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CYTOKINE PROFILE AND STRESS-RESPONSE SIGNALING MARKERS IN RAT SPLEEN AFTER LEIURUS MACROCTENUS VENOM ADMINISTRATION

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This study evaluated time-dependent changes in inflammatory mediators and stress-associated signaling molecules in the spleen of rats after a single intramuscular administration of L. macroctenus venom at a dose corresponding to the median lethal dose. Venom exposure induced an early increase in pro-inflammatory cytokines within one hour, followed by delayed elevation of anti-inflammatory cytokines at 24 hours. Concurrent increases in nuclear factor kappa B, hypoxia-inducible factor 1 alpha, as well as heat shock proteins 60 and 70 indicated activation of inflammatory transcriptional pathways and cellular stress responses. By the end of the observation period (72 hours), most parameters approached control values. These findings demonstrate coordinated and reversible immune modulation in splenic tissue and highlight the involvement of secondary lymphoid organs in systemic responses to scorpion envenomation.

Key words: Leiurus macroctenus, scorpion venom, spleen, rats, cytokines, heat shock proteins, nuclear factor kappa B, hypoxia-inducible factor 1 alpha.

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ЦИТОКІНОВИЙ ПРОФІЛЬ ТА СИГНАЛЬНІ МАРКЕРИ СТРЕС-ВІДПОВІДІ У СЕЛЕЗІНЦІ ЩУРІВ ПІСЛЯ ВВЕДЕННЯ ОТРУТИ LEIURUS MACROCTENUS

У дослідженні оцінено динаміку змін рівня запальних медіаторів і сигнальних молекул, асоційованих із відповіддю на стрес, у селезінці щурів після одноразового внутрішньом'язового введення отрути L. macroctenus у дозі, що відповідала середній летальній дозі. Вплив отрути зумовлював раннє підвищення рівня прозапальних цитокінів протягом першої години з подальшим зростанням протизапальних цитокінів через 24 години. Одночасне підвищення рівнів ядерного фактора каппа В, індукованого гіпоксією фактора 1 альфа, а також білків теплового шоку 60 і 70 свідчило про активацію транскрипційних механізмів запальної відповіді та клітинних стресових реакцій. Наприкінці періоду спостереження (72 години) більшість досліджуваних показників наближалася до контрольних значень. Отримані результати демонструють координовану та зворотну імунну відповідь у тканині селезінки та підкреслюють участь вторинних лімфоїдних органів у системній реакції організму на отруєння отрутою скорпіона.

Ключові слова: Leiurus macroctenus, отрута скорпіона, селезінка, щури, цитокіни, білки теплового шоку, ядерний фактор каппа В, індукований гіпоксією фактор 1 альфа.

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Scorpion venom is a complex biological mixture composed primarily of low-molecular-weight neurotoxic peptides, enzymes, bioactive amines, and other modulatory components capable of targeting voltage-gated ion channels and disrupting neurohumoral regulation [1, 12]. Although the acute neurotoxic manifestations of envenomation are well established, increasing evidence indicates that scorpion venoms also initiate profound systemic inflammatory reactions involving dysregulated cytokine production, oxidative stress, and activation of intracellular signaling pathways [9, 11].

Experimental in vivo models of scorpion envenomation have shown that venom exposure triggers rapid elevation of pro-inflammatory mediators accompanied by leukocyte activation, enhanced vascular permeability, and immune cell infiltration into peripheral tissues [3, 4]. These systemic immune disturbances are frequently associated with oxidative imbalance and multi-organ dysfunction, suggesting that the pathophysiology of envenomation extends beyond primary neurotoxicity and involves coordinated inflammatory networks.

Deeper insights into the underlying cellular and molecular mechanisms have been gained from controlled in vitro studies demonstrating that venom components activate pro-inflammatory signaling pathways, including nuclear factor kappa B (NF-κB) and inflammasome-dependent cascades, amplified inflammatory mediator production [15]. However, despite substantial progress in characterizing systemic and cellular responses, organ-specific immune dynamics remain insufficiently defined. A detailed analysis of tissue-level inflammatory and stress-associated alterations is therefore necessary to better understand the integrated pathophysiological response to scorpion envenomation.

