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Research article

Muscle Tissue Response to *Vipera berus berus* and *Vipera berus nikolskii* Venoms: A Study on Matrix Metalloproteinases and Cytokine Modulation

Tatyana Synelnyk[1]*, Yuriy Sogujko[2], Kostiantyn Maievskyi[3], Serhii Shchypanskyi[1], Maryna Taranushych[1], Tetiana Vovk[1], Tetiana Halenova[1], Nataliia Raksha[1] and Olexiy Savchuk[1]

[1]Educational and Scientific Center "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv, Kyiv, Ukraine
[2]Andrey Krupinsky Lviv Medical Academy, Lviv, Ukraine
[3]National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine

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Abstract

Vipera berus, also known as the common adder, is a snake species widely distributed in Europe and parts of Asia. Its venom is a mix of enzymatic and non-enzymatic components causing both local and systemic effects including edema, hemorrhage, myonecrosis, gastrointestinal disorders, and rarely – rhabdomyolysis, coagulopathy and hypovolemic shock. Matrix metalloproteinases (MMPs) and cytokines, being inflammatory mediators, participate both in the development of these reactions and in tissue regeneration after acute damage. The current study was aimed at analyzing the concentrations of important mediators of tissue damage, inflammation and regeneration, namely, MMP-1, -2, -3, -8, -10, TIMP-1 (a tissue inhibitor of MMPs), and pro- and anti-inflammatory cytokines, in rat muscle after intraperitoneal injection of venoms from two subspecies of adders – *V. berus berus* and *V. berus nikolskii* — to clarify their role in the local response of skeletal muscle to the studied venoms. Changes in muscle tissue were revealed, namely, significant increase in almost all MMPs including MMP-2, as well as TIMP-1, IFN-γ and anti-inflammatory IL-4 and IL-10 content. Moreover, a tendency for some pro-inflammatory cytokines levels to decline 24 h after venom administration was observed, which may be a sign of inflammation suppression and the beginning of reparative processes associated with extracellular matrix remodeling and formation of an optimal environment for muscle regeneration. However, to establish the exact mechanisms of the changes we found, further studies are needed. The ability of *V. berus nikolskii* venom to induce more pronounced changes was also shown. Our results may be useful for the development of differentiated and more effective treatment strategies for the consequences of bites by these snakes, which include myonecrosis

Keywords: *Vipera berus*; venom; matrix metalloproteinases; cytokines; muscle

*Corresponding author: E-mail: synelnykt@gmail.com
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1. Introduction

Up to 5.5 million cases of snakebite are registered annually in the world, resulting in 2.7 million cases of envenomation and about 100000 deaths more common in poor rural areas of tropical and subtropical regions of Africa, Asia, South and Central America (Resiere et al., 2022; Seifert et al., 2022; Kumar et al., 2023). According to the Poisoning Severity Score, about 80% of snakebite envenomations correspond to the G-0 ("dry" bites) and G-1 (local edema) grades, whereas the symptoms of moderate (G-2 grade with spread of edema and long-term general symptoms) or severe envenomation (G-3 grade with life-threatening symptoms) are observed in 10%-30% of cases (Garkowski et al., 2012; Czajka et al., 2013). Rarely, envenomation can lead to blindness and amputations (Gutiérrez et al., 2017). In 2017, the World Health Organization classified snakebite into Category A of the Neglected Tropical Diseases list, underscoring the global importance of the problem (Seifert et al., 2022).

The only venomous snakes in Europe belong to *Vipera* genus of *Viperidae* family (Nicola et al., 2021; Palamarchuk et al., 2023). The species *Vipera berus* (Linnaeus, 1758) is the most common viper in Europe, and its two subspecies, *V. berus berus* and *V. berus nikolskii*, cause more accidents than any other species of the genus *Vipera* (Tasoulis & Isbister, 2017; Damm et al., 2021; Siigur & Siigur, 2022). About 70% of reported *V. berus* bites cause no or very slight effects in humans, and death is rare. In moderate and severe cases, envenomation leads to both local and systemic effects caused by venom components such as snake venom (sv) phospholipases A2 (svPLA2s), metalloproteinases (svMPs), serine proteinases (svSPs) and C-type lectins (CTL/Snaclec), L-amino acid oxidases (LAAOs), and some other proteins and peptides (Gutiérrez et al., 2018). Local effects include edema, pain, hemorrhage, and myonecrosis, while the most common systemic symptoms are gastrointestinal disorders, rarely thrombosis, and bleeding caused by consumption coagulopathy, which can lead to hypovolemic shock (Resiere et al., 2022; Bittenbinder et al., 2023).

Myonecrosis is a common local manifestation of envenomation by most viper bites that is often accompanied by hemorrhage, blistering and edema and is associated with inflammatory cell infiltrate and changes in the levels of pro-inflammatory mediators (Costet, 2016). In some cases, systemic myotoxicity – rhabdomyolysis – develops (Xiao et al., 2017). It is more typical for exotic snakebite envenomation, but can also occur in severe viper bites, particularly in children (Denis et al., 1998; Czajka et al., 2013). Myonecrosis-damaged muscle tissue eventually undergoes regeneration, when some extracellular matrix (ECM) components are degraded by matrix metalloproteinases (MMPs), while other proteins are synthesized to form an environment favorable to myoblast proliferation and differentiation. However, specific viper venom components, affecting blood vessels and ECM components in muscle tissue, can lead to impairment progression and weakening of regenerative processes (Gutiérrez et al., 2018).

