Current Applied Science and Technology Vol. 23 No. 6 (November-December 2023)

Research article

Proteolytic Homeostasis in the Tissue of the Spleen and the Heart of Rats Injected with the Venom of *Vipera berus berus* and *Vipera berus nikolskii*

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Received: 18 October 2022, Revised: 28 November 2022, Accepted: 18 April 2023

DOI: 10.55003/cast.2023.06.23.015

Abstract

Keywords

viper;
Viperinae;
venom;
proteolysis;
toxicity

Species of the subfamily Viperinae, known to be Old World vipers, *Vipera berus berus* and *Vipera berus nikolskii*, are highly distributed in Europe and show their venomous effect, leading to proteolysis, thrombocytopenia, neurotoxicity, and haemorrhage state in susceptible organism. A shift in the protein balance underlies the envenoming in the targeted organs and in the whole organism. Thus, a study of the influence of the *V. berus berus* and *V. berus nikolskii* venoms on the protein systems of the spleen and the heart was conducted in order to single out the impact of toxins on the metabolic pathways in the targeted organs. The study included an investigation of the amount of total proteins, and their redistribution and connection with toxicity. The results prove the assumption of proteolytic activation and the emergence of the toxicity state, showing a decrease in the level of proteins, changes in protein composition, redistribution of enzymatic profiles, and an increased level of low molecular weight molecules.

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1. Introduction

Members of the snake family Viperidae cause the majority of snakebites all over the world, demonstrating the status of one of the most venomous vipers [1-4]. Three hundred and seventy-four (374) different species belong to the family and they are divided into three subfamilies within the entire family: Azemiopinae (2 species) and Crotalinae (271 species) – "pit vipers", and Viperinae (101 species) – "true vipers" or "pit-less vipers" (data taken from www.reptile-database.org).

The consequences of the snakebites by vipers of this family are different, depending on the molecular content of each viper's venom. The molecular content has a great impact on the action mechanisms, leading to different onsets and biochemical pathway alterations in the affected organisms. It is therefore necessary to understand the role of each compound of the poison [5].

In general, snakes venom consists of a highly complex mixture of enzymatic and non-enzymatic proteins, peptides and components with low molecular weight, which are responsible for the immobilization and initial digestion of the susceptible organism [2, 3, 6, 7]. Recently, an important breakthrough in the sphere of identifying the content of venoms was recorded, based on novel omics approaches, made it possible to distinguish the molecular components of the viper venoms [8-11]. Taking into account the results of these investigations, the major toxic molecules present in the venom of the Vipera genus are metalloproteinase (svMP), phospholipase A_2 (PLA_2), snake venom serine protease (svSP) and C-type lectin-related protein (CTL) [6]. However, the prevalence of each of them differs in various species of this genus. The amount of secondary toxins is far more unequivocal: vipers from Vipera genus are described as having high concentrations of cysteine-rich secretory protein (CRISP) and minor amounts of disintegrin (DI) [6]. Moreover, the presence of other minor toxic molecules was examined in the viper venoms [6, 12-14]. These molecules determine the hemolytic, neurotoxic, proteolytic, lipolytic and cytotoxic properties of a snakebite, which can be associated with coagulopathy, myonecrosis, vasoconstriction, tachycardia and bronchospasm [7, 10, 12, 15]. The effect of a viper bite on its prey directly depends on the toxins that are contained in the venom. Thus, knowledge of the molecular content of a poison is needed to analyze and predict the possible impact.

Snakes of the Vipera genus with the biggest distribution in Europe and in Ukraine (vicinity of Kharkiv), Central and South Russia, Romania and Moldova are *Vipera berus berus* and *Vipera berus nikolskii*. Compounds in the toxins of these vipers are responsible for the hemo-, proteo-, phospholipido- and fibrinolytic effects, with neuro- and cytotoxic action on the organism [7, 13, 16-18]. Because of these effects, the prey upon organism enters a state of toxicity. The main causative factors of envenoming in *V. berus berus* venom are PLA_2, svSP and svMP, which may act together with the minor ones: natriuretic peptides (NP), aspartic proteases (AspP), CRISPs, CTLs, L-amino-acid oxidases (LAAOs), DI and Kunitz-type protease inhibitors (KTPi) [12, 13, 19]. While *V. berus nikolskii* venom is characterized with a high amount of PLA_2 and svSP and lower content of CTLs, svMP and vascular endothelial growth factors (VEGF) [6, 14].

