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Inflammatory and stress biomarker changes in rat liver after *Leiurus macroctenus* envenomation

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Abstract

Scorpions of *Leiurus macroctenus* species are very dangerous, but to date the effect of their venom on the content of cytokines and stress biomarkers in the liver is understudied. This work aimed to analyse pro-inflammatory, anti-inflammatory and stress biomarkers content in rat liver after *L. macroctenus* envenomation. For the first time, a significant increase in the content of both pro-inflammatory cytokines: Tumour Necrosis Factor-α (TNF-α), interleukins-1β, -6, -8 (IL-1β, IL-6, IL-8), and anti-inflammatory cytokines: interferon-γ (IFN-γ), interleukins-4, -10 (IL-4, IL-10), along with an elevation in the content of Hypoxia-Inducible Factor-1 alpha (HIF-1α), transcription factor Nuclear Factor-kappa B (NF-κB), and heat shock proteins (HSP60 and HSP70) was shown. These findings may indicate that *L. macroctenus* envenomation is associated with hepatic inflammatory/stress responses of the cellular microenvironment in the rat liver against the background of noticeable changes in the innate immune response, which, in turn, lead to the development of further pathological consequences in response to scorpion venom action. Notably, a decrease in the content of all analysed parameters was detected 72 hours after venom injection, which may indicate resolution in the liver tissue.

Introduction

Several cases of scorpion stings are recorded annually in tropical and subtropical countries causing more than 3,000 deaths from scorpion envenomation.[1] The lethality of scorpion venoms is mainly due to the ability of their neurotoxins to alter the functional properties of ion channels in excitable tissues, which leads to various symptoms such as pain, fever, shock, vomiting, dermatitis, hyperthermia, haemorrhage, cardiac dysfunction, pulmonary edema.[1-3] Multiple organ failure can develop in severe envenomation and lead to death in the absence of medical intervention.[1,2] Understanding the mechanism of scorpion venom action helps in developing antivenoms and improving clinical management of scorpion stings, which are a significant public health issue.[1-3] Scorpions belonging to the genus *Leiurus* ("deathstalker") are among the most dangerous Arthropoda. Previously considered monospecific to *L. quinquestriatus*, the genus has been taxonomically reclassified in recent years.[4,5] Currently it contains 22 species, each with a distinct geographic range.[4,5] The specific morphological and morphometric features of *L. macroctenus*, which are distinct from other species in the genus, were described in 2014, with subsequent confirmation of its existence at the genetic level.[6,7] To date, the composition of the venom of this scorpion has not yet been established. The venom of this genus is a powerful combination of neurotoxins,[8,9] chlorotoxins, and charybdotoxins.[2,10] The analyzed venoms of related species are generally characterized by the presence of the following components: a mix containing disulfide-bridged peptides (neurotoxins targeting voltage-gated ion channels) and nondisulfide-bridged peptides (amphipathic molecules that exhibit antibacterial, antifungal or antiviral effects), chlorotoxins, charybdotoxins, enzymes and their inhibitors, free amino acids, biogenic amines, serotonin, histamine, mucoproteins, nucleotides, lipids, inorganic salts, and other components.[2,5,11] Despite these data, it should be noted that venoms of even closely related species can differ significantly in both their quantitative and qualitative composition.[4,12]

The liver plays a pivotal role in immune modulation, owing to the high abundance of resident myeloid (Kupffer cells, neutrophils or macrophages) and lymphoid (natural killer cells, T cells or B cells) immune cells.[13] The liver is also affected by scorpion venoms, as it has been shown that the main lesions resulting from hepatotoxicity are edema, necrosis and/or apoptosis of hepatocytes, haemorrhage, and infiltration of inflammatory cells.[13,14] The mechanisms by which venom components from different organisms activate immune-mediated pathways of the liver damage are still not clearly established.[13-15]

