia. Homogenization was

carried out using 50 mM Tris-HCl (pH 7.4) buffer with the addition of 140 mM NaCl and 1 mM EDTA. Buffer volume (in grams) was five times higher than the mass of isolated organs. The resulting crude homogenate was centrifuged at 600 g for 15 min, with the supernatant collected, followed by re-centrifugation at 15000 g for 15 min to remove the nuclear and mitochondrial fractions. Obtained homogenate aliquots were frozen in liquid nitrogen for storage. Protein concentration was measured by the Bradford method (Bradford, 1976).

Cytokines, transcription factors and heat shock proteins immunoassay

The levels of pro- and anti-inflammatory cytokines (interleukins (ILs) IL-1β, IL-4, IL-6, IL-8, IL-10, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ)) as well as the content of their regulators – NF-κB, HIF-1 α-subunit (HIF-1α), HSP-60, HSP-70 – in the rat adrenal gland homogenates were quantified by enzyme-linked immunosorbent assay (Commercial ELISA kits, Biotrak ELISA System, Healthcare, USA) using 96-well, high-binding microplates, according to the standard instructions for soluble proteins (Crowther, 2020).

Briefly, the samples were diluted to 1 μg/mL in 0.05 M Tris-HCl buffer (pH 7.4) and incubated in sterile ELISA plate wells overnight at 4°C. After incubation, unbound antigen was removed by washing the wells with immobilization buffer. Non-specific binding was blocked by incubation with 5% skim milk for 1 h at 37 °C. Following blocking, plates were washed again with buffer containing 0.1% Tween-20, and incubated with the corresponding primary antibodies (Santa Cruz Biotechnology, USA) (1:3000) for 1 h at 37°C. Next, plates were washed with buffer containing 0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, USA) (1 : 3000) for 1 h at 37°C. After that, wells were again washed with 0.1% Tween-20 buffer, and the peroxidase reaction was visualized by incubating with 0.4 mg/mL o-phenylenediamine (OPD) substrate (Sigma-Aldrich, USA), diluted in 0.05 M phosphate-citrate buffer, in the presence of 30% H_2O_2. The reaction was stopped in 10 min by adding 100 μL 1 M H_2SO_4. The optical density of the samples was measured on a microplate reader (μQuantTM, BioTek Instruments, Inc.) at a wavelength of 492 nm.

Statistical analysis

Data entry and statistical analysis were performed using Microsoft Excel (Microsoft Office) and Statistica ver. 8.0 for Windows. Prior to analysis, data distribution was assessed for normality using the Shapiro-Wilk test. Homogeneity of variances was evaluated using Levene's test. Since the data met the assumptions of normality and homogeneity of variances, group differences were analyzed using one-way analysis of variance (ANOVA). When a significant overall effect was detected, post-hoc analysis was performed to identify between-group differences. Comparisons of all experimental groups (1, 3, 24, and 72 h after venom administration) with the control group were conducted using Dunnett's post-hoc test. In addition, a predefined pairwise comparison between the 72 h and 24 h groups was performed using a planned contrast with Bonferroni correction. Results are presented as mean ± SEM. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Cytokine profile analysis

It was established that the intramuscular injection of *L. macroctenus* venom caused a gradual increase in the content of both pro-inflammatory and anti-inflammatory cytokines in the rat adrenal glands. In particular, the levels of all studied pro-inflammatory cytokines exhibited an initial rise within 1 h post-administration, peaking at 24 h. In 72 h after envenomation, their content declined compared to 24 h, but still exceeded the control values (Fig. 1).

In 1 h following venom injection, IL-8 and IL-6 levels demonstrated a more pronounced rise (by 34% and 25%, respectively, relative to the control group; $p < 0.001$), while TNF-α, IL-1β, and IFN-γ content increased to a lesser extent, ranging from 11 to 15% ($p<0.01$ for TNF-α, $p < 0.05$ for IL-1β, and $p<0.001$ for IFN-γ).

By 3 h, IL-8 continued to exhibit the greatest increase (by 55%; $p < 0.001$), with IL-1β, IL-6, and IFN-γ levels exceeding control values by 40-44% ($p < 0.001$), and TNF-α by 24% ($p < 0.001$).

At 24 h, IL-8 content showed the greatest increase (79%; $p < 0.001$), whereas TNF-α showed the smallest increase (34%; $p < 0.001$). At the same time, IL-1β, IL-6, and IFN-γ levels were 51-53% higher than the control values ($p < 0.001$). Thus, throughout the experimental timeline, IL-8 content consistently exhibited the most pronounced alterations, except at 72 h, when IL-1β showed the largest rise (32% above control; $p < 0.001$), followed by IL-6 (27%; $p < 0.001$).

By 72 h post-venom administration, IL-8 content decreased by 30% ($p < 0.001$) from its 24-h peak but still remained 24% above the control level ($p < 0.001$). The decline in the levels of other pro-inflammatory cytokines at 72 h ranged from 12 to 18% compared to their corresponding 24-h values ($p < 0.001$).