Thus, proteolysis activation is one of the key processes involved in the venomous state provoked by the action of either *V. berus* or *V. berus nikolskii* affecting the whole organism of a prey [17, 18]. The main reason of examining the impact of venoms on certain organs and systems can be explained through the demand of understanding of the direct and/or indirect impact on the components of proteolytic systems. This information can extend the knowledge about the pathology of snakebites and provide the possibilities for drugs, therapeutics and antivenoms development. The heart was chosen as a targeted organ of the investigation, based on the data about cardiovascular effect of snake toxins [20-23] and the necessity of examining the impact of viper venoms on the protein profile of the heart. The spleen plays an important role in ensuring the proper functioning of the immune system. Therefore, there is a great interest in studying the alterations of the molecular content of the organ after the exposure to viper venoms, which can possibly affect the physiological

state of the body, leading to the effects on the immune system. Therefore, the examination of the impact of the *Vipera berus berus* and *Vipera berus nikolskii* venoms on the spleen and the heart was conducted to extend the available and evident data in this sphere.

2. Materials and Methods

2.1 Animal care and handle

Albino nonlinear male rats that were used in a trial were first kept in the animal facility of Taras Shevchenko National University of Kyiv for acclimation for 7 days and then kept at constant temperature (22±3°C), humidity (60±5%) and light (12 h light/12 h dark cycle), being fed standard rodent food and water *ad libitum*.

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).

The experimental rats were randomly divided into three groups of ten animals in each group. One group of animals was injected intraperitoneally (i.p.) with semi-lethal dose (LD50) (1.576 µg· g^{-1} of body weight) of *Vipera berus berus* venom in saline solution and another group was injected i.p. with LD50 (0.972 µg· g^{-1} of body weight) of *Vipera berus nikolskii* venom in saline solution, respectively. The remaining group was injected i.p. with saline solution, being used as a control. Injections and the measurement of the dose was performed according to Shitikov *et al*. [24]. Cervical dislocation was used to kill animals after 24 h of the exposure to the venoms and the collection of organs under investigation (spleen and heart) was done immediately.

2.2 Venom

The experiments were conducted on the targeted organs of rats using the venoms of *V. berus berus* and *V. berus nikolskii* obtained from the V.N. Karazin Kharkiv National University (Kharkiv, Ukraine). Lyophilized crude venoms were kept at -20°C, then dissolved in saline immediately before experiments. They were centrifuged at 10.000 g for 15 min and the supernatant was used.

2.3 Preparation of homogenates of organs

The temperature conditions for the isolation of organs and homogenate preparation were between 1ºC and 4ºC. Tissue homogenization was performed in 50 mM Tris-HCl buffer solution (pH 7.4) containing 140 mM NaCl and 1 mM EDTA. The volume (mL) of the used buffer was 5 times greater than the mass (g) of the isolated organs. After that, the total homogenate was subjected to a series of centrifugations at 600 g for 15 min and at 15,000 g for 30 min, respectively, using the supernatant fraction after the first stage.

2.4 Determination of protein concentration

Protein concentration was determined by the Bradford method [25].

2.5 Disc electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide gel following the method of Laemmli [26]. SDS-PAGE was performed using Mini-Protean Tetra System (Bio Rad, USA) at 19 mA for stacking and 36 mA for separating gels. Samples for electrophoresis were prepared by mixing with sample buffer (0.05 M Tris, pH 8.8, 2% SDS, 5% sucrose, and 0.02% bromophenol blue) at a ratio of 1:1 (v/v). The samples were heated at +90 ºC for 1 min before loading into the gel. The total amount of proteins was 20 µg per well. The gels were stained with 2.5% Coomassie brilliant blue R-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15% (v/v) isopropanol for 30 min. Excess stain was washed from the gels by placing them in boiling water containing 5% (v/v) acetic acid. The gels were photographed with a digital camera and the obtained electrophoregrams were analyzed by TotalLab 2.04 (TotalLab Ltd., UK). The molecular weights of proteins and the number of fractions were calculated automatically by TotalLab 2.04.