Various mediators are involved in immune-inflammatory processes that play a key role in the biological disorders and tissue damage caused by scorpion venom. The exact triggering mechanism is multifactorial and is associated with the action of venom enzymes, which increase tissue permeability, causing a systemic inflammatory response, as venom toxins can easily penetrate all organs and tissues. In addition, bioactive molecules are generated, such as histamine, kinins, cytokines, as well their regulators: different transcription and growth factors.[15-17] The latter are also formed due to the increased activity of metallo- and serine-proteases, which activate endogenous signalling molecules and latent forms of toxins and modulate cytokine production.[3,15,16,18] Unfortunately, to date, no studies have quantified the role of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukins (IL-1β, IL-8, IL-6), and anti-inflammatory cytokines such as interferon-γ (IFN-γ), interleukins (IL-4, IL-10), and their regulators, transcription factors and growth factors such as transcription factor Nuclear Factor-kappa B (NF-κB), Hypoxia-Inducible Factor-1 alpha (HIF-1α), and the heat shock proteins HSP60 and HSP70 in the hepatic inflammatory response induced by *L. macroctenus* envenomation.[12,16,19,20] Taking into account the fact that liver injury is a common symptom in the severe scorpion envenoming, this work aimed to analyse the pro-inflammatory, anti-inflammatory and stress biomarkers content in rat liver after *L. macroctenus* envenomation.

Materials and Methods

Ethics

All experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive on 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by the Local Ethics Committee (protocol № 2 approved 19.08.2021).

Scorpion collection and maintenance

The study involved 10 sexually mature scorpions of the species *L. macroctenus*. All individuals were previously found and identified by Mark Stockmann (the owner and founder of the Stockmann Scorpion Breeding Facility, Hörstel, Germany) and kept in a private collection in Ibbenbüren (Germany). The animals were kept in individual boxes (10×5×5 cm) with a layer of sand ("Desert Sand", Exo Terra, HAGEN Deutschland GmbH & Co, Hagen, Germany) 1 cm thick on the bottom. Each box had a source of water, which was changed weekly. The boxes were placed in standard conditions for acclimatization (25-35°C, 50-60% humidity, sunlight). The appropriate level of aeration was maintained by holes in the boxes. Each scorpion was fed one *Shelfordella lateralis*

cockroach once a week. In case of refusal of food, the remains of cockroaches were collected after 2 days, and the boxes were thoroughly cleaned once a month.

Venom milking

Scorpion venom was obtained using the Ozkan method[21] with modifications.[22] After proper fixation of a scorpion, electrodes with a voltage of 24 V were applied to the telson and cephalothorax of the animal for 5 s. At this time, the end of the telson was directed into a sterile tube for venom collection. Depending on the amount of venom obtained, the number of contacts of scorpions with the electrodes varied from 1 to 10. The minimum interval between venom sampling from each scorpion was 2 weeks. The collected venom samples were frozen and stored at -20°C.

Venom injection and organ homogenization

Only male rats were used in order to minimize biological variability associated with sex-dependent hormonal fluctuations, particularly those related to the estrous cycle in females, which may affect cytokine production, stress responses, and inflammatory signalling pathways. Since the primary objective of this study was to characterize venom-induced hepatic inflammatory and stress biomarker dynamics, reducing endocrine-related variability allowed for more consistent interpretation of the biochemical outcomes. For the scorpion venom envenomation model, 90 male non-linear albino rats (age 2 months, 180 ± 3 grams) were used. The control group of animals, consisting of 10 rats, was injected intramuscularly with 0.5 mL of saline solution (0.9%); the experimental group (80 rats) was injected with 0.5 mL of venom solution (0.08 ± 0.01 mg/kg of body weight).[2] The method for determination of acute Lethal Dose-50 (LD50) levels of venom in rats, was reported for *Androctonus crassicauda* species, modified by Yaqoob *et al*.[22] and determined in our previous study.[2] Due to the LD50 value, 40 out of 80 animals died, so the total number of rats included in the study was 40: exactly 10 animals were taken for each time point (1 h, 3 h, 24 h and 72 h). The animals were euthanized by cervical dislocation at specific time intervals (1, 3, 24 and 72 hours after injection). Immediately after this, the animals were dissected and the livers of the animals were removed. Liver homogenization was performed at +4°C using manual homogenizer. To the organ sample was added 50 mM Tris(hydroxymethyl) aminomethane-HCl (Tris-HCl) buffer (pH 7.4) with 130 mM NaCl and 1 mM Ethylenediaminetetraacetic Acid (EDTA) in a mass ratio of organ to buffer of 1:5. The resulting homogenate was centrifuged for 15 min at 600 g, after which the resulting supernatant was centrifuged again for 15 min at 15,000 g. Aliquots of the resulting supernatant were frozen and stored in liquid nitrogen.

