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Original paper

Indirect effect of substances of the hemophagous parasite *Hirudo verbana* on the immune system of the host rats

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ABSTRACT. A positive relationship between the hematophagous parasite-medicinal leech and the host (animals and humans) has been partially shown by many authors. But only direct influence was shown, and how they can influence indirectly has not been studied by anyone. Therefore, it became relevant to analyse the immunological parameters of blood and histological analysis of the central lymphoid organ of female rats on the 30th day after the birth of their offspring, as well as the same indicators in their offspring on the 30th and 60th days of ontogenesis under the indirect influence of saliva substances of the hematophagous parasite *Hirudo verbana*. The parasite attachments were carried out to male rats before mating; after four attachments, males were added to the females. After pregnancy, the females were removed. The analysis of indicators was carried out according to the goal. We counted the number of erythrocytes and leukocytes, analysed the leukocyte blood count, studied the phagocytic and metabolic activity of neutrophils, histologically analysed the thymus, carried out a statistical analysis. Under the indirect influence of the substances of the parasite saliva, in experimental female rats and their offspring, in comparison with the control group, a significant increase in the number of leukocytes and erythrocytes is observed. The leukocyte blood count in the experimental group remains unchanged in females and their offspring in comparison with the control. When analysing the thymus in the experimental group, the number of lymphocytes significantly increases in females and their offspring. Substances of the hematophagous parasite are able to indirectly positively influence the general condition of pregnant female rats, and later their offspring. It should be noted that the parasite did not directly affect females and their offspring.

Keywords: hematophagous parasite, *Hirudo verbana*, rats, thymus, immunological parameters of blood

Introduction

Application of the hematophagous leech parasite has been used for centuries in medicine and veterinary medicine for the prevention and treatment of many diseases. The same effect was observed in nature, although it was noted in few places. During this time, the connection between the parasite and the host became more and more consolidated and developed. They have adapted to each other, their immune systems have become more resilient. And now the parasite is able to survive with the help of the host, and the host with their help also receive positive health effects. In the last decade, many scientific studies have shown the positive effects of the parasite in the prevention and treatment of many animal diseases. For example, experiments on laboratory rats have shown an increase in body weight and many important organs, hemoglobin [1], improved liver parameters [2] and improved wound healing [3]. In veterinary medicine: with uterine endometritis in small domestic animals [4–7], with intoxication and hematomas in dogs [8,9]. In horses, dogs and cats with hip dysplasia, acute and chronic arthritis, diseases associated with inflammation of the tendons and fasciae, diseases of the spine, and scar treatment [10]. In agriculture: in cows in the treatment of mastitis to increase milk yield and physiological blood parameters [11–14], in goats to improve physiological parameters, milk yield, fertility of offspring [15]. In our previous preclinical studies, we obtained a positive effect from the direct effect of medicinal leeches on the organism of female rats [16–18]. These parasites are also currently widely used in medicine for the prevention

and treatment of many diseases in humans. For example, in the treatment of ischemic heart disease, heart failure of A–B stages, atherosclerotic cardiosclerosis, postinfarction cardiosclerosis, cardialgia, discirculatory atherosclerotic encephalopathy, hypertension of 1–3 stages, in the prevention of ischemic stroke and many others. Patients with transient seizures, in the complex treatment of cardioembolic stroke in the acute period, encephalopathy [19,20], with acute disorders of cerebral circulation [21], venous congestion, in plastic and reconstructive surgery, osteoarthritis, migraine, with diabetic foot, skin diseases, priapism, complications of cancer and wound treatment, in the treatment of female diseases and the prevention of infertility [22–33]. Also, researchers have received positive results on the negative effect of medicinal leeches on *Mycobacterium tuberculosis* [34]. As a result of such a wide spectrum of action of the parasite substances, it became relevant to analyze the immunological parameters of the blood and histologically analyse central lymphoid organ of the female rat on the 30th day after the birth of their offspring, as well as the same indicators in their offspring on the 30th and 60th days of ontogenesis under the indirect influence of saliva substances of *Hirudo verbana* Carena, 1820.

Materials and Methods

Animals

The experiment involved 80 laboratory outbred rat pups: 40 females and 40 males and 20 adult male and female rats weighing 250–300 g. Also were taken 120 parasite-hematophages of *Hirudo verbana* Carena, 1820, obtained in the Laboratory of Cell and Organism Biotechnology of Zaporizhzhya National University. The studies used laboratory animals that passed the quarantine regime and did not have external manifestations of the disease. The animals were equally divided into two groups – control and experimental.

Experiment scheme

H. verbana with an average weight of 1.1±0.2 g was placed once every 3 days (4 times) on the coccygeal zone of adult laboratory outbred sexually mature male rats 7 months old. No manipulations were performed with adult males and females from the control group, as well as females from the experimental group. Then, three weeks after the last attachment of leeches, males of both groups were placed with females for mating. After pregnancy, females were removed from males in the control and experimental groups. The offspring obtained from females were fed up to 30 days and taken from their mothers.

Morphological and histological parameters

Blood was taken from females after the birth of offspring on the 30th day, and from the offspring on the 30th and 60th days. The resulting blood was diluted with heparin 1:10. The total number of leukocytes, erythrocytes and leukocyte blood count were analyzed by conventional methods. Phagocytic and metabolic activity of neutrophils was studied according to the methods developed by us [35,36].

The thymus in females and offspring was taken at the same time as blood, was fixed in a 10% solution of neutral formalin, paraffin blocks were made with the parts of the organ placed in them and performed according to the generally accepted technique. Using a Thermo Scientific HM 325 microtome, sequential sections of the entire thymus with a thickness of 4–6 μm were made. To study the structural components of the thymus gland, histological sections were stained with hematoxylin and eosin. Details of the histological structure of organs were studied using a cytomorphological complex based on an Axiocam digital camera and a Carl Zeiss Primo Star microscope. In which the number of cells per unit area was determined in the cortical and medullar substances of the thymus. Computer software analysis of histological slides was performed using the ZEISS ZEN 2011 software.