Studied venom administration also caused a rise in the levels of anti-inflammatory cytokines, with a maximum observed at 24 h (Fig. 2). IL-4 content increased to a greater extent, exceeding control level by 30%, 37%, 43%, and 26% at 1, 3, 24, and 72 h, respectively ($p < 0.001$). In contrast, the corresponding levels of IL-10 were 5%, 12%, 17%, and 7% above the control value (p

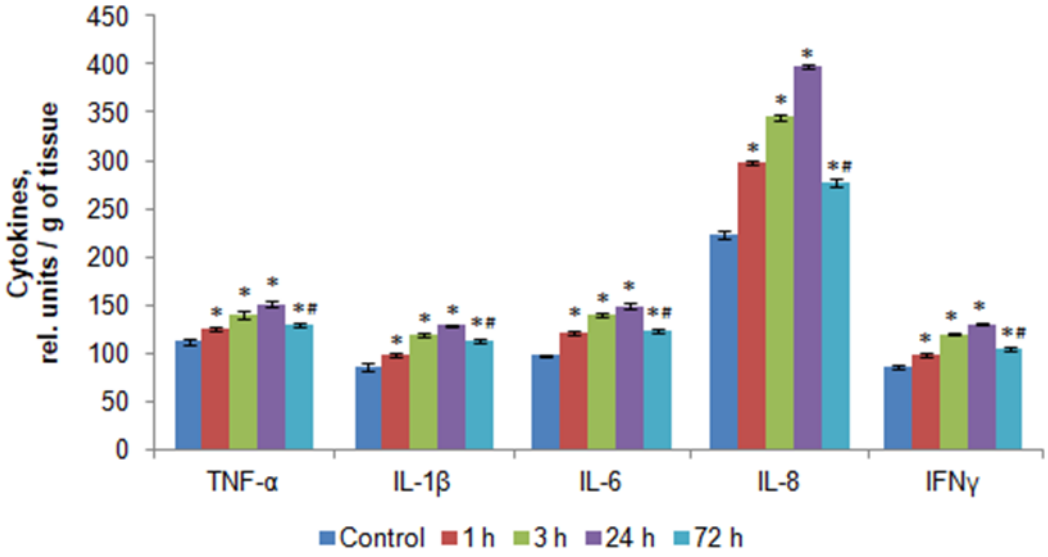

Fig. 1. *Pro-inflammatory cytokine levels in the adrenal glands of rats after Leiurus macroctenus envenomation. Results are presented as mean ± SEM (n = 10). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test for comparisons with the control group and a Bonferroni-corrected planned comparison between the 72 h and 24 h groups. * p < 0.05 vs. control, # p < 0.05 vs. 24 h*

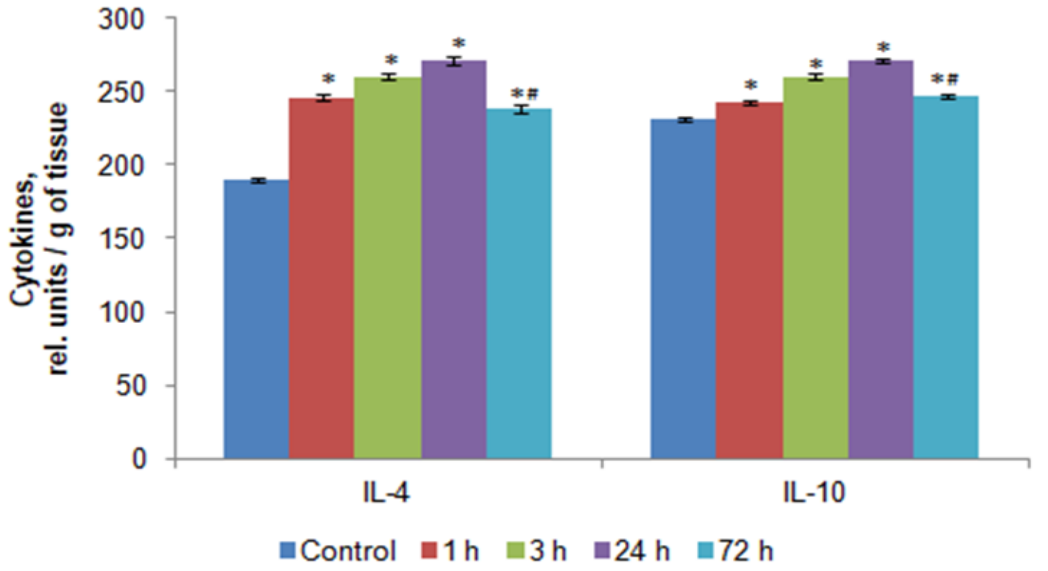

Fig. 2. *Anti-inflammatory cytokine levels in the adrenal glands of rats after Leiurus macroctenus envenomation. Results are presented as mean ± SEM (n = 10). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test for comparisons with the control group and a Bonferroni-corrected planned comparison between the 72 h and 24 h groups. * p < 0.05 vs. control, # p < 0.05 vs. 24 h*

< 0.001). At 72 h, IL-4 content declined by 13%, while IL-10 levels - by 9% compared to the 24-h peak (p < 0.001).