Protein content quantification

Protein concentrations were measured by Bradford protein assay.[23]

Enzyme-linked immunosorbent assay

To determine the content of TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, HIF-1α, NF-κB, HSP60, HSP70 in rat liver homogenates, experiments were performed in 96-well microplates made of polystyrene, which are characterized by the ability to sorb soluble protein macromolecules, according to the standard method for soluble proteins.[24] For the dilution of the homogenates, a 50 mM Tris-HCl buffer (pH 7.4) was used, which contained 130 mM NaCl. That is, the homogenate was diluted so that the protein concentration was 10 μg/mL, and 100 μL was taken as a sample for application to the wells of the microplate. Then, the samples applied to the wells of the microplate were incubated at +4°C overnight. At the next stage, after incubation, the wells were washed with a buffer with the following composition: 50 mM Tris-HCl buffer (pH 7.4) containing 130 mM NaCl and 0.05% Tween-20. To block nonspecific binding sites, a 5% solution of skim milk was applied to the wells and incubated on a shaker at low amplitude at +37°C for 1 hour. After washing with the same buffer as in the previous washing step, primary antibodies (dilution of which was 1:3,000) to the corresponding antigens were added. Depending on the antigen under investigation, primary antibodies obtained from mouse or goat blood were used: goat polyclonal IgG for TNF-α (sc-1351), IFN-γ (sc-9344), IL-1β (sc-1251), IL-6 (sc-1265), HSP60 (sc-1052), HSP70 (sc-1060); mouse monoclonal IgG for HIF-1α (sc-13515), IL-8 (sc-8427), IL-4 (sc-80094), IL-10 (sc-8438), NF-κB (sc-8414) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA). The microplates were incubated on a shaker at low amplitude at +37°C for 1 hour. After a series of washes with the appropriate buffer (50 mM Tris-HCl buffer (pH 7.4) containing 130 mM NaCl and 0.05% Tween-20), the appropriate secondary antibodies (anti-mouse A9044, anti-goat A5420) conjugated to horseradish peroxidase (dilution 1:25000) (Sigma-Aldrich, St. Louis, MO, USA) were applied. They were incubated on a shaker at low amplitude at +37°C for 1 hour. Then they were washed again with buffer as in the previous stage. To visualize the binding of secondary antibodies, 100 μL of o-phenylenediamine (OPD) solution (0.4 mg/mL) was added to the wells, which was prepared in citrate buffer (pH 5.0) containing 0.013% H_2O_2. After 10 min, the peroxidase reaction was stopped by adding 100 μL of 1M H_2SO_4. Next, the absorbance was measured on a microplate reader (μQuantTM, BioTek Instruments, Inc, Santa Clara, USA) at a wavelength of 492 nm. The content of the studied parameters was expressed in relative units, calculated as the optical density values normalized to the total protein content in the liver tissue.

Statistics

Our results were tested for normal distribution using the Shapiro-Wilk test. Homogeneity of variance was assessed using Brown-Forsythe's test for equality of variances. Then, the significance of differences between the means of experimental groups was determined by One-Way Analysis Of Variance (ANOVA) with Bonferroni's multiple comparisons test, performed in GraphPad Prism 9 (GraphPad Software Inc., Boston, USA). All tests were two-sided. The analysis was performed on raw values, and "fold-change" was only a method of presentation. Values present in figures are expressed as mean ± Standard Error of Mean (SEM). When $p<0.05$ differences between groups were considered statistically significant.

Results

We have observed a significant increase in pro-inflammatory cytokines content in rat liver after *L. macroctenus* envenomation compared to the control group: TNF-α (1.1-, 1.2-, 1.4- and 1.2-fold increment ($F =549.4$, $p\leq0.001$) in 1-72 h of envenomation, respectively), IL-1β (1.2-, 1.4-, 1.5- and 1.3-fold raising ($F=649.6$, $p\leq0.001$) in 1-72 h after injection, respectively), IL-8 (1.3-, 1.6-, 1.8- and 1.2-fold increment ($F=839$, $p\leq0.001$) in 1-72 h of envenomation, respectively) and IL-6 (1.3-, 1.4-, 1.5- and 1.3-fold increase ($F=579.3$, $p\leq0.001$,) in 1-72 h after injection, respectively). Moreover, peak levels were detected in the 24 hours after venom injection, with further decrement of TNF-α, IL-1β, IL-8 and IL-6 content (Figure 1).

