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ANALYTICAL AND QUANTITATIVE ASSESSMENT OF THE STATE OF THE SPLEEN AND IMMUNE PROCESSES IN RATS UNDER ADMINISTERING OF VIPERA BERUS VENOM

Bobr A.M. ✉ **Analytical and quantitative assessment of the state of the spleen and immune processes in rats under administering Vipera berus venom.**
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ABSTRACT. Background. The spleen can maintain the body's stability in response to the physical and chemical factors of the environment. However, the organ's immune status reserves suffer significantly under their chronic influence. They are depleted due to violating the morphology and functions of key structural elements. In this regard, more and more scientific studies are focusing on the pathophysiological pathways of spleen damage under the influence of adverse factors of various genesis. **Objective.** Determination of morphometric parameters of changes in the spleen of rats under the influence of Vipera berus venom. **Methods.** To assess the toxic effect of the venoms of Vipera berus berus and Vipera berus nikolskii snakes on the general morphology of the spleen and the manifestation of antigen-dependent immune processes in it, we compared discrete and continuous variables in rats from the control and two experimental groups. **Results and conclusion.** It was established that the pathological effect of Vipera berus nikolskii venom on the red pulp leads to a set of processes that increase its total area, thus equalising the ratio of white pulp to red pulp in this group. The most pronounced increase in the share of lymph nodes relative to the red pulp is in rats exposed to Vipera berus berus poison, indicating intensive lymphocyte proliferation processes precisely under the influence of this toxin. The hemolytic solid effect of the venom of Vipera berus nikolskii leads to the destruction of formed blood elements and the accumulation of their particles, which are also phagocytised by macrophages. This adds another load factor to phagocytising cells, leading to their overload and disruption of the normal processes of metabolism and exocytosis.
Key words: snake venom, rats, spleen, morphometry**.**

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Introduction

Among all poisonous animals, snakes attract the special attention of mankind. Such interest is because cases of poisoning by their toxins are prevalent and belong to unsolved health problems worldwide, as they cause numerous fatal consequences. According to WHO estimates, 81,000 to 138,000 people die from snake bites every year, and another 400,000 victims have severe complications or even disability [1, 2, 3, 4, 5]. However, despite this, snake toxins are also considered valuable sources for the production of various medicines [6, 7, 8, 9].

The spleen is one of the most reactive organs, and it shows a quick response to the action of damaging factors during the early stages of their influence. It is an essential centre for developing specific immunological reactions, as numerous cell populations ensure the stability of the body's internal environment. The spleen can maintain the body's strength in response to the physical and chemical factors of the environment. However, the organ's immune status reserves suffer significantly under their chronic influence. They are

depleted due to violating the morphology and functions of critical structural elements. In this regard, more and more scientific studies are focusing on studying the pathophysiological pathways of spleen damage under the influence of adverse factors of various genesis [10, 11].

To assess the toxic effect of the venoms of Vipera berus berus and Vipera berus nikolskii snakes on the general morphology of the spleen and the manifestation of antigen-dependent immune processes in it, we compared discrete and continuous variables in rats from the control and two experimental groups.

The study aims to determine the morphometric parameters of changes in the spleen of rats under the influence of Vipera berus venom.

Materials and methods

Morphological changes in the spleens of animals, reflecting the course of immune processes in these organs, were evaluated by comparing the ratio of the white and red pulp areas in the spleens of rats of the three studied groups. An increase in the average area occupied by the components of the white pulp may indicate an increase in the activity of the spleen in this group. This indicator was assessed on eight digital images obtained from 4 animals from each of the three groups, with a light microscope at magnifications of x200 and x400; the preparations were stained with eosin and hematoxylin. To correctly establish the relationship between the points of the digital image and the micrometres, measuring scales of 0,1 and 0,05 mm were applied to the image when taking pictures.

Based on the logic of immune processes in the spleen, the area of white pulp nodules was measured. Their fate was determined relative to the red pulp that surrounds them from all sides, reflecting the course of such reactions in this immune organ as antigen-dependent differentiation of B- and T- lymphocytes in response to the influence of external negative factors (in our case, toxins), the dedifferentiation of these cells into lymphoblasts, the formation of differentiated plasma cells capable of producing antibodies from B-lymphocytes, etc.