To calculate the molecular weights of proteins, the Natural High-Range SDS-PAGE Standards (Bio-Rad Laboratories, Inc, Hercules, California, USA) were used.

2.6 Enzyme electrophoresis

Enzyme electrophoresis was performed according to the method of Ostapchenko *et al*. [27]. The basis of the method is connected to the possibility of cleaving specific substrates under the action of cleavage enzymes. Thus, in this approach the separating gel of 12% concentration was polymerized with substrate proteins (collagen, fibrinogen and gelatin) at a concentration of 1 mg · mL^{-1}. Samples were not preheated to prevent loss of enzymatic activity of the proteins.

After process of electrophoresis, the gels were washed in a 2.5% Triton X-100 solution for 1 h, removing the remains of SDS, and incubated in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl for 12 h under 37ºC. The gels were stained and fixed according to the standard electrophoresis protocol (Section 2.5). The qualitative composition and amount of active protease fractions were determined using the TotalLab 2.04 program (TotalLab Ltd., UK). Enzymes with a known molecular weight (23 kDa trypsin; 36 kDa mini-plasmin; 84 kDa plasmin) were used as marker proteins to calculate the molecular weight of active enzymes.

2.7 Determination of the content of low-molecular-weight molecules

Nikolaychuk method [28] was used to isolate the fraction of low-molecular-weight molecules (LMWM). All manipulations during the experiment were carried out on ice and a 15 min endurance was performed with the samples after each manipulation. The step of protein precipitation was conducted by mixing the samples with an equal volume of 1.2 M $HClO_4$, which was followed by centrifugation for 15 min at 10,000 rpm. After that, the supernatant was centrifuged again under the same conditions after achieving pH 7.0 by adding 5 N KOH to the supernatant. The third step of protein precipitation included transfer of previously obtained supernatant to microtubes and addition of 96% ethyl alcohol in a ratio of 1:5. After that, samples were centrifuged for 15 min at 10,000 rpm.

The determination of molecules of different structures was performed by the identification of the optical density of the samples at the wavelengths of 210 nm (indicating the presence of peptides), 238 nm (indicating mainly the presence of non-aromatic peptides), and 254 nm (indicating the presence of non-aromatic sulfur-containing molecules, as well as purine bases and some nucleotides). The level of LMWM was expressed as rel. units per g of tissue.

2.8 Statistical analysis

Statistica 8.0 software was used to perform statistical analysis. The data of biochemical estimations were reported as mean±SEM for each group (n = 10). The Kolmogorov-Smirnov test was used to verify the normal distribution of results. Statistical analyses were performed using a one-way analysis of variance (ANOVA). Differences were considered to be statistically significant when $p < 0.05$.

3. Results and Discussion

At the first stage, the investigation of the influence of snake venom on the content of total protein and the protein profile in the tissues of the heart and spleen was performed in order to estimate the impact of venom on the organism of prey. Protein balance is analyzed as a possible primary target of the action of snake venom as there are a lot of proteinases found in the venom of many snakes.

Estimation of the total protein balance of the spleen and the heart after the influence of viper *V. berus berus* and *V. berus nikolskii* venoms revealed a reduction in the amount of proteins in both targeted organs, spleen and heart. Under the action of *V. berus berus* or *V. berus nikolskii* venoms, Figure 1 shows the negative influence of the venoms on the protein balance of the organism. The level of total proteins in the organs, which was expressed as mg per g of the targeted tissue, was significantly affected by *V. berus nikolskii* venom, reducing it by 59% in spleen and by 24% in heart (Figure 1). The impact of *V. berus berus* venom on the spleen is estimated to reduce the level of total protein by 17%, while the protein amount in the heart was slightly decreased (Figure 1). So, a bigger impact on the spleen, compared with the heart, was shown.