Among the organs particularly attractive for studying toxin-driven immune reactivity, the spleen occupies a central position. As a major secondary

lymphoid organ, the spleen filters blood-borne antigens and coordinates innate and adaptive immune responses through its diverse populations of macrophages, dendritic cells, T and B lymphocytes, and monocytes, which are actively mobilized during systemic inflammation. These features make it a sensitive indicator of toxin-induced immune activation.

Leiurus macroctenus, a newly described representative of the family Buthidae, is recognized as a potentially hazardous species due to its high toxicity and expanding geographic range. Experimental studies have demonstrated that its venom exerts pronounced neurotoxic and systemic effects, including alterations in cardiovascular function, induction of oxidative stress, and tissue-specific inflammatory responses in target organs such as the kidneys and liver [7, 8]. These findings indicate that, beyond its acute neurotoxic activity, the venom of L. macroctenus can trigger complex multisystem pathophysiological reactions. However, the splenic immune and stress-associated responses to envenomation with this species remain poorly characterized.

The purpose of the study was to determine time-dependent changes in inflammatory mediators and stress-associated signaling molecules in the spleen of rats following a single intramuscular administration of L. macroctenus venom at a dose corresponding to the median lethal dose (LD_{50}).

Materials and methods. Adult Leiurus macroctenus scorpions (n=10) were maintained individually in transparent plastic containers (10×5×5 cm) containing a 1-cm layer of natural sand substrate (“Desert Sand Exo Terra”, Hagen, Germany). Each container was supplied with a drinking bowl (with distilled water refreshed weekly) and aeration holes. Scorpions were kept under stable environmental conditions (25–35 C, 50–60 % humidity) and a natural photoperiod corresponding to the local day-night cycle (approximately 14 h light/10 h dark during the experimental period). Each animal was fed once per week with a single Shelfordella lateralis cockroach.

Venom was collected by electrostimulation using a custom-built electrical stimulator, according to the method described by Ozkan and Filazi and modified by Yaqoob et al. [13]. Briefly, after fixation, electrodes were positioned on the cephalothorax and telson. A 24 V electric current was applied to the base of the telson for 5 s, while the tip was directed into a sterile vial. The number of stimulations (up to 10) depended on the volume of venom obtained. The venom was aliquoted and stored at −20 C, and milking was performed at two-week intervals. Maintenance of scorpions and venom collection were carried out at the National Pirogov Memorial Medical University (Vinnytsia, Ukraine).

Experimental studies on rats were conducted at the Educational and Scientific Centre “Institute of Biology and Medicine”, Taras Shevchenko National University of Kyiv (Kyiv, Ukraine), where animal housing, experimental procedures, biomaterial processing and analysis of the obtained data were performed.

A total of 90 male Wistar rats (2 months old, 180±5g) were used in this study. Only male rats were used to avoid the potential influence of hormonal fluctuations associated with the estrous cycle in females, which may affect inflammatory responses and cytokine production. Throughout the entire experimental period, the animals were housed under standard vivarium conditions (20–24 C, 30–70 % humidity, 12-h light/dark cycle) with ad libitum access to standard chow and water.

To ensure unbiased distribution, animals were randomly allocated to experimental and control groups using computer-generated random numbers (RAND function in Microsoft Excel). The experimental group consisted of 80 rats that received a single intramuscular injection of scorpion venom dissolved in 0.9 % saline (Yuria-Pharm, Ukraine) at a dose of 0.08 mg/kg (0.5 mL), corresponding to the LD_{50} value [5]. Experimental animals were further divided into four subgroups according to the time of euthanasia after venom administration: 1 h, 3 h, 24 h, and 72 h. To ensure sufficient statistical power, 20 animals were initially allocated to each experimental subgroup. Due to the expected mortality from venom exposure (approximately 50 %), 10 surviving animals per subgroup were included in the final analysis.