MMPs participate in ECM molecule metabolism, playing an important role in ECM remodeling during embryogenesis, morphogenesis, angiogenesis, and wound healing as well as in the development of numerous pathologies (Slapak et al., 2020; Chang, 2023). Beside the direct effects on ECM components, MMPs also modulate the functions of numerous bioactive molecules involved in proliferation, chemoattraction, and migration, and regulate signaling pathways involved in inflammation (Ansari et al., 2013). Tissue inhibitor of metalloproteinases-1 (TIMP-1) is the main physiological inhibitor of already activated MMPs; other important MMP regulators are cytokines, which acting

through appropriate signaling cascades change the expression of the genes encoding these enzymes, as well as the genes of their regulators.

To date, the mechanisms underlying local tissue damage in *V. berus berus* and *V. berus nikolskii* bite envenomation have not been sufficiently elucidated. Also, little is known about the role of ECM remodeling and inflammatory phenomena in the pathogenesis of such envenoming. Since acute muscle damage is a common symptom in *Viperidae* bites envenomation, the aim of this study was to analyze the concentrations of important mediators of tissue damage, inflammation and regeneration, namely, MMP-1, -2, -3, -8, -10, their inhibitor TIMP-1, and cytokines in muscle tissue after intraperitoneal injection of *V. berus berus* and *V. berus nikolskii* venoms.

2. Materials and Methods

2.1 Animal models and experimental design

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 of 24 November 1986 for the Care and Use of Laboratory Animals (86/609/EEC) and were approved by the Local Ethics Committee and confirmed by the Bioethical Commission of the NSC "Institute of Biology and Medicine" of Taras Shevchenko Kyiv National University (protocol № 2 approved on 19.08.2021).

A total of thirty rats used in this study were randomly divided into three groups (n=10). The control group was injected intraperitoneally (i.p.) with 0.5 mL of 0.9% saline solution. The first experimental group was injected i.p. with a semi-lethal dose (LD50) of *V. berus berus* venom in saline solution (1.576 $\mu g \cdot g^{-1}$ of body weight), and the second one – with LD50 of *V. berus nikolskii* venom in saline solution (0.972 $\mu g \cdot g^{-1}$ of body weight). The selected LD50 values were based on findings published by Shitikov et al. (2018). The animals were euthanized by cervical dislocation 24 h post venom administration, and muscle tissues were subsequently collected for analysis.

2.2 Venom source and preparation

Freeze-dried venom samples of both *V. berus berus* and *V. berus nikolskii* were procured from V.N. Karazin Kharkiv National University, Ukraine. These were stored at a temperature of -20°C until needed. The venom was dissolved in saline before each experiment, followed by centrifugation (10,000 g, 15 min). The resulting supernatant was then used for further stages of the study.

2.3 Muscle sample preparation

To obtain a muscle tissue homogenate, the hind limb muscle tissue of each rat was immediately collected after each animal had been sacrificed. Then 1 g of muscle tissue was homogenized in 9 mL of ice-cold Tris-buffered saline (TBS; 50mM Tris-HCl, 140mM NaCl; pH 7.4), and the buffer amount (in grams) was 5 times greater than the mass of isolated tissue. The obtained crude homogenate was centrifuged (600 g, 15 min, 4°C) with further supernatant collection and re-centrifugation (15000 g, 30 min, 4°C). The supernatant liquid was collected and immediately used for the measurements. Protein concentration was determined by the Bradford method (Bradford, 1976).

2.4 Matrix metalloproteinases, tissue inhibitor of metalloproteinases-1, and cytokines immunoassay

The enzyme-linked immunosorbent assay (ELISA) technique was employed to measure levels of MMPs, TIMP-1 and cytokines in muscle tissue samples according to the standard instructions for soluble proteins (Crowther, 2000).

The samples were first diluted to 10 μg·mL^{-1} with TBS (pH 7.4) and incubated overnight at 4°C in sterile ELISA plate wells. This was followed by well washing with TBS to remove unbound antigen. To block the plate wells, incubation with 5% non-fat dry milk solution for 1 h at 37°C was used. Then plates were washed again with TBS containing 0.05% Tween-20, with further incubation with the corresponding primary antibodies against MMP-1,-2,-3,-8,-10, TIMP-1, TNF-α, and IL-1β,-4,-6,-8,-10 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) (1:3000) for 1 h at 37°C. Next, the plates were washed with TBS containing 0.05% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) (1:6000) for 1 h at 37°C. After that, the wells were washed again with TBS containing 0.05% Tween-20 and incubated with 0.4 mg·mL^{-1} o-phenylenediamine, diluted in 0.05M citrate-phosphate buffer, in the presence of H_2O_2 to visualize the reaction. The reaction was stopped 10 min later by adding 100 μL 1M sulfuric acid. The optical density of the samples was measured by a microplate reader (μQuantTM, BioTek Instruments, Inc, USA) at a wavelength of 492 nm (Raetska et al., 2017; Koval et al., 2018). Results were expressed in rel. units·g^{-1} of muscle tissue.

2.5 Statistical analysis

Data entry and analysis were performed using MS Excel (MS Office) and Statistica 8.0 software for Windows. The data of biochemical estimations were reported as mean±SEM for each group (n = 10). After testing for normality (by Kolmogorov-Smirnov), a one-way analysis of variance (ANOVA) was used to compare the means among different groups. Differences were statistically significant when $p<0.05$.