Also, it was shown that envenomation had impact on anti-inflammatory cytokines content in rats livers too compared to the control group: IFN-γ content was higher by 1.2, 1.4, 1.5 and 1.2 fold ($F=768.8$, $p\leq0.001$) in 1-72 h after injection, respectively; IL-4 content elevated by 1.3, 1.4, 1.4 and 1.3 fold ($F=867$, $p\leq0.001$) in 1-72 h of envenomation, respectively; and at the same time, IL-10 content was higher by 1.1, 1.1, 1.2 and 1.1 fold ($F=603$, $p\leq0.001$) in 1-72 h of after venom injection, respectively). As well, we found that generally peak levels were observed in the 24 hours after venom injection, with further reduction of IFN-γ, IL-4 and IL-10 content (Figure 2).

In addition, our study results revealed increased content of hypoxic factor HIF-1α with 1.1-, 1.1-, 1.2- and 1.1-fold increase ($F = 613.8$, $p \leq 0,001$) in 1-72 h after injection, respectively, transcription factor NF-κB with 1.1-, 1.2-, 1.3- and 1.2-fold increment ($F = 511.4$, $p \leq 0,001$) in 1-72 h of envenomation, respectively (Figure 3), and the heat shock proteins HSP60 and HSP70 with 1.1-, 1.2-, 1.1- and 1.1-fold increment ($F = 601.5$, $p \leq 0,001$), and 1.1-, 1.1-, 1.2- and 1.1-fold raising ($F = 755.3$, $p \leq 0,001$) in 1-72 h after injection, respectively (Figure 4). For these stress biomarkers, the highest levels were observed 3 hours (for HSP60) and 24 hours (for NF-κB, HIF-1α and HSP70)

after venom injection, with a subsequent decrease in the content of NF-κB, HIF-1α, HSP60 and HSP70 (Figures 3 and 4).

Discussion

Pro-inflammatory cytokines IL-1, IL-6, and TNF-α protect the body from exogenous pathogens, but when overexpressed, they can have harmful effects. In contrast, anti-inflammatory cytokines such as IL-4, IL-10 and others are extremely important for inhibiting the acute inflammatory response. And, accordingly, when overproduced, they can suppress the body's immune function.[3,15,16,19] Pro-inflammatory cytokines have been shown to induce neutrophil migration to the site of injury during the first few hours of the acute inflammatory response, with a peak between 4 and 6 hours. At the same time, 24-48 hours after envenomation, neutrophils begin to produce chemoattractants that direct blood monocytes to the site of inflammation, leading to macrophage infiltration. In addition, neutrophils induce the synthesis of pro-inflammatory cytokines, reactive oxygen species, and proteinases, locally damaging tissues after venom administration. Overexpression of pro-inflammatory cytokines, such as TNF-α, induces the synthesis of pro-inflammatory M1 macrophages, peak levels of which have been shown to occur 1-3 days after injury.[13,16,25,26] These macrophages, which efficiently remove apoptotic neutrophils and necrotic cell debris from damaged tissue, are also capable of secreting growth factors and pro-inflammatory cytokines. Following neutrophil apoptosis, IL-10 expression increases, leading to the formation of anti-inflammatory M2 macrophages. The latter produce IFN-γ, IL-4 and -10, changing the pro-inflammatory microenvironment to an anti-inflammatory one.[26,27] We observed an increase in the content of both pro-inflammatory and anti-inflammatory cytokines (peak levels were observed 24 hours after venom injection), which may indicate a change in the phenotype of the predominant macrophage population in hepatocytes, since changes in the cytokine expression pattern are an important indicator of such a process.[3,16,25,26]

We further analysed the levels of the transcription factors NF-κB and HIF-1α, as these proteins are important regulators of cytokine biosynthesis. We found an increase in their levels after venom exposure, the peak of which coincided with the maximum detected changes in cytokine levels (24 h). This suggests a potential link between persistent inflammatory activity and the immune response. It is known that NF-κB also affects the classical signalling pathways of pro-inflammatory cytokines involved in the pathogenesis of the tissue response to *L. macroctenus* envenomation. At the same time, TNF-α is able to regulate the synthesis of NF-κB.[19,28] The increase in TNF-α and NF-κB levels observed in rat liver after venom injection suggests a potential link between persistent inflammatory activity and the immune response.[29] At the same time, the increase in NF-κB content

in rat liver homogenates exposed to scorpion envenomation may also be caused by the action of reactive oxygen species. They are highly produced in the tissues of envenomed animals and can cause an increase in NF-κB expression through oxidative modulation of a number of signalling pathways.[3,16]