Quantification of transcription factor levels

The levels of the studied transcription factors in the adrenal glands of rats injected with *L. macroctenus* venom exhibited a dynamic pattern characterized by a gradual increase, reaching a maximum at 24 h, followed by a decrease up to 72 h (Fig. 3).

NF-κB content demonstrated a more pronounced elevation, exceeding the control value by 14% at 1 h, 19% at 3 h, and 31% at 24 h (p < 0.001). In comparison, HIF-1α levels showed increases of 8%, 12%, and 15% at 1, 3, and 24 h, respectively, versus the control level (p < 0.001). By 72 h, NF-κB levels decreased by 12% relative to the 24-h peak (p < 0.001); however, they remained elevated by 15% compared to the control group. And HIF-1α content, while decreasing by 4% compared to its 24-h level (p < 0.001), remained 10% higher than the control value (p < 0.001).

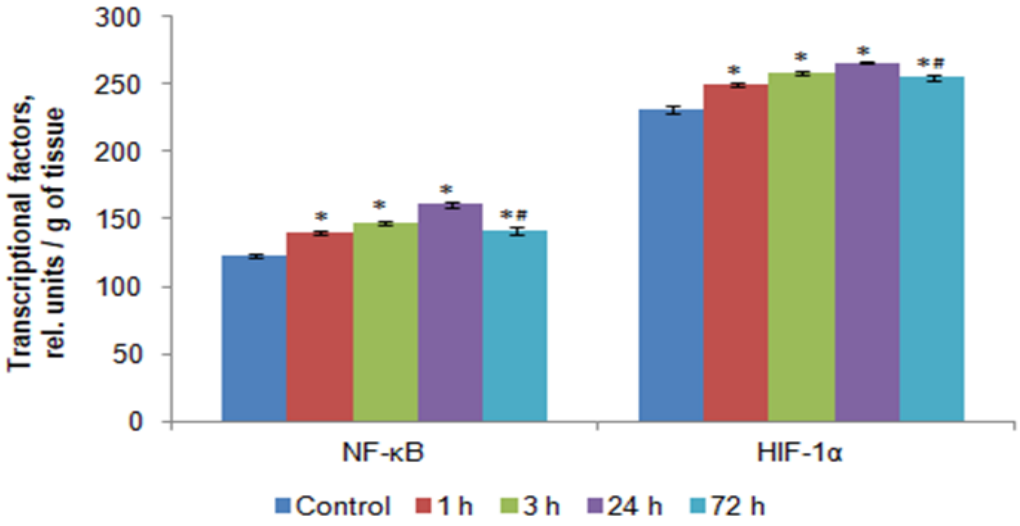

Fig. 3. *Transcriptional factors content in the adrenal glands of rats after Leiurus macroctenus envenomation. Results are presented as mean ± SEM (n = 10). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test for comparisons with the control group and a Bonferroni-corrected planned comparison between the 72 h and 24 h groups. * p < 0.05 vs. control, # p < 0.05 vs. 24 h*

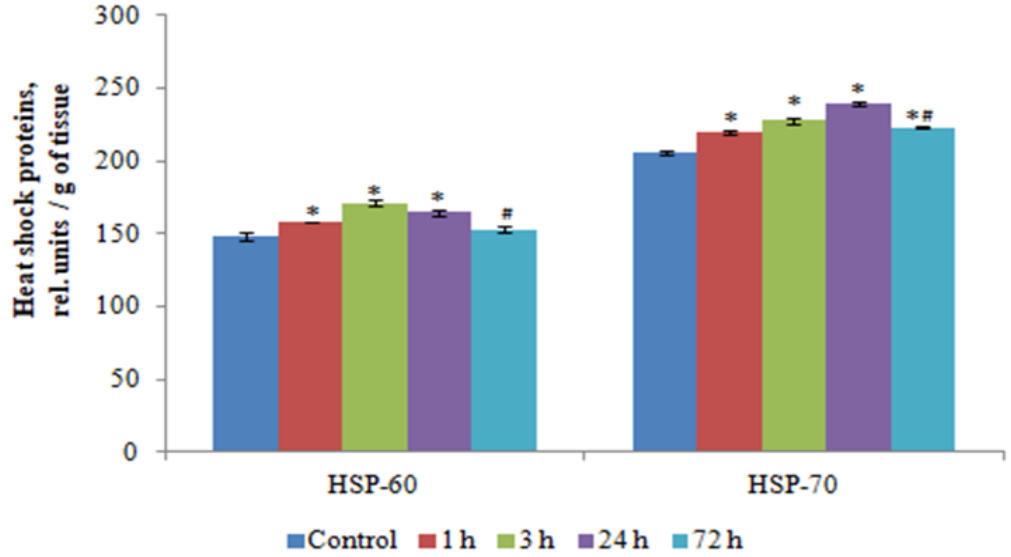

Fig. 4. *Heat shock proteins content in the adrenal glands of rats after Leiurus macroctenus envenomation. Results are presented as mean ± SEM (n = 10). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test for comparisons with the control group and a Bonferroni-corrected planned comparison between the 72 h and 24 h groups. * p < 0.05 vs. control, # p < 0.05 vs. 24 h*