The potential negative impact of externally introduced poisonous factors on the functioning of the parenchymal and stromal components of the spleen was assessed using a discrete parameter - the number of macrophages containing the inclusion of lipofuscin pigment was counted since this pigment is a direct product of histiocytes' struggle with the toxic effect of reactive oxygen species on the components cells After all, it is known that the accumulation of toxins in tissues leads to an imbalance in the homeostasis of reactive oxygen species (ROS). In contrast, the liver and spleen are the main targets of this increased oxidative stress [12]. Also interesting is that, for example, in mice, low doses of toxicants are toxic to the immune system but relatively safe for other organs [13].

The "Cell counter" plugin was used in Fiji: ImageJ program to determine the number of macrophages with lipofuscin inclusions. Counting was performed on digital images of preparations stained with eosin and hematoxylin, which helped us verify the presence of brown-gold lipofuscin inclusions in macrophages. Five digital images from each of the four animals in three groups (control and two experimental) were processed at a magnification of x1000. Accordingly, we analysed 20 digital images for each group, which increased the statistical reliability of the measurements. All data were further processed in Excel.

Results and discussion

Since when checking measurements from control and experimental groups of animals for normality of distribution, the graphs did not correspond to a Gaussian curve; we used the non-parametric Mann-Whitney test to determine the reliability of differences between groups.

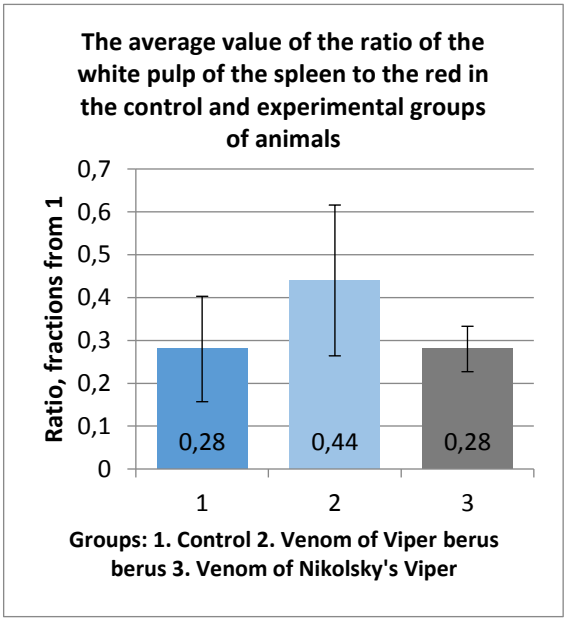

Fig. 1. The average value of the proportion of white pulp of the spleen to red with standard deviation in the control group, the group with the injection of Vipera berus berus venom and Vipera berus nikolskii. * - The difference from the control group is significant at p≤0,05.

According to our measurements, no significant difference relative to the control in the ratio between the white and red pulp of the spleen was observed for any of the experimental groups (Fig. 1). Nevertheless, we observed a tendency to increase in the proportion of white pulp for animals exposed to Vipera berus berus venom. If for the control group of rats, the average value of this indicator was 0,277 (first quartile 0,207; third quartile 0,343), then for the group whose animals were exposed to Vipera berus berus poison, it is 0,443 (first quartile 0,396; third quartile 0,54).

At the same time, the standard deviation of this parameter in the experimental group is slightly more

significant than in the control group, which indicates the heterogeneity of the growth processes of the white pulp within the red pulp in this group (Figs. 1, 2). These results correspond to our morphological description of the spleen parenchyma of animals from this experimental group. The increase in the area of the white pulp relative to the red pulp was facilitated by the general disorganisation of its structure, which was manifested, in particular, in the vagueness of the contours of the lymphoid nodules, due to which their marginal zone was almost impossible to differentiate. In addition, the growth of the white pulp was facilitated by an increase in the size of the germinal centres with an increase in the number of lymphoblasts in them. Just such a reaction is physiological when toxins enter the body with the blood because rejuvenation and active proliferation with a predominance of young lymphoblasts over mature B-lymphocytes accompany the body's responses to the negative impact of antigens.