The cellular response to hypoxia is mediated by The Hypoxia-Inducible transcription Factor (HIF) family. In addition, HIF-1 is also involved in the regulation of the immune response. Through HIF-1, hypoxia significantly alters the gene expression profile of macrophages that accumulate in hypoxic areas, inducing the production of many pro-inflammatory cytokines and chemokine genes.[16,19,30] At the same time, pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, as well as growth factors and bacterial products, can induce HIF under normoxic conditions In particular, TNF-α and IL-1β are able to modulate HIF expression through NF-κB activation.[19,30-32] Thus, the increased content of HIF-1α in rat liver may indicate that the immune system tried to adapt to the envenomation by activating angiogenesis and related tissue repair mechanisms.[13,33]

In addition, the cellular and immune response to the venom resulted in the activation of HSPs (chaperones): HSP60 and HSP70. Their main functions, in addition to preventing aggregation of misfolded proteins and protecting cells from stress, are to regulate immune and inflammatory responses by interacting with cytokines during the repair of damaged tissue .[17,34] The increase in HSP60 and HSP70 content in rat liver after *L. macroctenus* venom injection may indicate that the immune state after venom administration is characterized by complex interactions in acute stress response pathways. Moreover, the modulation of HSP levels reflects the transition from acute cellular stress to probable long-term immune adaptation in scorpion envenomation syndrome.[17,34] At the same time, a decrease in the level of all analysed indicators was detected 72 hours after the introduction of the venom, which may be a sign of the beginning of liver tissue recovery.[14,15,35]

Thus, our results suggest that *L. macroctenus* venom may potentially contain components with pro- and anti-inflammatory properties that affect both the cytokine profile and their regulators, altering the innate immune response, which in turn may cause complex effects on the rat liver.[14-16,19]

Therefore, it can be concluded that there is potential damage to liver tissue after venom injection. The liver, responsible for detoxifying venom toxins and endotoxins produced during envenomation , is subjected to extremely stressful conditions, as both the action of toxins and inflammatory effects can lead to damage to the integrity of its tissue, as well as possible disturbances in the functioning of the hepatobiliary system overall.[13-15,19]

We focused on a limited set of cytokines and stress-related biomarkers, which, although informative, do not provide a comprehensive picture of the entire inflammatory and metabolic response to *L. macroctenus* venom. Additionally, no histological or biochemical analyses (such as

serum alanine aminotransferase, aspartate aminotransferase, or bilirubin measurements) were performed to assess the structural and functional state of the liver following envenomation. Such tests would allow a deeper evaluation of the relationship between molecular changes and organ-level pathology. Finally, only male rats were used in this study, so that possible sex-related differences in venom response were not analyzed. Despite these limitations, our findings provide valuable insight into the dynamics of pro- and anti-inflammatory cytokines and stress biomarkers in hepatic tissue after *L. macroctenus* envenomation. They may form the basis for future studies combining molecular, histological, and functional assessments, which may be useful for developing more effective treatment protocols for scorpion envenomation syndrome .[1,2,16,19,36]

Conclusions

To conclude, for the first time, we have examined the pro-inflammatory, anti-inflammatory and stress biomarkers content in rat liver after *L. macroctenus* envenomation. In the liver tissue, a significant increase in the content of both pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) and anti-inflammatory cytokines (IFN-γ, L-4, IL-10), along with an elevation in the content of hypoxic factor HIF-1α, transcription factor NF-κB, and heat shock proteins (HSP60 and HSP70) was detected. Our results may indicate that *L. macroctenus* envenomation can provide inflammation response in the rat's liver that can potentially lead to the liver tissue impairment. Moreover, in 72 h the values of the studied parameters tended towards normalizing, approaching the control values, which may be a sign of the beginning of resolution in the liver tissue. However, the precise mechanisms of the changes we observed require additional research.