Determination of heat shock protein content

An initial slight increase in the levels of both studied HSPs was established after *L. macroctenus* venom administration. Specifically, at 1 h post-administration, both HSP-60 and HSP-70 content exceeded the corresponding control values by 7% ($p<0.05$ for HSP-60, and $p < 0.001$ for HSP-70) (Fig. 4). Subsequently, the levels of studied HSPs continued to rise, with HSP-60 peaking at 3 h (an increase by 16% from the control level; $p < 0.001$) and HSP-70 at 24 h (by 17%; $p < 0.001$). Thereafter, the content of both proteins began to decline. In particular, HSP-60 level at 24 h decreased relative to its 3-h maximum, yet remained significantly higher than control levels (by 11%; $p<0.001$), and by 72 h returned to the control value. HSP-70 content at 72 h showed a 7% decrease from its 24-h peak ($p < 0.001$), but remained 8% above control levels ($p < 0.001$).

DISCUSSION

Scorpion venom is a complex mixture of peptides, proteins, and other substances. Its principal constituents responsible for common envenomation symptoms are

neurotoxins targeting Na^+, K^+, Ca^{2+}, and Cl^- channels, as well as enzymes (serine proteases, metalloproteinases, phospholipases, hyaluronidases), enzyme inhibitors, and peptides exhibiting diverse biological and pharmacological activities (Mabunda *et al*., 2024 and Gunas *et al*., 2023a,b, 2024a). It is well established that upon entering the organism, venom components diffuse into various tissues, causing damage. Existing studies indicate that venom neurotoxins alter ion channel functions, provoking a massive release of catecholamines and acetylcholine. This leads to the development of typical envenomation symptoms described in the literature, including hypertension, agitation, hyperthermia, cardiac arrhythmias, muscle spasms, diarrhoea, hypersalivation, and vomiting, among others (Mabunda *et al*., 2024; Gunas *et al*., 2024a). Hyaluronidases and metalloproteinases degrade extracellular matrix (ECM) components, thereby accelerating toxin diffusion, while phospholipases act as hemolytic agents that disrupt cell membranes, leading to tissue necrosis and haemorrhage. Additionally, metallo- and serine proteases activate latent forms of toxins and endogenous signaling molecules (Mabunda *et al*., 2024; Gunas *et al*., 2024a and Petricevich, 2010). Furthermore, a recently identified scorpion venom L-amino acid oxidase (LAAO) serves as a source of reactive oxygen species (ROS), which can induce apoptosis in vascular endothelial cells, causing hemorrhage and edema, and also alter the course of intracellular signaling cascades, thereby contributing to tissue damage (Mabunda *et al*., 2024; Zhang and Zhang, 2016 and Magalhães *et al*., 2021).

The ability of venoms from various scorpions, including species phylogenetically related to *L. macroctenus (e.g., Leiurus abdullahbayrami* and *L. quinquestriatus*), to induce an inflammatory response is well-established (Borges and Lomonte, 2024 and Fereidooni *et al*., 2023). Immune response activation can be initiated by neurotoxins, which, by modulating ion channels, may stimulate the release of inflammatory mediators. Moreover, by activating pro-inflammatory signalling cascades, scorpion venom components can up-regulate cytokine production, recruit and activate immune cells, and stimulate the synthesis and activation of endogenous matrix metalloproteinases (MMPs). In turn, MMPs influence cytokine gene expression and promote the release of latent growth factors from ECM, exacerbating tissue damage. Nevertheless, some other venom components may exhibit immunosuppressive properties, thereby inhibiting the inflammatory response (Gunas *et al*., 2023a, 2024a). Thus, the first step of the present study was to analyze the cytokine profile in the adrenal glands of rats envenomed by *L. macroctenus* venom to assess venom's ability to induce an inflammatory response in this organ.

The findings revealed that intramuscular injection of *L. macroctenus* venom led to a gradual increase in the content of both pro- and anti-inflammatory cytokines in rat adrenal glands, with levels peaking at 24 h and subsequently decreasing by 72 h without returning to control values (Fig. 1). Throughout the experimental timeline, except for the 72 h time point, IL-8 content exhibited the most pronounced alterations, while the levels of TNF-α displayed the least. The changes in IL-1β, IL-6, and IFN-γ levels were similar, specifically at 3 and 24 h after venom administration. Regarding anti-inflammatory cytokines, IL-4 levels increased more than IL-10 levels (Fig. 2).