The reduction of the amount of total proteins can be associated with the activation of the degradation processes, hyperactivation of proteolytic systems, and catabolic processes or alterations in protein-synthetic processes due to the direct action of enzymes or effectors present in snake venoms. It can also be the result of changes in tissue homeostasis, in particular, in the development of oxidative stress and inflammatory processes in response to the complex action of snake venoms [29-31]. Although changes in protein-synthetic processes are considered to be possible for the reduction of proteins, there are several cautions about the validity concerning the differences in the time frames of synthetic mechanisms transformation and the time spent in this experiment. Worth claiming that the influence of *V. berus nikolskii* venom on the targeted organs is far more notable, compared to *V. berus berus* one, which may probably reflect the difference in the composition of the venom of these snakes [32]. Reduction of the protein amount greatly influences the homeostasis of the body, leading to alterations in the physiological functioning of the body. Understanding the possibilities of the existence of several mechanisms that underlie the decline of protein amount, further investigation needs to be conducted in order to confirm or refute each of the assumptions, which include hyperactivation of proteolytic systems or changes in the protein-synthetic processes of the organ.

The obtained data, which indicates the reduction of total protein content in targeted organs under the influence of *V. berus berus* and *V. berus nikolskii* venoms, aroused the interest in examining the reasons for the protein balance shift, and raised the question of analyzing the qualitative protein composition of the tissues. Thus, a second step of determining the impact of viper venoms on the rat spleen and heart protein balance by disk electrophoresis in PAGE in the presence of SDS was performed, and a qualitative protein profile of targeted organs was measured (Table 1). The redistribution of protein molecules seen as reduction of molecular weight was identified in both studied organs under the action of both *V. berus berus* and *V. berus nikolskii*

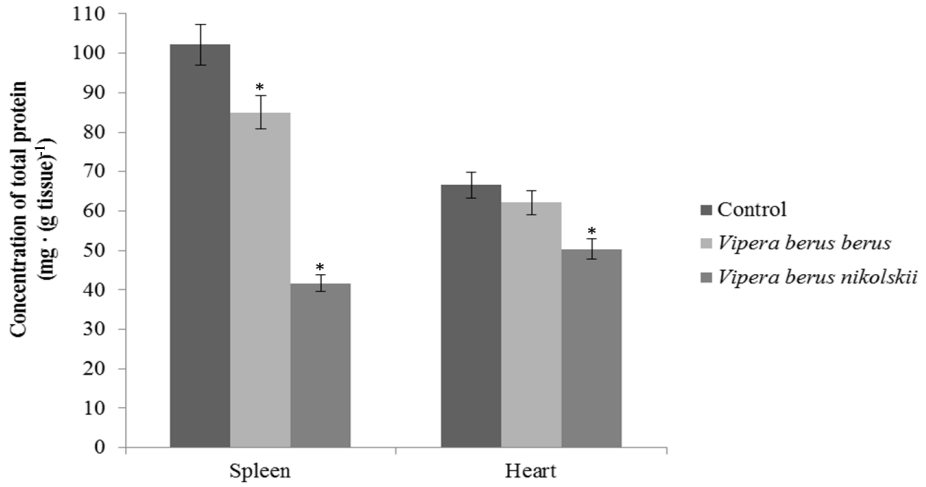

Figure 1. The total protein content in the spleen and the heart under the influence of *Vipera berus berus* and *Vipera berus nikolskii* viper venoms (mg · (g of tissue)$^{-1}$)
* – $p<0.05$ comparing to the control

Table 1. Protein profile of heart and spleen tissues under the influence of *Vipera berus berus* and *Vipera berus nikolskii* viper venoms

	Control		***Vipera berus berus***		***Vipera berus nikolskii***	
	Band, %	**Number of Fractions**	**Band, %**	**Number of Fractions**	**Band, %**	**Number of Fractions**
			Spleen			
≥ 150 kDa	–	–	–	–	–	–
150-100 kDa	5.29	2	8.96	2	–	–
100-67 kDa	–	–	–	–	–	–
67-35 kDa	23.42	3	28.99	4	11.31	3
35-10 kDa	31.6	3	62.06	4	53.46	5
≤ 10 kDa	39.68	2	–	–	35.23	4
			Heart			
≥ 150 kDa	–	–	–	–	–	–
150-100 kDa	–	–	–	–	4.35	1
100-67 kDa	5.69	1	7.66	1	5.27	2
67-35 kDa	13.26	3	27.46	1	28.33	3
35-10 kDa	49.69	4	33.89	2	21.85	1
≤ 10 kDa	31.35	4	30.99	3	40.21	1

venoms. The increased amount of proteins, almost doubled compared to the control tissues, and protein fractions under the influence of both viper venoms in spleen was found to be in the range of 10-35 kDa, whereas heart tissue underwent a reduction of proteins in this range, showing an increased percentage of protein molecules in 35-67 kDa. Furthermore, the appearance of a protein fraction in the range of 100-150 kDa was observed in tissues of the heart under the influence of *V. berus nikolskii*, whilst the controlled tissues did not present such proteins. Furthermore, the number of fractions of proteins was altered in both studied organs.