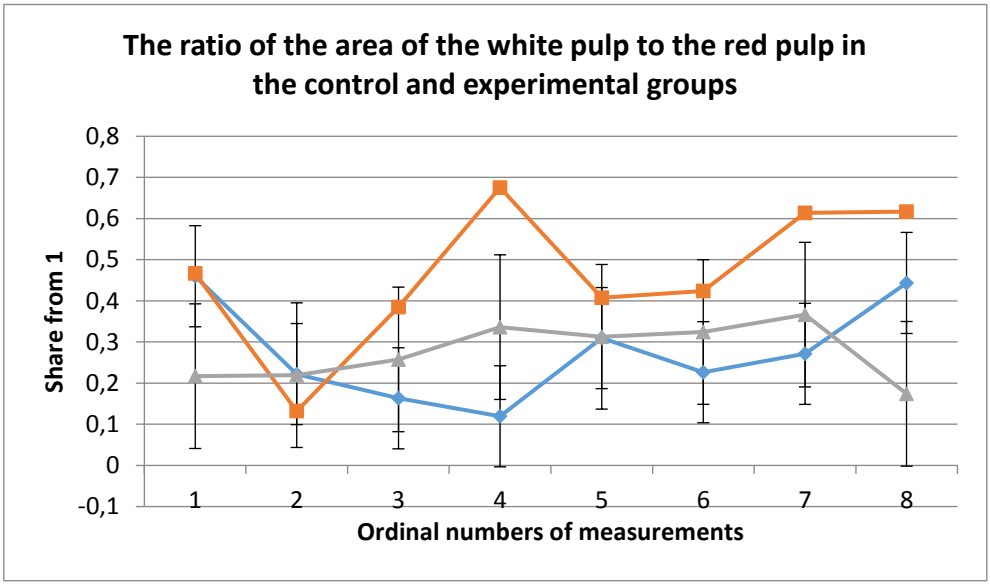

Fig. 2. Individual indicators of the proportion of white pulp of the spleen to red with standard deviations. Row 1 – control group; row 2 – a group with an injection of Vipera berus berus venom; row 3 – a group with an injection of Vipera berus nikolskii venom.

At the same time, in the red pulp of the spleen of animals from this group, the changes were not so pronounced as to affect its total area. After all, according to our morphological study, the zones of the destruction of red pulp tissues and haemorrhages in them, although they were present, were of a relatively limited nature. All these data together indicate the activation of the white pulp of the spleen of rats in response to the action of Vipera berus berus venom toxins.

Let's talk about changes in the group's white and red pulp ratio with the introduction of Vipera berus nikolskii venom. This indicator practically does not differ from that in the control group. Here, it is 0,278 (first quartile 0,229; third quartile 0,321) with a mean of 0,277 in controls. When interpreting these results, it is essential not to forget that we are not talking about absolute but about relative indicators; that is, here, it is necessary to take into account changes not only in the white but also in the red pulp - when the areas of both of these components of the parenchyma increase, the ratio between them will not change dramatically, which does not indicate the absence of pathological processes in them.

During the morphological study of the white pulp of rats from this group, we noted a decrease in the number of lymphocytes in the marginal zone and a general increase in the number of lymphoblasts, which generally corresponds to the normal reaction of the immune organ to the entry of an adverse external factor into the body - an antigen. These processes lead to an increase in the proportion of white pulp, but in the case of this group, a significant degree of red pulp impression led to the invariance of the overall ratio.

It is also worth paying attention to the fact that the area of the white pulp is not only affected by the processes of mitosis of white cells and an increase in their number but may also decrease somewhat due to the death of a part of them by apoptosis. During the morphological examination, we found lymphocyte proliferation processes in the white pulp and their death, with typical signs of apoptosis: the cells faded, and their nuclei had signs of pyknosis. These processes generally did not contribute to the growth of this parenchyma component.

When studying the state of the red pulp of animals with the simulation of acute intoxication with Vipera berus nikolskii viper venom, we noted foci of haemorrhage due to the violation of vascular integrity due to the vasotoxic effect of the venom. Presumably, due to the poison's hemotoxic effect, the veins' lu-

mens expanded, filled with aggregated formed elements of the blood. As is known, the intravascular component of the red pulp also affects its total area, so whole blood in the vein lumens and stasis of formed blood elements added to the expansion of the red pulp area.

Thus, according to the results of the evaluation of the ratio of white pulp to red in the control group and groups with the simulation of acute intoxication with two types of poisons, we found a tendency to increase the share of white pulp in the group affected by Vipera berus berus venom, which we associate with the active course of immune processes in it. At the same time, the significant destructive effect of Vipera berus nikolskii venom on both white and red pulp led to an increase in the area of both and had little effect on the ratio between them. At the same time, taking into account the data of the morphological description, it becomes clear that the significant effect of the venom components of this viper on the blood and blood vessels in the composition of the red pulp is more aggressive than in the other experimental group.