The study observed that the increase in pro- and anti-inflammatory cytokine levels in the adrenal glands of rats may reflect the ability of *L. macroctenus* venom components to induce an inflammatory response in this endocrine organ. Specifically, it is well established that the early inflammatory stage is characterized by infiltration of the inflammatory focus by neutrophils and pro-inflammatory M1 macrophages. According to the literature, IL-8, secreted by resident macrophages and other cells, acts as a chemoattractant for neutrophils, facilitating their recruitment and activation at the injury site (Moradzadeh Roozbehani *et al*., 2023; Chávez-Galán *et al*., 2015). M1 macrophages exacerbate inflammation by producing IL-1β, IL-6, TNF-α, and IFN-γ, and they efficiently eliminate damaged tissue debris. As the inflammatory focus is cleared of pathogens and damaged cells, macrophage polarization shifts toward anti-inflammatory M2 Cells. M2-macrophages secrete IL-4 and IL-10, which control pro-inflammatory cytokine activity and suppress inflammation – specifically, IL-4 is known to block the synthesis of IL-1, IL-6, IL-8, and TNF-α, while IL-10 can inhibit the production of IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-8, and IL-12 (Moradzadeh Roozbehani *et al*., 2023; Gunas *et al*., 2023a and Chávez-Galán *et al*., 2015). In addition, these cells also release growth factors with immunomodulatory, angio- and mitogenic properties, participating in the regulation of later stages of the inflammatory response and regenerative processes (Chávez-Galán *et al*., 2015). Elevated IFN-γ levels are known to promote macrophage polarization toward an M1 phenotype, while high IL-10 content induces M1-M2 transition (Cheng *et al*., 2021). Moreover, because stimuli promoting macrophage polarization often coexist, macrophages can simultaneously express genes of both phenotypes, exhibiting a more pro-inflammatory (M1-like) profile during early inflammation and a more anti-inflammatory (M2-like) profile in later stages (Strizova *et al*., 2023). However, despite the observed shifts in cytokine levels, confirming a classic inflammatory reaction requires complementary histopathological assessments. Future studies should be conducted to identify cellular infiltration and pathomorphological alterations in the adrenal glands under the action of *L. macroctenus* venom, providing

direct morphological evidence to support these molecular findings.

Overall, the balance between pro- and anti-inflammatory cytokines determines the progression or resolution of inflammation and the potential for tissue repair. Existing studies suggest that IL-1β, IL-6, and TNF-α are essential for effective pathogen defense, but become harmful when produced in excess. In turn, IL-4 and IL-10 are critical for suppressing acute inflammation, but their over-release can impair immune function. IFN-γ, despite being a pro-inflammatory cytokine, exhibits pleiotropic properties and, like anti-inflammatory cytokines, can prevent excessive immune system activation and tissue damage (Petricevich, 2010; Matkivska *et al.*, 2024).

Currently, data on cytokine content in mammalian adrenal gland tissue following scorpion envenomation is extremely limited. A number of studies report high serum levels of pro-inflammatory cytokines in humans and experimental animals envenomed by *Tityus serrulatus*, *Centruroides noxius*, *Buthus occitanus*, and *L. quinquestriatus* (Moradzadeh Roozbehani *et al.*, 2023; Gunas *et al.*, 2023a; Razi Jalali *et al.*, 2015; Saidi *et al*., 2013). However, the cytokine profile of blood serum does not necessarily reflect that of individual tissues. Increased pro-inflammatory cytokine production by macrophages has also been observed *in vitro*, with peak levels of IL-1α, IL-1β, and TNF-α at 12 h, IL-6 at 24 h, and IFN-γ at 72 h following envenomation by *Tityus serrulatus* (Petricevich and Lebrun, 2005).

Intriguingly, as previously reported, the cytokine profile in several other organs of rats injected with *L. macroctenus* venom did not indicate a classical inflammatory response. Importantly, these results were obtained from tissues collected from the same animals used in the current study, allowing a direct comparison of the responses of different rat organs to the studied venom (Matkivska *et al.*, 2024; Gunas *et al.*, 2024a). Specifically, kidney tissue exhibited increased IFN-γ levels, no significant alterations in IL-1β and IL-8 content, and reduced levels of IL-6 and TNF-α. At the same time, content of anti-inflammatory IL-10 and IL-4 was elevated, peaking 24 h post-administration. Thus, in the kidneys, the studied venom induced a significant imbalance in pro- and anti-inflammatory cytokine levels during the immune response (Matkivska *et al.*, 2024). Similar bidirectional alterations in pro- and anti-inflammatory cytokine levels were observed in the lungs of rats envenomed by *L. macroctenus*: a reduction in IL-1β, IL-6, IL-8, and TNF-α, coupled with an increase in IL-4, IL-10, and IFN-γ. All observed alterations peaked at 24 h post-injection, with levels trending toward normalization by 72 h, approaching control values (Gunas *et al.*, 2024a).