The control group consisted of 10 rats that received an equivalent volume of 0.9 % saline intramuscularly and were euthanized 1 h after injection under the same conditions. A single control group was used for comparison across all experimental time points because saline administration is not expected to induce significant time-dependent alterations in the inflammatory and stress-response markers assessed during the study period.

At the end of each designated observation period, animals were euthanized by CO_2 inhalation in accordance with institutional animal care guidelines.

The study was conducted between September 2021 and February 2022. All procedures complied with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and were approved by the Bioethics Committee of Taras Shevchenko National University of Kyiv (protocol No. 2, dated August 19, 2021).

Immediately after euthanasia, spleens were removed and rinsed in cold saline. The tissue was homogenized in 50 mM Tris-HCl buffer (pH 7.4) containing 140 mM NaCl and 1 mM EDTA, using a tissue-to-buffer ratio of 1:9 (w/v). The homogenates were centrifuged twice: first at 600 g for 5 min to remove cellular debris and nuclei, and then at 15,000

g for 15 min to pellet the mitochondrial fraction. Aliquots of the resulting supernatants were snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Total protein concentration was determined using the Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, USA) according to the manufacturer's instructions.

The levels of pro- and anti-inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-4, IL-6, IL-8, and IL-10), as well as stress/response markers (NF-κB, HIF-1α, HSP60, and HSP70), in spleen homogenates were quantified using enzyme-linked immunosorbent assay (ELISA) according to a standard protocol for soluble proteins [8]. Briefly, the homogenates were diluted to 1 μg/mL in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.14 M NaCl and incubated overnight at 4 °C in high-binding 96-well plates. After washing, nonspecific binding sites were blocked with 5 % non-fat dry milk for 1 h at 37 °C. Plates were then incubated with the corresponding primary antibodies (Santa Cruz Biotechnology, USA) for 1 h at 37 °C, washed, and subsequently incubated with HRP-conjugated secondary antibodies (Sigma-Aldrich, Germany) under the same conditions. Color development was achieved using o-phenylenediamine substrate (0.4 mg/mL in citrate-phosphate buffer containing H_2O_2); the reaction was stopped with 1 M H_2SO_4, and absorbance was measured at 492 nm using a μQuan™ microplate reader (BioTek Instruments, Inc., USA).

Data were analyzed using GraphPad Prism 8.0 (GraphPad Software, Inc., USA). The normality of data distribution was assessed using the Shapiro-Wilk test, and the homogeneity of variances was evaluated with Bartlett's test. Differences among groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test (each time point vs. control). Data are expressed as mean±SD. Differences were considered statistically significant at $p<0.05$.

Results of the study and their discussion. To evaluate tissue-level immune responses to scorpion envenomation, we analyzed the dynamics of inflammatory mediators and stress-associated signaling molecules in rat spleen tissue. Particular attention was paid to the early and delayed phases of the response following venom administration. Changes in inflammatory mediators and stress-related signaling markers were assessed in spleen homogenates of rats after a single intramuscular administration of scorpion venom at different time points (1 h, 3 h, 24 h, and 72 h). The results are presented as relative units per gram of tissue and compared with the control group.

The obtained results demonstrated that scorpion venom triggered a marked pro-inflammatory response in spleen tissue (Fig. 1).

TNF-α increased at the early post-injection time point and remained elevated during the subsequent hours ($p<0.05$); however, at later measurements its level gradually declined, approaching control values by the end of the observation period. In contrast, IFN-γ exhibited a delayed elevation pattern: its concentration was significantly higher than in controls at 3 h after venom administration ($p<0.05$) and remained elevated at 24 h ($p<0.05$), whereas by 72 h it returned to values comparable to the control group. Similarly, to TNF-α, IL-1β, IL-6, and IL-8 increased within the first hour after scorpion venom injection and remained elevated up to 24 h ($p<0.01$); however, by 72 h their levels declined toward those of the control group. The levels of anti-inflammatory cytokines also exhibited time-dependent changes (Fig. 2).