3. Results and Discussion

The current study provides a comprehensive evaluation of the impact of venom from two subspecies of vipers, *V. berus berus* and *V. berus nikolskii*, on various markers of tissue remodeling and inflammation in muscle tissue.

First, the concentration of MMPs and their physiological inhibitor TIMP-1 in the rat muscles after *V. berus berus* and *V. berus nikolskii* venom injection was analyzed. MMPs are Ca^{2+}-dependent zinc-containing endopeptidases involved in ECM remodeling during embryo-, morpho- and angiogenesis, as well as under wound healing. They also modulate the functions of bioactive molecules involved in proliferation, chemoattraction, and migration, participating in metastasis, and the inflammatory response. To date, 23 MMPs have been identified in humans: MMP-1, -8 and -13 belong to collagenases, MMP-2 and MMP-9 - to gelatinases. MMP-3, -10 and -11 are representatives of stromelysins (Slapak et al., 2020). They are produced by various cells, including neutrophils and macrophages, as inactive zymogens, and their gene expression is regulated by pro-inflammatory cytokines, growth factors and hormones. Their activation occurs through a limited proteolysis of their zymogens by a number of serine proteases or already

activated MMPs; however, due to TIMPs and other inhibitors, their proteolytic activity in healthy tissues is usually very low (Ansari et al., 2013).

In our study, a rise in MMPs and TIMP-1 content was established in the rat muscles after injection of both viper subspecies venoms compared to the control values (Figure 1). The detected differences were statistically significant in all cases, except for MMP-10 when *V. berus berus* venom was administered: its level did not significantly differ from the control values, but a tendency toward a rise was observed. The increase we found was more pronounced under *V. berus nikolskii* venom injection (32% (MMP-1), 41% (MMP-2), 38% (MMP-3), 25% (MMP-8 and -10), and 19% (TIMP-1)) compared to *V. berus berus* envenoming (18% (MMP-1 and -3), 23.5% (MMP-2), 15% (MMP-8), 11.5% (TIMP-1). Therefore, the greatest changes were related to MMP-2 content, and the slightest ones – to TIMP-1 concentration, and MMP-1, -2, and -3 levels in the muscle tissue of rats after *V. berus nikolskii* venom administration significantly exceeded those in animals subjected to injection of *V. berus berus* venom – by 11.5%, 14%, and 16%, respectively, which may be explained by the differences in biochemical composition of the venoms of these subspecies.

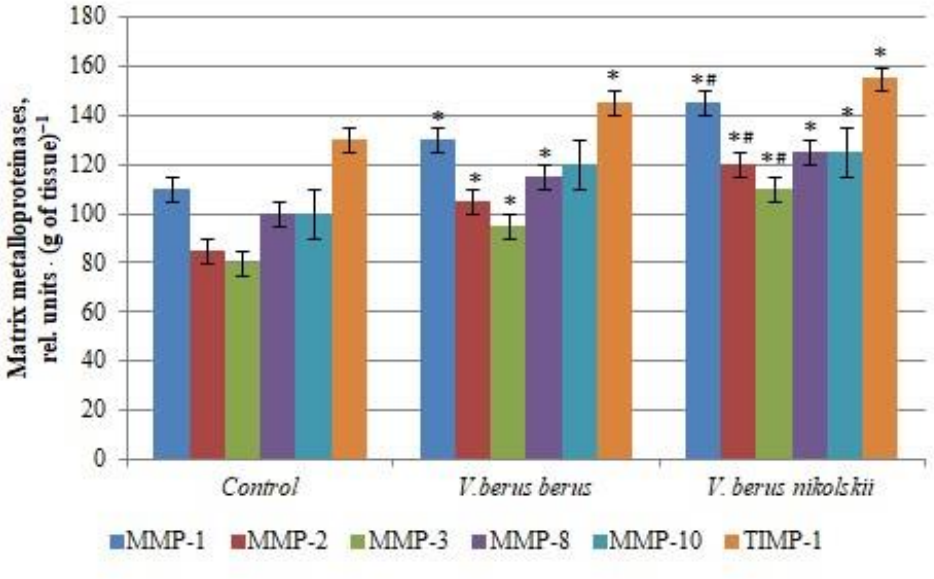

Figure 1. Matrix metalloproteinases and their physiological inhibitor content in the muscles of rats injected with snake venom. Results are presented as mean ± SEM (n=10)
* $p < 0.05$ vs. control, # $p < 0.05$ vs. *V. berus berus*

While the venoms of most other species of *Viperidae* family are enriched with svMPs, the main components of the studied venoms are represented by different types of svPLA2s (Table 1) (Kovalchuk et al., 2016).

The key components of *V. berus berus* venom determining its hemolytic, proteolytic and cytotoxic properties include svSPs, svMPs, LAAOs and cysteine-rich secretory proteins (CRISPs) in addition to svPLA2s (Bocian et al., 2016; Al-Shekhadat et al., 2019; Palamarchuk et al., 2023). *Vipera berus nikolskii* venom is characterized by a

higher content of svPLPA2s (it is considered to be one of the snake venoms most enriched with these enzymes). It also has specific svPLA2s isoforms that are absent in *V. berus berus* venom. LAAO and CRISP levels are much lower in *V. berus nikolskii* venom, and it also lacks disintegrins, bradykinin potentiating peptides and C-type natriuretic peptides. Vascular endothelial growth factor is the most common (8%) among the non-enzymatic proteins in *V. berus nikolskii* venom (Bocian et al., 2016; Kovalchuk et al., 2016).