Overall, the impact of scorpion venoms on the adrenal gland remains one of the least-studied aspects of medicine. While signs of HPA-axis activation by scorpion venoms (such as elevated serum levels of corticosterone and adrenocorticotropic hormone, and cortical cell hyperplasia) have been documented in mouse models exposed to *Aegaeobuthus nigrocinctus*, *Androctonus australis hector*, *Tityus serrulatus*, and *Hottentota gentili*, a broader understanding is needed. Notably, venoms from other animals, including snakes, spiders, and bees, exhibit marked toxicity towards organs of the HPA axis, predominantly the adrenal glands. It is manifested by adrenal cortical cell hyperplasia, vascular dilation, cortical hemorrhages, and mitochondrial dysfunction, and is accompanied by alterations in stress hormone levels (Kobzina-Didukh, 2024).

The peculiarities of the cytokine profile of the adrenal glands of rats envenomed with *L. macroctenus* venom that we observed may be a consequence of the unique microenvironment of this organ. It is well-established that the adrenal gland contains various cell types, including hormone-producing adrenocortical and chromaffin cells, their progenitors, as well as neuronal, glial, endothelial, and immune cells. Evidence from existing studies indicates that all these cells influence one another's functions, either directly or via paracrine signalling, by secreting diverse biologically active substances such as steroid hormones, catecholamines, cytokines, neurotransmitters, and neuropeptides (Kanczkowski *et al.*, 2017). According to literature data, scorpion venom, rich in highly toxic components, causes severe acute pain, which can be considered a physical and psychological stressor capable of activating HPA-axis and triggering the release of adrenal stress hormones (Santhosh *et al*., 2016). It has also been reported that scorpion venom sodium channel toxins can trigger catecholamine release, leading to the development of an 'autonomic storm' (Dokur *et al.*, 2017). Moreover, as noted in the literature, resident or infiltrating immune cells can influence hormone-secreting cells via cytokine and chemokine receptors, thereby altering their endocrine function (Yang *et al.*, 2017 and Kanczkowski *et al.*, 2017). Thus, the adrenal gland microenvironment under scorpion envenomation is likely enriched in glucocorticoids and catecholamines, which possess powerful immunomodulatory effects (Yang *et al.*, 2017). While existing studies suggest that glucocorticoids have potent anti-inflammatory properties, this control of inflammation is thought to occur primarily under basal conditions. Under certain circumstances, these hormones can actually exacerbate inflammation – a phenomenon referred to as glucocorticoid resistance (Agil *et al.*, 2013). Similarly, catecholamines are also capable of multiple immunomodulatory effects. Specifically, it has been reported that, upon binding to β2-adrenergic receptors, these hormones promote IL-10

secretion, reduce TNF-α production by macrophages, and inhibit ROS production by neutrophils. However, through their interaction with α-adrenergic receptors, catecholamines can exacerbate inflammation by increasing TNF-α release from macrophages (Kanczkowski *et al.*, 2016). Moreover, non-immune cells of adrenal gland may also serve as a source of pro-inflammatory cytokines. For example, adrenal cortical cells are known to express several TLRs, allowing them to produce cytokines in response to VAMPs (Kanczkowski *et al.*, 2016).

Thus, the changes in the adrenal gland cytokine profile observed in our study likely reflect neuroendocrine-immune crosstalk within the adrenal microenvironment under the action of *L. macroctenus* venom. However, to provide direct evidence of this potential interplay between stress hormones and immune signals, further research is needed. While the present study focused on levels of inflammatory markers, measuring stress hormones fell outside its scope. Therefore, future studies should emphasize temporal profiling of intra-adrenal glucocorticoid and catecholamine content to evaluate the potential linkage between the levels of these adaptive hormones and cytokines. Furthermore, the use of glucocorticoid receptor antagonists and/or adrenergic blockers would be essential to determine if blocking these hormonal signals abrogates the observed cytokine shifts. Overall, such experiments would be crucial for fully elucidating the role of neuroendocrine-immune crosstalk in the response of this endocrine organ to the studied venom.

The next step of the present study focused on evaluating the levels of key regulators of cytokine production in the adrenal glands of *L. macroctenus* venom-injected rats. Transcriptional factors NF-κB and HIF-1α control the production of a wide range of proteins, including pro-inflammatory cytokines, and are thus considered the most important orchestrators of the inflammatory response. It was shown that the temporal changes in both NF-κB and HIF-1α levels were consistent with the observed cytokine dynamics, with NF-κB demonstrating a more pronounced elevation (Fig. 3). These findings align with results from other studies: a similar significant increase in NF-κB and HIF-1α levels, peaking at 24 h, was observed in kidney and lung tissues of rats envenomed by *L. macroctenus* (Gunas *et al.*, 2024a and Matkivska *et al.*, 2024).