All of these data demonstrate the confirmation of the assumption of direct proteolytic system activation in the studied organs under the influence of *V. berus berus* and *V. berus nikolskii* venoms. We assume that the appearance of a new fraction of proteins in the molecular weight range

of 100-150 kDa in the heart under the action of *V. berus nikolskii* poison results from the formation of covalent complexes of certain degraded proteins or aggregation of enzymes with their inhibitors in response to the action of snake venoms. Such a shift in a tissue protein profile and protein content indicates a serious imbalance, strongly suggesting that the injection of snake venoms leads to the onset of the body dysfunction.

Maintaining the stability of the protein composition of tissues is an important requirement for ensuring the proper functioning of the body. Thus, the changes in the protein profile in the tissues of the heart and spleen that we discovered together with the background of a decrease in the total protein content indirectly indicate a violation of the general homeostasis and lead to serious consequences. Sometimes such changes can lead to the development of pathological states.

As far as the activation of proteolytic processes was shown in the spleen and in the heart under the influence of *V. berus berus* and *V. berus nikolskii* venoms, the study of the enzymatic profile of targeted organs was required as a second step of the assay. It was conducted by the zymography. Different substrate specificity (gelatinolytic, fibrinogenolytic and collagenolytic) was measured and the results are as follows.

Total proteolytic activity was determined with the use of gelatin as a substrate. An increase of the active proteolytic molecules capable of breaking down gelatin was observed in the spleen under the action of both viper venoms, and this increase was in the range of 35-67 kDa, but the amount of enzymes decreased in the rnge of 67-100 kDa (Figure 2A). However, changes of the amount of enzymes of this specificity in the molecular weight of 10-35 kDa are not as obvious: the action of *V. berus berus* reduced the level by almost twice, while exposure to *V. berus nikolskii* resulted in the enhancement of the percentage of these molecules, causing the huge reduction of proteins in the molecular weight equal to 67-100 kDa to some extent (Figure 2A). The qualitative composition of active proteases in the 10-35 kDa and 35-67 kDa ranges increased after the influence of *V. berus nikolskii* from 1 for the control to 2, while the impact of *V. berus berus* resulted in an expanse of the fractions in the 35-67 kDa molecular weight range from 1 for the control to 3. The amount of fractions of active proteolytic enzymes with the molecular weight in the range of 67-100 kDa decreased under the action of both viper venoms (Figure 2A).

In general, such changes indicate the activation of certain proteolytic enzymes or the formation of non-physiologically degraded forms of enzymes. Such processes, activated by the impact of the studied venoms, result in alterations of the metabolism of affected cells together with the enhancement of the activity of physiologically inactive enzymes, and an increase in the level of altered and slightly degraded proteins with changed substrate specificity. All of these processes lead to the appearance and activation of non-physiological biochemical mechanisms, which are responsible for the further development of the intoxication processes.

Fibrinogenolytic activity, which corresponds to the presence of blood stream enzymes and can be an indication of a pathological process, was not detected in the controlled tissues. However, both spleen and heart tissues under the influence of *V. berus berus* and *V. berus nikolskii* venom show the appearance of enzymes with the molecular weight equal to 10-35 kDa and 35-67 kDa, which are capable of cleaving fibrinogen (Figure 2B).

Thus, after evaluating the data towards the presence or absence of active proteolytic enzymes with fibrinogenolytic activity in tissues and taking into consideration the correlation of the appearance of active enzymes with substrate specificity towards fibrinogen in the tissues with the pathological processes, it can be claimed that the *V. berus berus* and *V. berus nikolskii* venoms' impact on the targeted organs and on the whole organism results in the alteration of the biochemical pathways, leading to the enhancement of pathological changes. Such a pathological shift is possible because of the synthesis of active fibrinogenolytic enzymes under the action of studied venoms, or with the creation of degraded/abnormal molecules with changed substrate specificity.