The ability of *V. berus nikolskii* venom to cause more pronounced changes in MMP content in muscle tissue is consistent with data on its greater toxicity compared to *V. berus berus* venom to frogs and mice when administered i.p. (Kovalchuk et al., 2016; Nicola et al., 2021). Data obtained in our laboratory earlier also showed that *V. berus nikolskii* venom injection into rats has a much more noticeable influence on the proteolysis system in the heart and spleen compared to *V. berus berus* venom (Palamarchuk et al., 2023). Thus, the greater effects of *V. berus nikolskii* venom are most likely associated with a higher content of enzymes with PLA2 activity and their specific set (Table 1).

svPLA2s convert erythrocyte and platelet membrane phospholipids into lysophospholipids, causing hemolysis, thrombocytopenia, and eicosanoids formation, as well as preventing activation of some procoagulants, thus showing anticoagulant effect. Various svPLA2s types are associated with symptom characteristics of snake envenomation such as pain, edema, bleeding, local and systemic myotoxicity, cyto-, neuro- and nephrotoxicity. Neurotoxic svPLA2s found in *V. berus nikolskii* venom can block neuromuscular transmission in vertebrates, causing acute neuro-muscle weakness and paralysis, respiratory depression and death. The myotoxic svPLA2s of this venom can lead to acute necrosis of skeletal muscle, and, in severe cases – to rhabdomyolysis (Garkowski et al., 2012; Xiao et al., 2017). In these processes, svPLA2s act on lipids of sarcolemma,

Table 1. The main components of *Vipera berus berus* and *Vipera berus nikolskii* venom (according to Bocian et al., 2016; Kovalchuk et al., 2016; Al-Shekhadat et al., 2019; Siigur & Siigur, 2022; Palamarchuk et al., 2023)

Component	***Vipera berus berus* (%)**	***Vipera berus nikolskii* (%)**
Phospholipases A2	25-60*	65
Serine proteases	15-16	19 (nicobin > 12%)
L-amino acid oxidases	7-9	0.1
Cysteine-rich secretory proteins	6-8	0.66
Metalloproteinases	0.5-17*	< 1
Bradykinin potentiating peptides	9.5	-
C-type natriuretic peptides	7.8	-
Other/peculiarities	C-type lectins (CTL)/Snaclec, Kunitz-type protease inhibitors (KUNs), disintegrins (DI)	CTL/Snaclec, KUNs No DI

*Such a wide range in this component content can be explained by the different protein identification methods used as well as by the differences in the geographical origin, sex,

age and/or food type of the studied snakes. The influence of these factors on venom composition has been actively studied and is currently proven (Siigur & Siigur, 2022).

altering its fluidity and permeability, which contributes to calcium influx into myocytes with further myofilament hypercontraction, mitochondrial dysfunction, and Ca^{2+}-dependent intracellular proteases activation leading to irreversible damage of muscle cells (Gutiérrez et al., 2017). Oxidative stress caused by mitochondrial dysfunction and/or venom LAAOs may be involved in myonecrosis mechanisms (Rucavado et al., 2002; Bocian et al., 2016 Resiere et al., 2022).

Under snake venom-induced myonecrosis, muscle fibers also suffer from both ischemia caused by vascular changes and increased intramuscular pressure due to edema (Gutiérrez et al., 2017). svSPs first act on blood coagulation, contributing to thrombus formation (Siigur & Siigur, 2022). But over time, the pool of procoagulants is depleted with consumption coagulopathy development, leading to systemic bleeding, hypovolemia, and cardiovascular shock (Gutiérrez et al., 2017). Destruction of ECM components by svMPs leads to connective tissue loosening and the release of peptides that cause additional tissue damage, participate in reparative processes, or serve as mediators of inflammation, contributing to pain, edema development and leukocyte infiltration (Gutiérrez et al., 2017; Seifert et al., 2022). Similar, svSPs can also destroy some ECM components, being involved in the local effects of envenomation (Gutiérrez et al., 2018). Hydrolysis of basement membrane collagen by hemorrhagic svMPs contributes to muscle tissue ischemia due to extravasation caused by capillary wall damage and increased interstitial pressure in muscle compartments (Resiere et al., 2022; Siigur & Siigur, 2022). Different svMPs can differentially affect platelet functions and can also promote activation of some procoagulants, which participate in consumption coagulopathy development (Teixeira et al., 2019; Resiere et al., 2022).

Thus, myonecrosis is typical seen with the studied venoms. As these venoms contain both myotoxic and hemorrhagic toxins, muscle damage results from both the direct action of the myotoxins on muscle fibers and the indirect effect of ischemia. ECM component destruction under snake envenomation is initially carried out by svMPs and svSPs. But the ability of svMPs to stimulate production of MMP-1 and -10 by macrophages, as well as to activate latent forms of various MMPs, is also known. There are also data indicating an increase in MMP-2 and -9 production by resident macrophages, fibroblasts and endothelial cells in skeletal muscles under myonecrosis development during the first hours after injection of *Bothrops asper* venom toxins (*Viperidae* family) (Rucavado et al., 2002). However, since svMPs content in the studied venoms is quite low (Table 1), it is likely that the increase in MMPs concentration in rat muscles we found demonstrated additional reasons. One reason is related to inflammation development, as immune cells infiltrating damaged muscle tissue produce cytokines and chemokines that are important regulators of MMP levels.