Determination of the number of tissue macrophages, which include lipofuscin granules, is indicative in assessing the level of oxidative stress in the tissues of the spleen in animals from experimental groups when simulating acute intoxication with two types of poisons because, as is known, the action of external toxins leads to an increase in the concentration of reactive oxygen species in tissues [13]. Standard distribution plots of the count data did not show a Gaussian curve, so we again used the Mann–Whitney test to compare groups.

When analysing this parameter, a statistically significant increase in the number of macrophages with lipofuscin inclusions was found, according to the Mann-Whitney test, not only in both experimental groups relative to the control group but also between the two groups in which poisoning was simulated (Fig. 3). In general, this indicates the benefit of increasing oxidative stress and combating reactive oxygen species.

If animals in the control group, on average, 6,2 cells with lipofuscin are observed in the field of view at x1000 magnification (first quartile 5; third quartile 7), then for the group whose animals were exposed to Vipera berus berus venom, the number of cells on average is 14,55 (first quartile 12; third quartile 16,25) (Fig. 3). A statistically significant increase in the number of cells with lipofuscin inclusions of a characteristic golden-brown shade indicates the accumulation of an excessive amount of aggregated proteins. It can be argued that this process is enhanced by oxidative stress caused by intoxication. It is known from the literature that protein aggregates are absorbed by macroautophagy and, as a result of the reaction with other cellular components, form lipofuscin, which accumulates in the lysosomal system of cells [14].

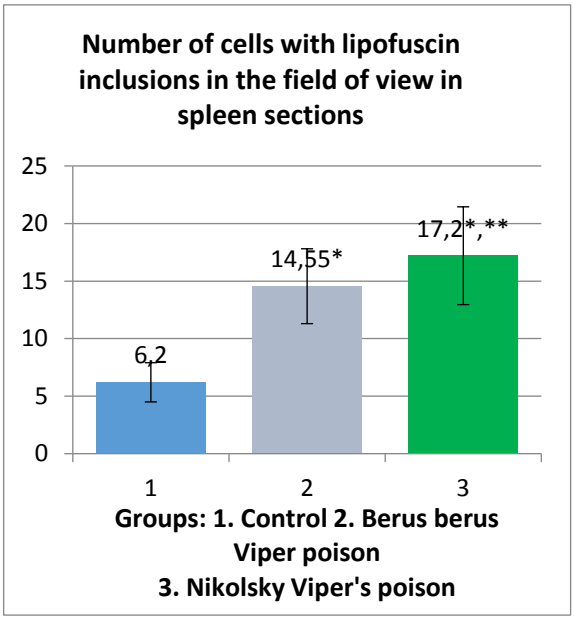

Fig. 3. The average value of the number of macrophages with lipofuscin inclusions in the field of view in the control and experimental groups. * - The difference from the control group is significant at p≤0,05; ** - from the experimental group with the introduction of Vipera berus berus venom is significant at p≤0,05.

The morphological description of the control and this experimental group helps to understand such morphometric study results precisely. In the group where rats were exposed to Vipera berus berus venom, numerous large-sized macrophages characterised by numerous appendages were observed in the red pulp. This morphology of these cells indicates their increased phagocytic activity. Cells try to absorb harmful particles from the environment, and lysosomes accumulate metabolic products of these particles, some of which are lipofuscin. The fact that at the same time, we noted signs of oedema, uneven staining of the cytoplasm of macrophages and accumulated remnants of phagocytosed material in them indicates that, probably, these cells did not always cope with an excessive amount of harmful substances could not digest everything absorbed and take it outside by exocytosis. Cells underwent pathological changes and accumulated undigested remnants of toxins, including the so-called "accumulation pigment" - lipofuscin.

In rats exposed to the potentially more toxic venom of Vipera berus nikolskii, an average of 17,2 cells with inclusions of lipofuscin pigment (first quartile 14,75; third quartile 18,5) are observed in the field of view at x1000 magnification, which is statistically significantly more of these cells not only in the control group but also in the group injected with Vipera berus berus venom, which once again confirms its higher toxicity. A statistically significant increase in the proportion of macrophages with lipofuscin inclusions shows the struggle of these cells with toxin particles and the consequences of increasing the level of reactive oxygen species in the tissues of the spleen as

one of the consequences of poisoning.