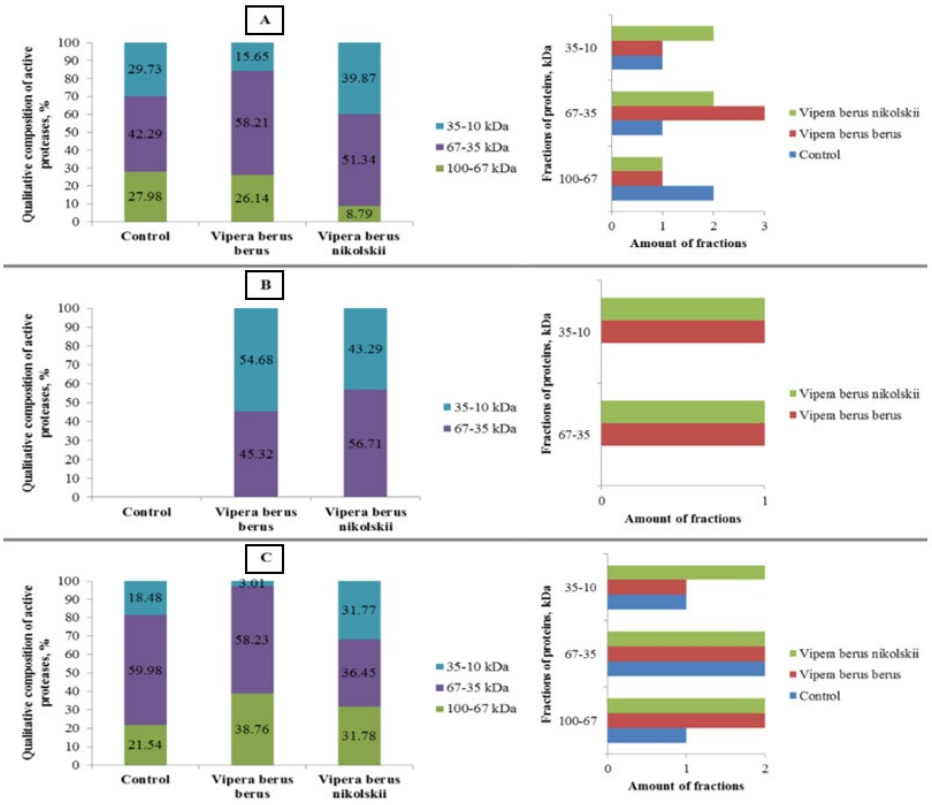

Figure 2. Qualitative composition and the amount of fraction of active proteases in the spleen under the influence of viper *Vipera berus berus* and *Vipera berus nikolskii* venoms with the use of different substrates: (A) gelatin, (B) fibrinogen, (C) collagen

Proteolytic molecules, showing a more specific collagenolytic activity, also experienced redistribution. The level of enzymes capable of breaking down collagen, with molecular weight of 10-35 kDa, significantly decreased after the influence of *V. berus berus*, but *V. berus nikolskii* action resulted in the enhancement of such molecules (Figure 2C). The action of both viper venoms resulted in the increase of the amount of proteins with molecular weight of 67-100 kDa (Figure 2C). However, the amount of proteolytic molecules having a molecular weight of 35-67 kDa decreased (Figure 2C). The amount of fractions of active proteolytic enzymes increased compared with control, in the range of molecular weight of 10-35 kDa and 67-100 kDa (Figure 2C).

Alterations in the enzymatic profile of the active proteolytic molecules, which demonstrate the ability to cleave collagen, show the direct impact of *V. berus berus* and *V. berus nikolskii* bites on the pathological enhancement of proteolytic processes that end with the activation of particular collagenolytic enzymes.

The analysis of the activity of proteolytic enzymes in the heart with the use of zymography with same substrates suggests a similar result to that found for spleen tissues. The results show a slight difference in the amount of fractions of active proteases and show an increase of molecules with molecular weight of 67-100 kDa and decrease of enzymes in 35-67 kDa range capable of breaking down gelatin (Figure 3A-C).

Previously obtained data showed significant alterations of protein balance demonstrating decrease of total protein amount and enzymatic profile redistribution, and thus proving the possible mechanisms for poisoning the targeted organs under the influence of viper *V. berus berus* and *V. berus nikolskii* venoms. Furthermore, the effects on the targeted organs can result in an intrinsic intoxication, which is associated with the appearance of low-molecular-weight molecules.