The increase in MMP content, in particular in MMP-2, as we established, may also indicate the beginning of skeletal muscle regeneration. Indeed, MMPs, by splitting ECM, facilitate the penetration of inflammatory cells into inflammation focus and promote the release of latent growth factors embedded in the matrix, which regulate proliferation and differentiation of myogenic cells (Rucavado et al., 2002; Gutiérrez et al., 2018). It is known that MMP-2, -9 and -10 are necessary for successful muscle regeneration: MMP-2 affects satellite cell activation and muscle fiber maturation, and also controls angiogenesis, while MMP-9 and -10 are involved in signaling pathways important for efficient regeneration (Alameddine & Morgan, 2016). Moreover, there is also evidence that the regulated expression of MMP-2, -9, MT1-MMP, and to a lesser extent MMP-1

and -13 is critical for muscle regeneration after injury by *Viperidae* venoms (Rucavado et al., 2002).

Normally, the rate of ECM remodeling is determined by a tightly regulated balance between expression and activity of MMPs, and their inhibition by TIMPs and other physiological inhibitors. Excessive and/or dysregulated MMPs expression can lead to uncontrolled ECM degradation and tissue damage. Thus, the increase in TIMP-1 content in rat muscle we found may be a compensatory phenomenon aimed at maintaining the balance between MMPs and their inhibitors.

To assess the role of inflammation in muscle injury after injection of the studied venoms, the cytokine profile of the rat muscle was further determined and analyzed. It was established that a statistically significant difference in pro-inflammatory cytokines content in rat muscles was related exclusively to IFN-γ, which was at concentration of 25% (*V. berus berus* venom injection) and 60% (*V. berus nikolskii* venom injection) higher than the control value. And its level in the muscles of rats under *V. berus nikolskii* venom influence was 28% higher than that in rats envenomed with venom of *V. berus berus* (Figure 2). At the same time, a tendency for the levels of IL-8 (under both venoms action), IL-1β (when *V. berus berus* venom was administered) and IL-6 (after *V. berus nikolskii* injection) to decrease was found. Thus, multidirectional changes in the content of different pro-inflammatory cytokines in rat muscle under administration of the studied venoms were revealed, and some features of these venoms action were detected.

To date, data on cytokines levels in muscle tissue and serum under *Viperidae* snake envenomation is limited to a few species (mainly *Bothrops* sp.), and relevant studies are few and quite contradictory. In particular, there are indications of a marked increase in IL-1β and -6 levels without changes in the TNF-α and IFN-γ content in the muscles of mice after intramuscular injection of myotoxic svPLA2s and hemorrhagic svMPs isolated from *B. asper* venom (Rucavado et al., 2002). Elevated serum IL-1β, -6, -10, TNF-α, and IFN-γ levels were detected after *B. jararaca* and *B. asper* venoms were injected in mice, and high levels of IL-6, -8, and TNF-α were found in serum of patients bitten by these snakes (Teixeira et al., 2019). There is also evidence that *V. berus* and *Crotalus v. viridis* venoms also induce IL-6 release (Lomonte, 1994).

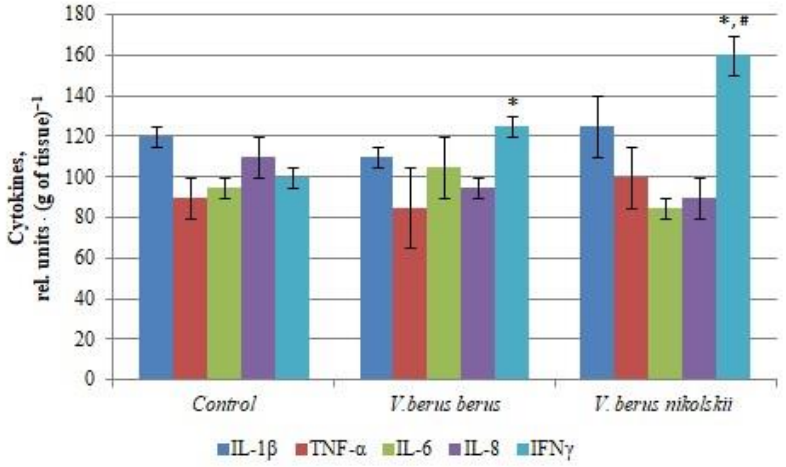

Figure 2. Pro-inflammatory cytokines content in the muscles of rats injected with snake venom. Results are presented as mean ± SEM (n=10)
* $p < 0.05$ vs. control, # $p < 0.05$ vs. *V. berus berus*

A possible explanation for the inconsistency of the tendency to decrease pro-inflammatory cytokine levels we found in the literature might be the different times of detection of changes. In fact, cytokine levels in our study were analyzed in rat muscle 24 h after venom injection, whereas according to the literature, pro-inflammatory cytokine content increased much earlier both at the injection site and in serum. Studies of the dynamics of local inflammation, carried out in mice using *Bothrops* sp. venom, showed that edema, caused by vascular permeability remodeling, developed within the first 30 min after *B. asper* and *B. atrox* venoms injection, peaked at 1-2 h, and then declines (Teixeira et al., 2019; Resiere et al., 2022). There is also evidence of a rapid increase in serum IL-6 after intramuscular administration of *B. asper* venom or its components (svPLA2s or svMPs) into mice, which peaks within 3-6 h and returns to baseline by 12 h (Lomonte, 1994).