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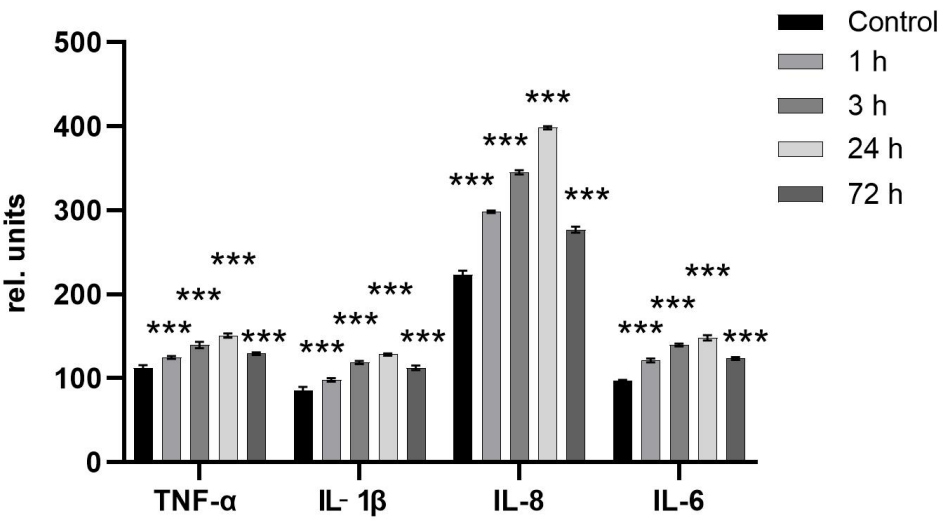

Figure 1. Pro-inflammatory cytokines tumour necrosis factor-α (TNF-α), and interleukins-1β, -8, -6 (IL-1β, IL-8, IL-6) content in rat liver after *Leiurus macroctenus* envenomation. The content of the studied parameters was expressed in relative units, calculated as the optical density values normalized to the total protein content in the liver tissue. Results are presented as mean±Standard Error of Mean (SEM) (n = 10). ∗∗∗ p≤0.001; the significance is expressed relative to the control.

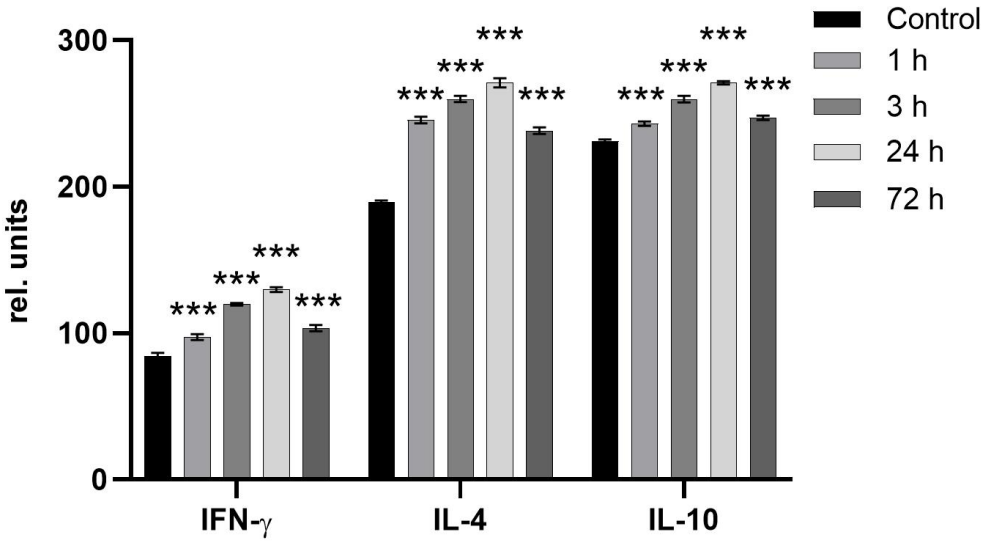

Figure 2. Anti-inflammatory cytokines interferon-γ (IFN-γ), and interleukins-4, -10 (IL-4, IL-10) content in rat liver after *Leiurus macroctenus* envenomation. The content of the studied parameters

was expressed in relative units, calculated as the optical density values normalized to the total protein content in the liver tissue. Results are presented as mean±Standard Error of Mean (SEM) (n = 10). *** p<0.001; the significance is expressed relative to the control.