The precise mechanisms by which the immune system detects venom components and triggers inflammation are not fully understood. However, several studies indicate that PRRs, particularly TLRs on immune cells, are involved in these processes. Although TLRs are typically activated by PAMPs and DAMPs, some of them, including TLR2, TLR4, and TLR9, can also detect venom components (VAMPs). Moreover, upon scorpion envenomation, TLRs may also be stimulated by endogenous danger signals released from damaged cells – DAMPs, or alarmins. And NF-κB activation is a key downstream consequence of TLR stimulation by VAMPs/DAMPs (Gunas *et al.*, 2024a). Additionally, the increase in NF-κB levels observed in present study may be attributed to ROS. Although ROS levels in the adrenal glands were not directly evaluated in the present study, ROS could be generated by certain venom components, such as LAAO, or by neutrophil NADPH oxidase at the inflammatory site. According to the existing literature, this mechanism is possible because ROS can activate NF-κB by oxidizing its inhibitor kinase or by modulating the ERK/MAPK signalling cascade (Mabunda *et al.*, 2024; Hong *et al.*, 2024).

The HIF family of transcription factors mediates cellular response to hypoxia. HIF-1 is a heterodimer consisting of an oxygen-sensitive HIF-1α subunit and HIF-1β (Gunas *et al.*, 2024a). Nearly all immune cells express HIF-1α. Under normoxia, iron-dependent prolyl hydroxylase (PHD) hydroxylates this subunit, which is then degraded by the proteasome. Conversely, oxygen deprivation impairs PHD function, preventing HIF-1α breakdown. As a result, HIF-1α dimerizes with HIF-1β and up-regulates hypoxia-inducible genes (Gunas *et al.*, 2024a; Watts and Walmsley, 2019 and Ramakrishnan *et al.*, 2014). Thus, the increase in HIF-1α levels observed in our study could be linked to the formation of hypoxic conditions within the damaged adrenal gland. Direct biochemical markers of hypoxia were not measured in our study. However, this potential mechanism is consistent with reports that venom protease-induced vascular damage and coagulopathy lead to ischemia (Matkivska *et al.*, 2023), which, in turn, triggers hypoxic responses. In addition, respiratory and cardiovascular complications of scorpion envenomation are known to induce acute hypoxia, which may contribute to HIF-1α stabilization (Gunas *et al.*, 2024a). Moreover, the elevated levels of pro-inflammatory cytokines we detected may reflect an inflammatory response, which is known to locally create hypoxic conditions due to the high metabolic activity of immune cells exceeding oxygen supply (Watts and Walmsley, 2019; Ramakrishnan *et al.*, 2014). It is well established that HIF-1 induces the expression of genes encoding proteins involved in hypoxic adaptation and also regulates the immune response. Through HIF-1 activation, hypoxia significantly alters gene expression in macrophages accumulating in hypoxic zones, inducing numerous genes encoding pro-inflammatory cytokines and chemokines. In turn, pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are capable of up-regulating HIF-1 even under normoxic conditions, and TNF-α and IL-1β achieve this through NF-κB activation (Gunas *et al.*, 2024a; Watts and Walmsley, 2019; Ramakrishnan *et al.*, 2014; Pham *et*

al., 2021 and Malkov *et al.*, 2021). Evidence also suggests an interplay among HIF-1α, TLR4, and inflammation: stimulation of TLR4 on macrophages (e.g., by VAMPs) activates signalling pathways that promote HIF-1α accumulation. Thus, both HIF-1α and NF-κB are important for TLR4-dependent pro-inflammatory cytokine expression, induced by venom components (Gunas *et al.*, 2024a). The influence of ROS may serve as an additional mechanism for increasing HIF-1α content. Specifically, it has been reported that ROS prevent HIF-1α degradation by modifying and inactivating PHD and reducing Fe^{2+} content (Iacobini *et al*., 2022). Furthermore, ROS upregulate HIF-1 by altering cellular signalling (Hong *et al.*, 2024; Bae *et al*., 2024; Iacobini *et al*., 2022).

Heat shock proteins HSP-70 and HSP-60 are the other regulators of cytokine production analyzed in the present study. It is well-known that the canonical roles of these HSPs are linked to intracellular protein folding and proteasomal degradation (Zininga *et al.*, 2018). Specifically, intracellular HSP-70 provides cytoprotection through its chaperone activity, supporting high-quality protein synthesis, and by modulating cell signaling, thereby exerting an anti-inflammatory effect (Alberti *et al.*, 2021). HSP-60, as a mitochondrial chaperonin, supervises protein folding and free radical activities within mitochondria (Singh *et al.*, 2024). However, existing studies suggest that beyond their intracellular functions, both proteins are actively secreted or released into the ECM from stressed or necrotic cells (Alberti *et al.*, 2021). Extracellular HSPs perform non-canonical functions and participate in the inflammatory response: acting as DAMPs, they activate TLR-2 and TLR-4 on immune cells, triggering NF-κB activation and subsequent pro-inflammatory cytokine synthesis (Zininga *et al.*, 2018; Alberti *et al.*, 2021; Murao *et al.*, 2021).

The present study demonstrated increased levels of both HSP-60 and HSP-70 in the adrenal glands of rats injected with *L. macroctenus* venom compared with the control group. However, the temporal characteristics of these changes varied significantly, with HSP-60 content exhibiting a considerably faster rise and subsequent decline than HSP-70 (Fig. 4).