Other data also indicate an increase in serum TNF-α 1 h after svMPs injection and no similar changes at later points in time (Teixeira et al., 2019). In general, IL-1, -6, and TNF-α are considered to be early or "alarm" proinflammatory cytokines responsible for the initiation and propagation of the inflammatory response induced by snake venom injection, or by venomous snake bite (Rucavado et al., 2002; Costet, 2016). Although most mentioned studies of acute muscle damage after snake envenomation have included intramuscular injection of the crude venom or its toxic components, with intraperitoneal administration, venom toxins rapidly enter the systemic circulation and reach various tissues, including muscles, causing their acute damage. Therefore, the time frames of pro-inflammatory cytokines release in the case of intramuscular and intraperitoneal venom administration generally coincide.

Skeletal muscle injury promotes the release of TNF-α and IL-1β, which in turn induces production of IL-6 and -8 by the vascular endothelium. These cytokines stimulate neutrophil migration to the injury site within the first few hours of the acute inflammatory response, with a peak between 4 and 6 h. Neutrophil production of pro-inflammatory cytokines, ROS and proteinases, as well as netosis and the formation of neutrophil extracellular traps are involved in the mechanisms of local tissue damage after snake venom injection (Resiere et al., 2022). Neutrophils also secrete chemoattractants, which direct blood monocytes to the inflammation site, resulting in macrophage infiltration that begins 24-48 h after venom administration. An excess of pro-inflammatory cytokines, in particular IFN-γ, promotes formation of pro-inflammatory M1 phenotype macrophages. They effectively eliminate necrotic cell debris and apoptotic neutrophils from the damaged tissue and secrete growth factors and pro-inflammatory cytokines. M1 macrophage infiltration peaks at 1-3 days after injury. Simultaneously, macrophage-mediated clearance of apoptotic neutrophils contributes to the secretion of transforming growth factor-β1 and IL-10, which promotes formation of anti-inflammatory M2 phenotype macrophages. Therefore, as soon as M1 macrophages reach their peak concentration, the pro-inflammatory microenvironment begins to transform into an anti-inflammatory one with a higher concentration of M2 macrophages secreting IL-4 and -10 (Ziemkiewicz et al., 2021; Wang & Zhou, 2022). Changes in cytokine expression are also important indicators of M1-to-M2 macrophage transition.

svPLA2s and svMPs are mainly responsible for the initiation of inflammation in damaged tissues under *Viperidae* snakes envenoming, while svSPs play a minor role (Costet, 2016). svPLA2s cause the generation of lipid mediators that act as a chemoattractants for leukocytes, modulate intracellular signaling cascades, and stimulate pro-inflammatory cytokine genes expression, while svMPs activate various types of cells and promote their secretion of chemokines, pro-inflammatory cytokines and MMP-1 and -10 (Teixeira et al., 2019; Resiere et al., 2022).

Both intracellular pattern recognition receptors and toll-like receptors (TLRs, mainly TLR-2 and -4) of resident cells including macrophages are involved in the early initial increase in pro-inflammatory cytokine content in muscle tissue after snake venom administration. They recognize venom-associated molecular patterns and trigger signaling pathways that culminate in pro-inflammatory cytokine gene expression. Damage-associated molecular patterns, or alarmins, such as fibrinogen or fragments of ECM proteins released from damaged skeletal muscles, can be also involved in TLR activation (Teixeira et al., 2019; Resiere et al., 2022; Zuliani, 2023).

Thus, as the tendency we revealed for the content of most pro-inflammatory cytokines in the rat muscle 24 h after envenomation by both snake subspecies to decrease may not reflect the levels of these compounds at earlier times, it can be assumed that an increase in pro-inflammatory cytokine content could occur earlier, within the 1st day after venom administration, which could not be detected in our study because we did not examine the content of these molecules during this time period. In this context, the rise in MMP levels we found may be a consequence of such increased cytokine production in the early stages of the inflammatory process. However, to establish the exact mechanisms of the changes we found, further studies such as investigation of studied compounds' contents at different time points during the 1st day after envenomation and estimation of the cellular infiltrate of damaged muscles are needed. Moreover, a decrease in pro-inflammatory cytokine content can be a sign of inflammation suppression and the beginning of reparative processes in the damaged muscles, involving anti-inflammatory cytokines and MMPs.

Our results also revealed an increased content of anti-inflammatory cytokines in rat muscle under *V. berus berus* and *V. berus nikolskii* venom administration compared to the control. IL-4 content was 23% and 41% higher, respectively, and IL-10 level was 26% and 48% higher, which can indicate reparative mechanism activation in muscle in response to envenomation (Figure 3). Moreover, IL-4 levels were 26% higher, and IL-10 content 17% higher after *V. berus nikolskii* envenomation compared to *V. berus berus* venom injection.