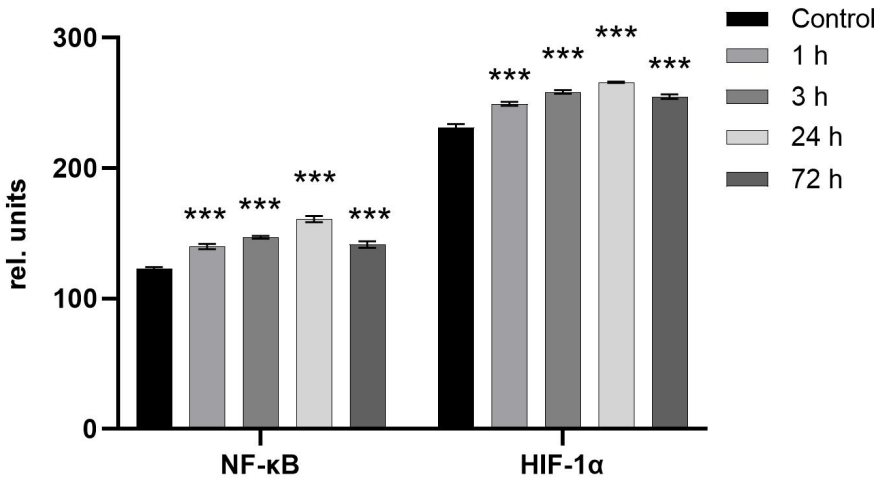

Figure 3. Transcription factor Nuclear Factor-kappa B (NF-κB), and Hypoxia-Inducible Factor-1 alpha (HIF-1α) content in rat liver after *Leiurus macroctenus* envenomation. The content of the studied parameters was expressed in relative units, calculated as the optical density values normalized to the total protein content in the liver tissue. Results are presented as mean±Standard Error of Mean (SEM) (n = 10). *** p<0.001; the significance is expressed relative to the control.

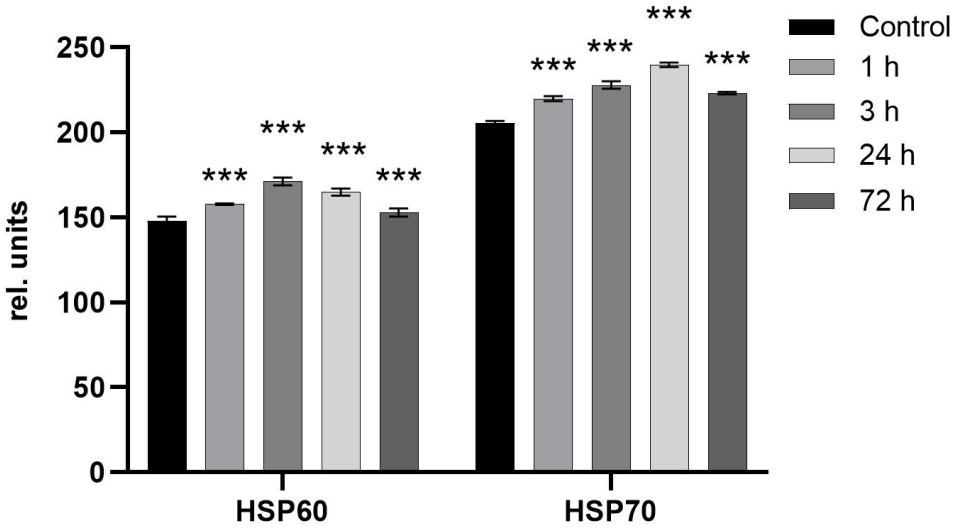

Figure 4. Heat shock proteins HSP60, and HSP70 content in rat liver after *Leiurus macroctenus* envenomation. The content of the studied parameters was expressed in relative units, calculated as the optical density values normalized to the total protein content in the liver tissue. Results are presented as mean±Standard Error of Mean (SEM) (n = 10). ∗∗∗ $p<0.001$; the significance is expressed relative to the control.

Contributions: Dzevulska Iryna designed the study, analysed data, and wrote the paper; Alevtyna Huet, Tetiana Halenova, and Tetiana Vovk performed research and statistical analysis, and wrote the paper; Nataliia Raksha and Haidai Olena designed and performed research, and offered critical revision of the manuscript; Maievskyi Oleksandr coordinated the research, provided supervision, and gave the final approval; Olexiy Savchuk coordinated and designed the research, and gave the final approval. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: the authors declare no conflict of interest.

Ethics approval: all experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive on 24

November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by the Local Ethics Committee (protocol № 2 approved 19.08.2021).

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