Currently, there is very little data on the levels of HSPs in tissues or biological fluids of experimental animals and humans envenomed by scorpions. It is well-established that HSP-60 and HSP-70 can be present both intracellularly and extracellularly in the adrenal gland (Zininga *et al.*, 2018). Furthermore, adrenal HSP content is known to increase under physiological stress and can be induced by ACTH (Pignatelli *et al.*, 2003). Specifically, as noted in the literature, elevated levels of HSP-70 mRNA were observed in the adrenal cortex of rats under immobilization stress, while a rise in the level of the corresponding protein was observed in models of acute heat, cold, immobilization, and combined stress (Sadek *et al.*, 2023). It's known that the expression of genes encoding HSPs is induced by the activation of heat shock transcription factors (HSFs), with HSF-1 being the most studied (Westerheide *et al.*, 2012). Various stressors, including heat, immobilization, or hypoxia, can activate HSF-1, often involving the HPA axis and/or the sympathetic nervous system, thereby inducing HSP expression in the adrenal glands. Additionally, high ROS levels promote HSF-1 nuclear translocation and DNA binding, thus positioning oxidative stress as a transcriptional regulator of HSPs synthesis (Sadek *et al.*, 2023).

The temporal differences between HSP-60 (peaking at 3 h) and HSP-70 (peaking at 24 h) we observed likely reflect distinct subcellular localization, specific functions, and/or differential regulatory pathways for these proteins during the venom-induced cellular stress response. It is well established that HSP-60 is primarily a mitochondrial chaperonin (Singh *et al.*, 2024); thus, the early HSP-60 peak likely represents an immediate organelle-specific defence against venom-induced mitochondrial dysfunction and oxidative stress. In addition, this rapid increase might also be linked to the formation of an extracellular HSP-60 pool resulting from mitochondrial damage. However, further research on adrenal redox status and mitochondrial function is warranted to verify these hypotheses. Conversely, the delayed HSP-70 peak at 24 h may suggest a broader, systemic cytoprotective response. In the adrenal glands, HSP-70 is known to support proteostasis, inhibit apoptotic cascades, and exert anti-inflammatory effects under various stress conditions (Sadek *et al.*, 2023; Li *et al.*, 2019; Blake *et al.*, 1991; Pignatelli *et al.*, 2003; Alberti *et al.*, 2021). Thus, the early HSP-60 response appears focused on immediate mitochondrial events, while the later HSP-70 elevation reflects a comprehensive effort to maintain cellular proteostasis and, potentially, regulate neuroendocrine-immune crosstalk.

Certain methodological considerations regarding the generalizability of our results should be noted. Our experiments were conducted exclusively on male albino rats to avoid the potential influence of sex-based biological differences on the obtained results. Additionally, only a single venom dose equivalent to the LD50 was used in this study to ensure a strong systemic reaction (Gunas *et al.*, 2024b). However, clinical envenomation cases often involve sub-lethal exposures, in which the adrenal response might be less pronounced or follow a different kinetic pattern. Thus, future research incorporating female cohorts and sub-lethal doses will be essential to broaden the clinical applicability of our findings. Finally, the precise biochemical profile of *L. macroctenus* venom has yet to be fully characterized. Alt-

hough scorpions were reared under standardized laboratory conditions to minimize ecological variability, potential batch-to-batch variation cannot be entirely ruled out. To address this aspect, future research will focus on profiling venom components and developing a standardization approach based on key molecular and biochemical markers. This will help elucidate the links between specific venom components and the observed molecular changes, thereby improving the reproducibility and comparability of future studies.

Conclusion

The present research revealed increased levels of both pro- and anti-inflammatory cytokines and their regulators – transcription factors (NF-κB, HIF-1α) and heat shock proteins (HSP-70, HSP-60) – in the adrenal glands of male rats injected with *L. macroctenus* venom. Such changes in levels of key molecular markers of inflammation may reflect the venom's ability to induce an inflammatory response in this endocrine organ. However, to gain a more comprehensive understanding of the mechanisms underlying the present findings, additional investigations should include histopathological assessments to evaluate potential cellular infiltration and other pathomorphological alterations in the adrenal glands, thereby providing direct evidence of a classic inflammatory response. Further studies could also focus on determining adaptive hormone levels to elucidate the interplay between the immune and endocrine systems, and on examining the pro- and antioxidant balance to clarify the role of oxidative stress in adrenal inflammation. Given the observed differences in the responses of different tissues to *L. macroctenus* venom, it would also be valuable to evaluate cytokine levels in serum and tissue homogenates, as well as to analyze the cellular infiltrate during the first hour post-venom administration. Finally, as scorpion venom may contain both pro- and anti-inflammatory constituents that exert complex effects on various tissues, studying the precise composition of *L. macroctenus* venom and the impact of its isolated components on rat tissues remains a priority for future research.

Conflict of interest

The authors declare that they have no conflict of interest.

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