Skeletal muscle regeneration after myonecrosis involves activation, proliferation, and fusion of myogenic satellite cells to form myotubes. After myonecrosis onset for 3-5 days, a large number of myotubes and small myofibers are usually observed. On the 7th day, most of the damaged muscle is replaced by small regenerating fibers that reach their normal diameter in a month. All these processes are regulated by cytokines, chemokines and growth factors released under conditions of inflammation. Moreover, the phenotype of the predominant population of macrophages is critical for shifting predominantly inflammatory processes towards regenerative ones (Gutiérrez et al., 2018). Indeed, M1 macrophages exacerbate inflammation, and contribute to further muscle repair as they phagocytize damaged tissue debris, whereas M2 macrophages suppress inflammatory responses and participate in ECM remodeling, angiogenesis, and repair of damaged tissues (Chávez-Galán et al., 2015).

Macrophage phenotype affects muscle repair processes by influencing myogenic satellite cells: M1 macrophages stimulate mainly satellite cells proliferation, whereas M2 macrophages their differentiation and myotubes formation (Ziemkiewicz et al., 2021). Elevated IFN-γ content promotes M1- polarization of macrophages, while high IL-10 levels inhibit this phenotype formation by suppressing the release of proinflammatory cytokines, and contributes M1-to-M2 macrophage transition several days after injury indicating a switch from a proliferative state to differentiation and myogenesis (Gehlert & Jacko, 2019).

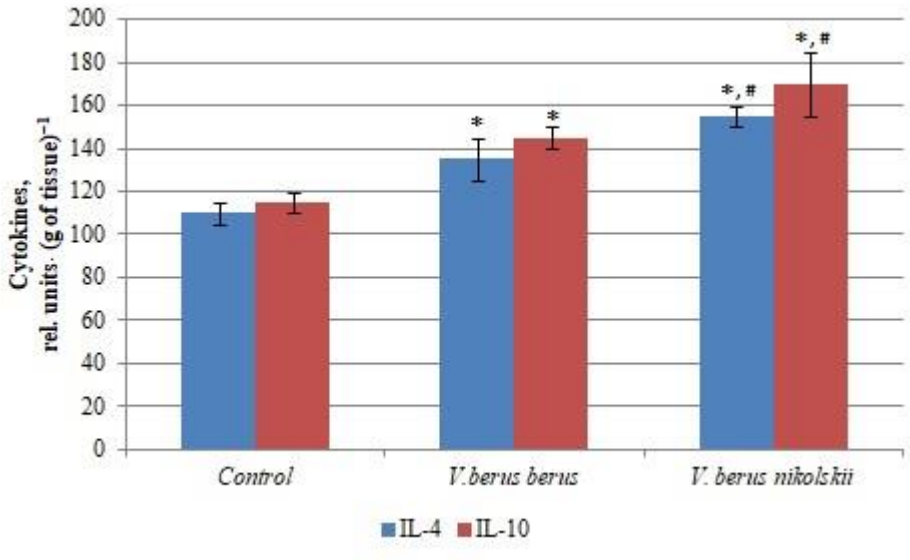

Figure 3. Anti-inflammatory cytokines content in the muscles of rats injected with snake venom. Results are presented as mean ± SEM (n=10)
* $p < 0.05$ vs. control, # $p < 0.05$ vs. *V. berus berus*

However, *in vivo* M1 and M2 stimuli often coexist, and macrophages can exhibit mixed M1/M2 phenotypes, being not strictly M1- or M2-polarized, but more likely M1- or M2-like, exhibiting a more “pro-inflammatory” phenotype in the early stage of inflammation and a more “anti-inflammatory” and “pro-regenerative” phenotype in the later stages (Wang & Zhou, 2022; Strizova et al., 2023).

Thus, the increase in anti-inflammatory cytokines content we found in combination with a high level of IFN-γ may indicate a change in the phenotype of macrophages infiltrating the damaged tissue, and a gradual transition from inflammatory processes to muscle regeneration. However, to establish the precise mechanisms of the changes we found, further studies are needed, such as evaluation of the cellular infiltrate of damaged muscles as well as histological studies. It should also be noted, if our hypothesis is correct, the rather early onset of reparative processes may be a specific sign of muscle tissue damage under studied venoms action. Indeed, successful reparative processes require preservation of vascular network integrity and the structure of the basement membrane separating the space where myoblasts fusion takes place. Thus, regeneration is successful after the action of mainly myotoxic venoms (as in many *Elapidae* snakes), which do not affect the vasculature. In contrast, the venoms of most *Viperidae,* including *Bothrops* and *Crotalus* genera, are enriched with svMPs, and their administration affects the vascular system and ECM, causing a significant impairment of regeneration (Gutiérrez et al., 2018). However, the venoms of *V. berus berus* and *V. berus nikolskii* are rich in svPLA2s and have only minor svMPs content (Figure 4, Table 1) (Bocian et al., 2016; Kovalchuk et al., 2016; Oliveira et al., 2022).

Thus, it can be expected that the regeneration of muscles injured by studied *Vipera* subspecies venoms, as with venoms of *Elapidae* snakes, would be more successful.