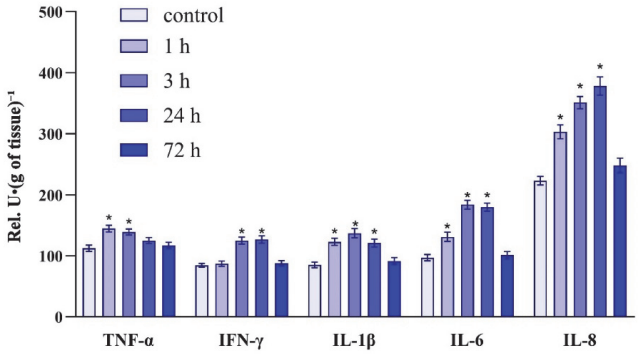

Fig. 1. Content of pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukins-1β, -6, and -8 (IL-1β, IL-6, IL-8), in the spleen of rats following Leiurus macroctenus envenomation. Results are presented as mean±SD (n=10); *indicates significant difference ($p<0.05$) from control group.

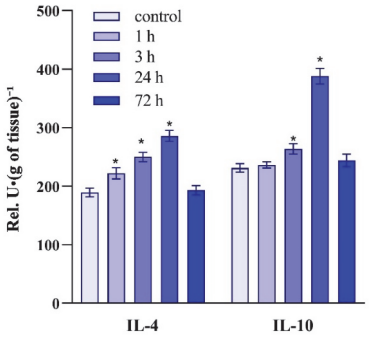

Fig. 2. Content of anti-inflammatory cytokines, including interleukins-4, and -10 (IL-4, IL-10), in the spleen of rats following Leiurus macroctenus envenomation. Results are presented as mean±SD (n=10); *indicates significant difference ($p<0.05$) from control group.

The highest levels were recorded at 24 h ($p<0.01$). This pattern was generally consistent with the dynamics of pro-inflammatory cytokines, which at this time point showed a tendency to decrease compared with earlier periods after envenomation. Notably, by 72 h the levels of anti-inflammatory cytokines declined and did not differ significantly from control values.

Our results showed that the stress-response markers HSP60 and HSP70 were upregulated in splenic tissue after venom exposure (Fig. 3).

Both proteins increased compared with controls during the post-injection period, with the highest recorded levels observed at 24 h ($p<0.05$). By the final time point, the levels of both HSP60 and HSP70 declined toward baseline, reaching values of the control group.

Finally, the signaling molecules HIF-1α and NF-κB were also elevated in splenic tissue following envenomation (Fig. 4).

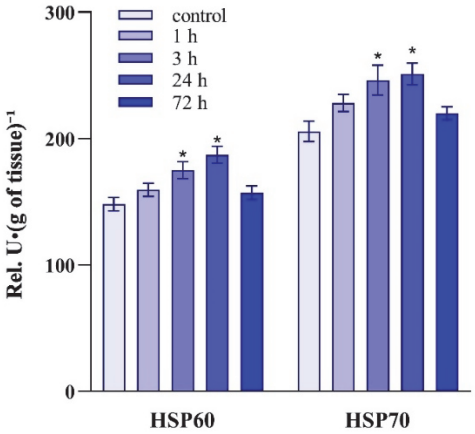

Fig. 3. Content of heat shock proteins (HSP60 and HSP70) in the spleen of rats following Leiurus macroctenus envenomation. Results are presented as mean±SD (n=10); *indicates significant difference ($p<0.05$) from control group.

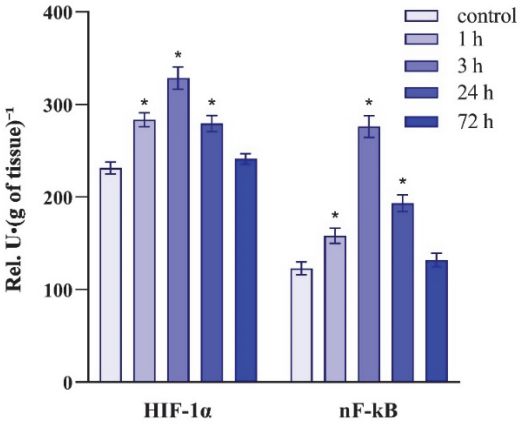

Fig. 4. Content of hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-κB (NF-κB) in the spleen of rats following Leiurus macroctenus envenomation. Results are presented as mean±SD (n=10); *indicates significant difference ($p<0.05$) from control group.