According to the results of a morphological study, this increase in the number of macrophages with lipofuscin inclusions can be explained by pathological processes in these cells that prevented the final digestion of absorbed foreign particles and contributed to the accumulation of this golden-brown pigment in them. In this group, as in the other experimental group, an increase in the size of macrophages and the number of their processes was observed. However, a morphological feature unique to this group is the foamy cytoplasm of these cells with phagocytosed remains of blood elements. Most likely, the death of blood cells as a result of the hemotoxic effect of Vipera berus nikolskii venom led to an overload of phagocytic cells of the spleen, which could not digest such a large number of particles of foreign origin and remnants of its cells, which led to swelling, slowing down of metabolic processes and accumulation of metabolic products.

It is also worth noting that the standard deviation is greater in both experimental groups than in the control group. Therefore, the number of macrophages increases unevenly in the spleen sections of animals from these groups.

Summary

In general, according to the results of processing discrete and continuous data of calculations and measurements in the spleen of animals of the control group and experimental groups with the simulation of acute intoxication with two types of poisons, it should be noted that the increase in the total area of the white pulp is characteristic of both experimental groups in comparison with the control group. Still, the pathological effect of Vipera berus nikolskii venom on the red pulp leads to a set of processes that increase its total area, thus equalising the ratio of white pulp to red pulp in this group. Therefore, the most pronounced increase in the share of lymph nodes relative to the red pulp is precisely in rats exposed to Vipera berus berus poison, indicating intensive lymphocyte proliferation processes precisely under the action of this toxin.

In contrast to the proportion of white pulp, the number of macrophages with lipofuscin inclusions is statistically significantly more significant according to the Mann-Whitney test for both experimental groups compared to the control group, which indicates a response to the toxic effect of both poisons not only of lymphocytes, but also macrophages, and the impact of toxins in excessive amounts on metabolic processes in cells. At the same time, the solid hemolytic effect of Vipera berus nikolskii venom leads to the destruction of formed blood elements and the accumulation of their particles, which are also phagocytised by macrophages. This adds another load factor to phagocytising cells, leading to their overload and disruption of the normal processes of metabolism and exocytosis. Cells swell and accumulate intermediate products of metabolism, undigested remains, including lipofuscin. Therefore, the number of macrophages with accumulations of lipofuscin in this group is significantly higher than that of the control group and the other experimental group.

Prospects for further development are related to the analysis of structural and functional changes in the spleen of experimental rats after the introduction of Vipera berus venom.

Information on conflict of interest

There are no potential or apparent conflicts of interest related to this manuscript at the time of publication and are not anticipated.

References

1. Bolon I, Durso AM, Mesa SB, Ray N, Alcoba G, Chappuis F, Ruiz de Castaneda R. Identifying the snake: first scoping review on practices of communities and healthcare providers confronted with snakebite across the world. PLoS One. 2020;15(3):e0229989. URL: https://doi.org/10.1371/journal.pone.0229989.

2. Di Nicola MR, Pontara A, Kass GEN, Kramer NI, Avella I, Pampena R, Paolino G. Vipers of major clinical relevance in Europe: Taxonomy, venom composition, toxicology and clinical management of human bites. Toxicology. 2021;453:152724. Doi: 10.1016/j.tox.2021.152724.

3. Gutiérrez JM, Calvete JJ, Habib AG, Harrison RA, Williams DJ, Warrell DA. Snakebite envenoming. Nat Rev Dis Primers. 2017;3:17063. Doi: 10.1038/nrdp.2017.63.

4. Hermansen MN, Krug AH, Tjønnfjord E, Brabrand M. Envenomation by the common European adder (Vipera berus): a case series of 219 patients. Eur J Emerg Med. 2019;26(5):362-365. Doi: 10.1097/MEJ.0000000000000577.

5. Jayakrishnan MP, Geeta MG, Krishnakumar P, Rajesh TV, George B. Snake bite mortality in children: beyond bite to needle time. Arch Dis Child. 2017;102(5):445-449. Doi: 10.1136/archdischild-2016-311142.

6. Alangode A, Rajan K, Nair BG. Snake antivenom: Challenges and alternate approaches. Biochem Pharmacol. 2020;81:114135. Doi: 10.1016/j.bcp.2020.114135.