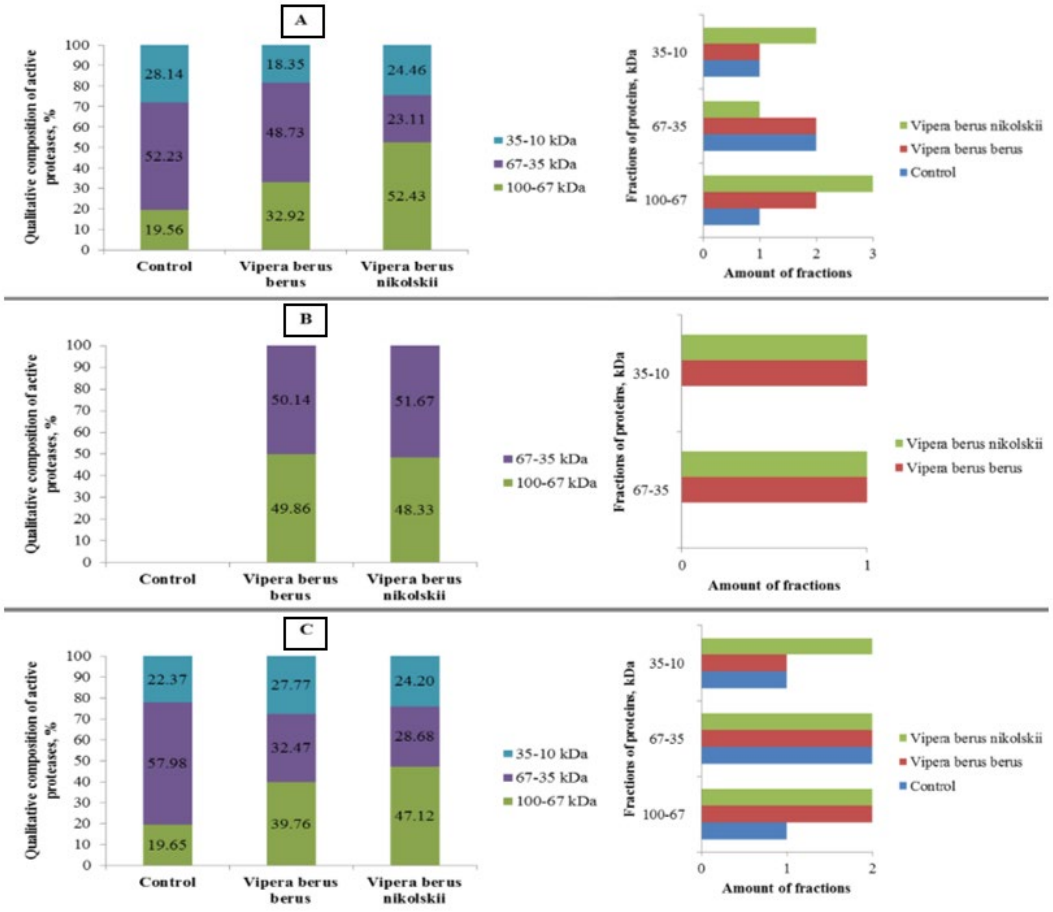

Figure 3. Qualitative composition and the amount of fraction of active proteases in the heart under the influence of viper *Vipera berus berus* and *Vipera berus nikolskii* venoms with the use of different substrates: (A) gelatin, (B) fibrinogen, (C) collagen

Therefore, the content of LMWM in the heart and spleen tissues of animals that received a semi-lethal dose of *Vipera berus berus* and *Vipera berus nikolskii* venoms was determined. Spectrophotometric assay shows the enhancement in the level of LMWM in targeted organs under the influence of both poisons in the spleen (Figure 4A) and the heart (Figure 4B). Since the composition of the fraction of LMWM is heterogeneous, measurements were carried out at different wavelengths – 210, 238, and 254 nm – in order to estimate the contribution of molecules with different chemical structures. A slight increase in the amount of LMWM that can be measured at 210 nm was observed under the influence of both venoms in the heart and spleen compared to the

control (Figure 4A-B). The number of molecules measured at 254 nm, which reflect the presence mainly of non-aromatic peptides, increased in the range of 8 to 14 times in both examined organs after the exposure to *V. berus berus* and *V. berus nikolskii* venoms (Figure 4A-B). Moreover, the greatest change in the amount of LMWM measured at 254 nm was a characteristic of the spleen (Figure 4A). However, the most significant change in the number of LMWM, compared with the control, was demonstrated in the spleen: the content of molecules determined at 238 nm was 47.9 and 37 times higher than the amount in the control group after injections of the venoms of *V. berus berus* and *V. berus nikolskii*, respectively (Figure 4A).