Both HIF-1α and NF-κB showed an increase within the first hour after venom injection, reaching peak recorded values at 3 h ($p<0.01$). Thereafter, their levels gradually declined and approached control values by the final time point (72 h).

The present study demonstrates that a single intramuscular administration of L. macroctenus venom induces a rapid and time-dependent immune response in the spleen, characterized by early activation of pro-inflammatory cytokines, subsequent engagement of anti-inflammatory mediators, and transient upregulation of stress-associated signaling pathways.

The early elevation of TNF-α, IL-1β, IL-6, and IL-8 within the first hour after venom administration is consistent with previously reported increased cytokine production following scorpion envenomation [3, 8, 11]. Such rapid changes likely reflect activation of resident splenic macrophages and dendritic cells, as well as recruitment of circulating immune cells.

The early elevation of TNF-α, IL-1β, IL-6, and IL-8 observed in the present study is consistent with previous reports demonstrating rapid increases in these cytokines following scorpion envenomation in both humans and experimental animals [3, 8, 10, 11]. Such rapid changes likely reflect activation of resident splenic macrophages and dendritic cells, as well as recruitment of circulating immune cells. IL-6 has been described as a marker of systemic inflammatory activation, whereas TNF-α and IL-1β contribute to amplification of inflammatory signaling and tissue injury [10, 11]. Increased IFN-γ levels have also been reported in experimental models, indicating involvement of Th1-associated immune responses during envenomation [10].

Notably, the increase in anti-inflammatory cytokines (IL-4 and IL-10) at 24 h coincided with a tendency toward reduction of pro-inflammatory mediators. This pattern suggests activation of compensatory regulatory mechanisms aimed at downregulating excessive inflammatory responses and maintaining immune homeostasis. Similar time-dependent cytokine changes have been reported in experimental scorpion envenomation, demonstrating dynamic regulation of inflammatory mediators during the course of intoxication [6]. The subsequent decline of both pro- and anti-inflammatory cytokines by 72 h may reflect partial restoration of immune balance in splenic tissue.

The observed upregulation of NF-κB and HIF-1α during the early post-envenomation period further supports the activation of transcriptional programs involved in inflammation and metabolic stress. NF-κB is a central regulator of cytokine gene expression and has been implicated in venom-induced inflammatory amplification [15]. Elevation of HIF-1α may indicate altered microcirculatory conditions and increased metabolic demand in activated immune cells, as well as crosstalk between hypoxic and inflammatory signaling pathways [2].

In parallel, increased levels of HSP60 and HSP70 at 24 h indicate activation of cellular stress-response mechanisms. Heat shock proteins function not only as molecular chaperones but also as danger-associated molecular patterns capable of modulating immune signaling [14]. Their transient elevation may therefore represent both a protective adaptation to venom-induced cellular stress and a potential amplifier of immune activation.

Limitations. The study was limited to a single experimental dose of venom and a relatively short observation period, which may not fully reflect longer-term systemic effects of envenomation.

Conclusion

A single intramuscular administration of L. macroctenus venom elicited a coordinated, time-dependent response in rat splenic tissue, combining early predominance of pro-inflammatory mediators with subsequent engagement of counter-regulatory cytokines. Concomitant elevations of NF-κB and HIF-1α, together with increased HSP60/HSP70, suggest activation of inflammatory transcriptional programs coupled to cellular stress adaptation. By the late observation time point, the assessed parameters generally approached control values, indicating attenuation of the venom-induced response by the end of the studied period. In combination with our previously reported structural and functional alterations in other organs induced by L. macroctenus venom, the present findings further support the concept that scorpion envenomation is associated with complex, organism-wide pathophysiological changes.

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Conflict of interest. The authors have no conflicts of interest to declare.

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