7. Herzig V, Cristofori-Armstrong B, Israel MR, Nixon SA, Vetter I, King GF. Animal toxins – nature's evolutionary-refined toolkit for basic research and drug discovery. Biochem Pharmacol. 2020;181:114096. URL: https://doi.org/10.1016/j.bcp.2020.114096.

8. Simoes-Silva R, Alfonso J, Gomez A, Holanda RJ, Sobrinho JC, Zaqueo KD, Soares AM.

Snake venom, a natural library of new potential therapeutic molecules: challenges and current perspectives. Curr Pharm Biotechnol. 2018;19(4):308-335. Doi: 10.2174/1389201019666180620111025.

9. Xiao H, Pan H, Liao K, Yang M, Huang C. Snake venom PLA2, a promising target for broad-spectrum antivenom drug development. Biomed Res Int. 2017;2017:6592820. Doi: 10.1155/2017/6592820

10. Feriani DJ, Sousa AS, Delbin MA, Ruberti OM, Crestani CC, Rodrigues B. Spleen tissue changes after restraint stress: effects of aerobic exercise training. Stress. 2021;24(5):572-583. Doi: 10.1080/10253890.2021.1895112.

11. Li Y, Du X, Zhao Y, Wang J, Wang J. Fluoride can damage the spleen of mice by perturbing Th1 / Th2 cell balance. Biol Trace Elem Res. 2021;199(4):1493-1500. Doi: 10.1007/s12011-020-02264-y.

12. Sang X, Zheng L, Sun Q, Li N, Cui Y, Hu R, Gao G, Cheng Z, Cheng J, Gui S. The chronic spleen injury of mice following long-term exposure to titanium dioxide nanoparticles. J. Biomed. Mater. Res. Part A. 2012;100:894–902. doi: 10.1002/jbm.a.34024

13. Reddy RV, Taylor MJ, Sharma RP. Evaluation of citrinin toxicity on the immune functions of mice. J. Food Prot. 1988;51:32–36. doi: 10.4315/0362-028X-51.1.32.

14. Höhn A, Grune T. Lipofuscin: formation, effects and role of macroautophagy. Redox Biology. 2013;1(1):140-144. https://doi.org/10.1016/j.redox.2013.01.006.

Бобр А.М. Аналітична і кількісна оцінка стану селезінки та імунних процесів у щурів за умов введення отрути гадюк виду Vipera berus.

РЕФЕРАТ. Актуальність. Селезінка здатна підтримувати стійкість організму у відповідь на дію фізичних та хімічних факторів навколишнього середовища. Однак, за умов їх хронічного впливу, резерви імунного статусу органу значно страждають та виснажуються внаслідок порушення морфології та функцій ключових структурних елементів. В зв'язку з цим серед наукових досліджень сьогодення з'являються все більше тих, що зосереджені на вивченні патофізіологічних шляхів ураження селезінки при впливі несприятливих факторів різного генезу. **Мета.** Встановлення морфометричних параметрів змін в селезінці щурів при впливі отрути гадюк виду Vipera berus. **Методи.** Для оцінки токсичного впливу отрут змій *Vipera berus berus* та *Vipera berus nikolskii* на загальну морфологію селезінки та прояви антиген-залежних імунних процесів у ній, нами було проведено порівняння дискретних і безперервних змінних у щурів з контрольної та двох експериментальних груп. **Результати та підсумок.** Встановлено, що патологічний вплив отрути *Vipera berus nikolskii* на червону пульпу призводить до сукупності процесів, які збільшують її загальну площу, таким чином вирівнюючи співвідношення білої пульпи до червоної у цій групі. Найбільш яскраво вираженим зростання частки лімфатичних вузликів відносно червоної пульпи є саме у щурів, що зазнали дії отрути *Vipera berus berus*, що свідчить про інтенсивні процеси проліферації лімфоцитів саме при дії цього токсину. Сильна гемолітична дія отрути *Vipera berus nikolskii* призводить до руйнування формених елементів крові і накопичення їх часточок, які теж фагоцитують макрофаги. Це додає ще один фактор навантаження на фагоцитуючі клітини, призводячи до їх перевантаження і порушення нормальних процесів метаболізму і екзоцитозу.

Ключові слова: отрута змій, щурі, селезінка, морфометрія.