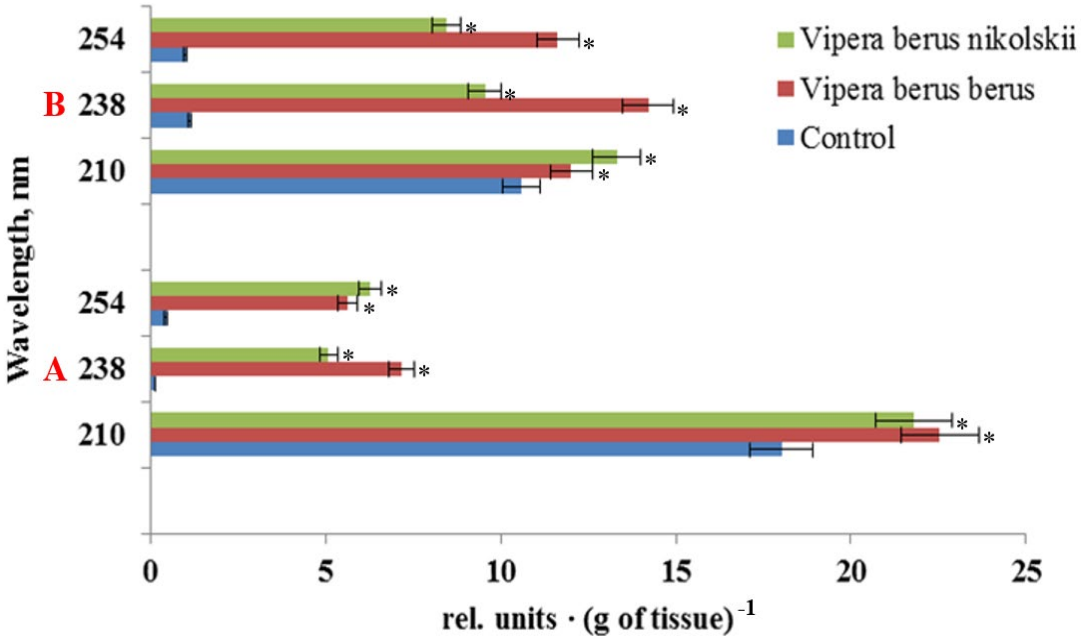

Figure 4. The level of low molecular weight molecules in the tissues of the spleen (A) and the heart (B) under the influence of viper *V. berus berus* and *V. berus nikolskii* venoms
* – $p<0.05$ comparing to the control

Then, the transformation of the normal physiological state of the organism into a very abnormal state one was explained. The change was associated with the emergence of fundamentally new ligand-receptor interactions occurred due to the changes in protein profile. Such change caused alterations in the normal functioning of the organism and onset of intrinsic intoxication in both organs.

4. Conclusions

From the results in this experimental work, it can be seen that both *Vipera berus berus* and *Vipera berus nikolskii* venoms have considerable impact on the spleen and on the heart of the rats, leading to the physiological alterations together with the activation of the pathological process and the redistribution of proteins. Such changes cause the formation or activation of the enzymatic molecules with shifted substrate specificity because of the partial degradation processes. Thus,

change in key biochemical pathways are boosted due to the creation of new ligands that are responsible for transformation of the physiological state of the organism or the action of newly formed molecules that are the triggers for toxicity. Furthermore, it is worth noting the multidirectional effects of the studied viper venoms on the whole organism. These effects are associated with the influence of the alterations on the targeted organs and thus on the metabolic system of the whole organism.

5. Acknowledgements

The authors would like to express their deepest gratitude to Zinenko Oleksandr for providing the venoms of *V. berus berus* and *V. berus nikolskii*.

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