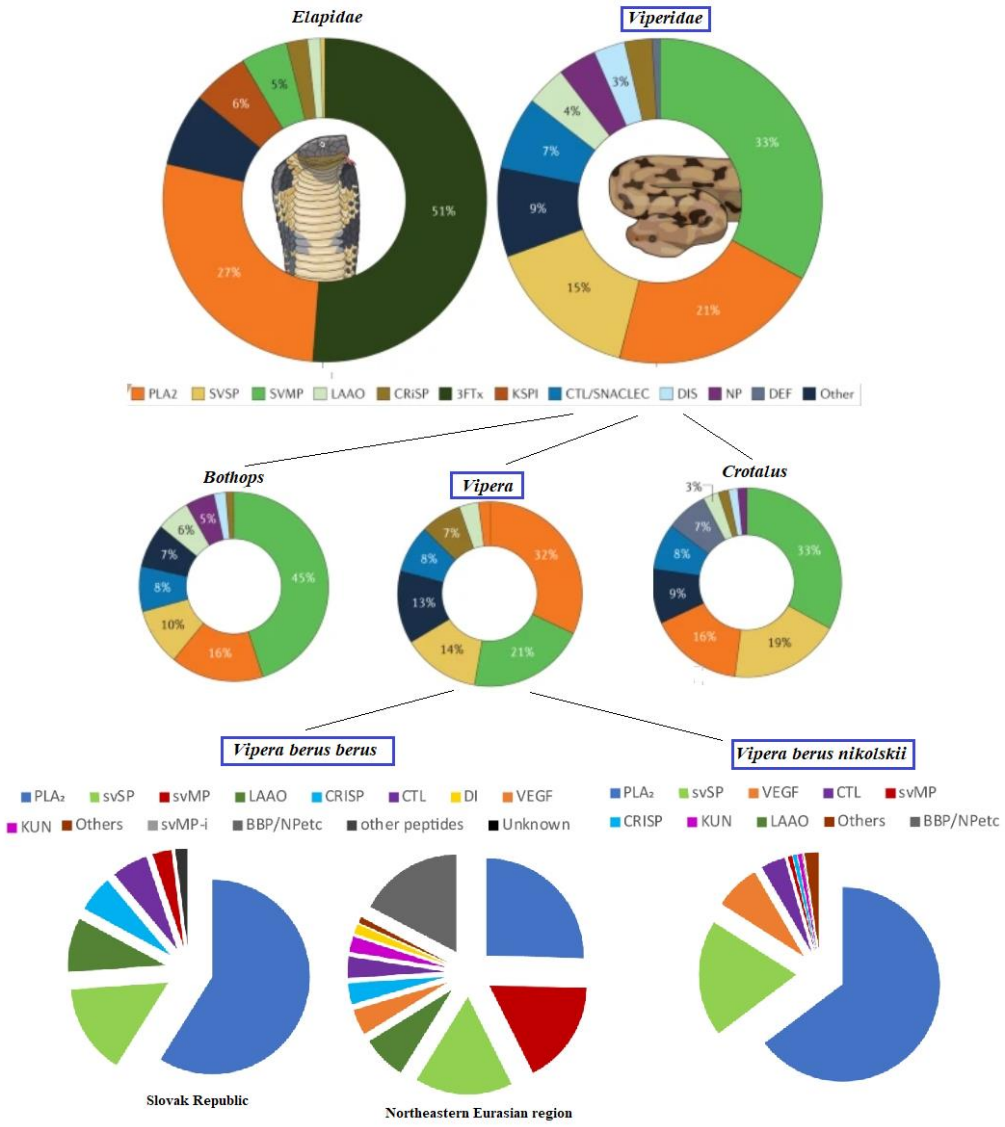

Figure 4. Venom composition of snakes from *Elapidae* and *Viperidae* families and the peculiarities of various viperides venoms (according to Bocian et al. (2016), Kovalchuk et al. (2016), Al-Shekhadat et al. (2019) and Oliveira et al. (2022), www.venomzone.expasy.org)

4. Conclusions

The intraperitoneal injection of *V. berus berus* and *V. berus nikolskii* venoms causes changes in muscle tissues, which are characterized by a significant rise in most MMPs, TIMP-1, IFN-γ and anti-inflammatory IL-4 and -10 content, and a tendency for some pro-inflammatory cytokines levels to decrease. The greatest changes observed were related

to MMP-2, IL-4 and -10 levels, and their simultaneous increase may be a sign of inflammation suppression and reparative process beginning associated with extracellular matrix remodeling and formation of optimal environment for skeletal muscle regeneration. Accordingly, the tendency for a decrease in the content of some pro-inflammatory cytokines in the rat muscle 24 h after envenomation we established can be also a sign of inflammation suppression and starting of the reparative processes in the injured muscle. However, to establish the exact mechanisms of the changes we found, further studies are needed. The ability of *V. berus nikolskii* venom to induce more pronounced changes was also revealed which underscores the uniqueness of the venoms from different viper subspecies and the envenomation effect dependence on their venom composition. Understanding the mechanisms of snake venom action can be applied in medicine, pharmacology, and biomedical research aimed at antidote development, new drug creation, and further study of inflammatory processes.

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6. Conflicts of Interest

The authors delcare that there is no conflict of interest in this study.

ORCID

Tatyana Synelnyk https://orcid.org/0000-0002-7131-0642
Yuriy Sogujko https://orcid.org/0000-0001-5294-1847
Kostiantyn Maievskyi https://orcid.org/0000-0002-3093-8396
Serhii Shchypanskyi https://orcid.org/0000-0002-6259-7784
Maryna Taranushych https://orcid.org/0009-0001-6938-6656
Tetiana Vovk https://orcid.org/0000-0002-0440-8922
Tetiana Halenova https://orcid.org/0000-0003-2973-2646
Nataliia Raksha https://orcid.org/0000-0001-6654-771X
Olexiy Savchuk https://orcid.org/0000-0003-3621-6981

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