Statistical analysis

Statistical processing of the obtained results was carried out using parametric statistical methods (Student's t-test), after checking the sample for normality of distribution, with the values presented in tables as X ± SD, where X is the sample mean, SD is the standard deviation of the mean, with the package IBM SPSS Statistics 21.0 applications (USA). Differences were considered significant at a significance level of $P<0.05$.

Bioethics

The manipulations with animals were carried out in accordance with the regulated rules and regulations for the treatment of laboratory animals:

Table 1. Hematological and immunological parameters of the blood of female rats after pregnancy and their offspring

Indicators		Control			Experiment		
		females	30 days	60 days	females	30 days	60 days
Number of leukocytes 10^9/l		5.01±0.4	4.0±0.3	4.55±0.5	7.9±0.5*	5.4±0.2*	14.1±0.7*
Number of erythrocytes 10^{12}/l		6.9±0.3	3.5±0.4	5.06±0.3	8.7±0.5*	5.07±0.3*	5.99±0.4*
Leukocyte blood count,%	Sigmentonuclear neutrophils	14.21±0.75	11.5±0.6	6.3±0.6	13.92±0.61	11.52±0.5	4.3±0.7
	Rod neutrophils	12.10±0.55	3.6±0.7	2.7±0.3	13.31±0.85	4.0±0.5	2.61±0.4
	Lymphocytes	73.47±0.84	84.7±1.2	90±2.1	72.52±0.93	84.3±2.2	92.1±3.0
	Eosinophils	0.22±0.03	0.20±0.01	1±0.07	0.25±0.03	0.18±0.03	0.99±0.03
Phagocytic index,%		50.31±1.1	74.21±1.2	70.33±1.2	82.11±1.4*	74.70±1.6	74.74±1.4*
Phagocytic number		3.41±0.05	4.64±0.07	4.33±0.05	3.74±0.08*	4.68±0.08	5.76±0.08*
NBT sp., %		38.21±1.7	61.69±2.1	42.8±3.2	52.04±1.4*	79.02±3.2*	72.79±4.1*
NBT l., %		46.34±1.8	67.93±1.9	48.28±2.1	61.05±2.1*	85.58±2.3*	74.27±2.7*

* – indicators that significantly differ from the control group ($P \leq 0.05$); sp. – spontaneous; l. – loading; NBT – nitro blue tetrazole test

principles of bioethics, legal norms and requirements in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Research and scientific purposes the Law of Ukraine on the protection of animals from cruelty.

Results

Before pregnancy in the experimental group of females there were no differences in all hematological and immunological parameters of blood compared to the control group of animals, which confirms their physiologically healthy state.

When studying the hematological parameters of blood in experimental females after pregnancy and in their offspring, a statistical increase in the total number of leukocytes and erythrocytes was revealed, within physiological limits ($P \leq 0.05$) (Tab. 1). This indicates a positive stimulating effect of the

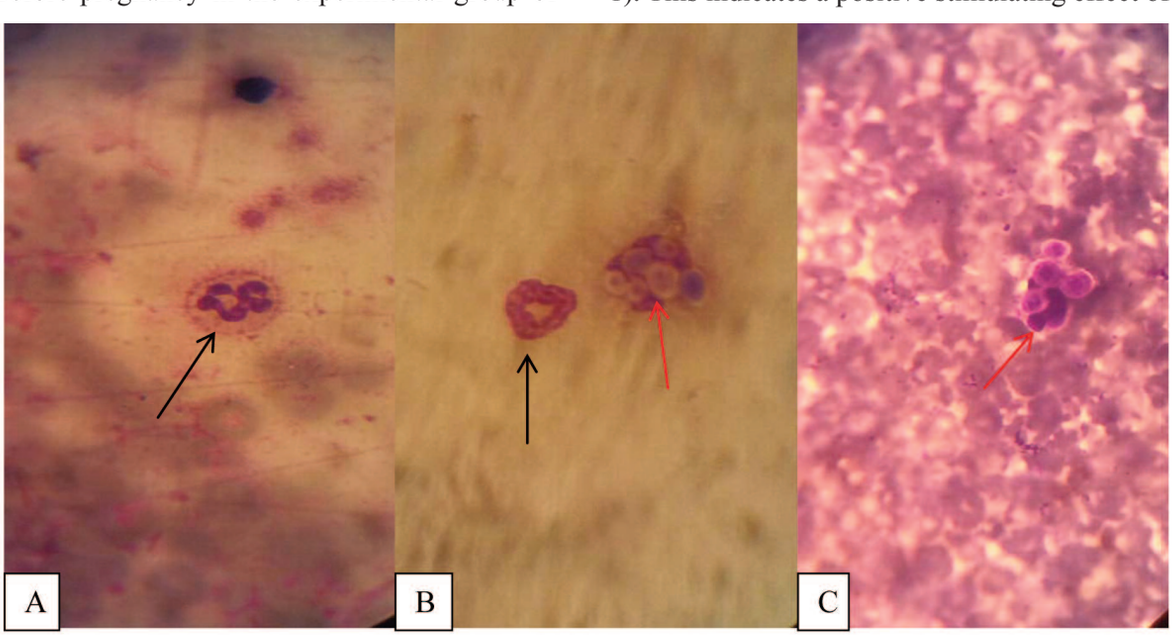

Figure 1. Phagocytosis of neutrophils: A – inactive, B and C – active neutrophils. Black indicates not activated, red indicates activated

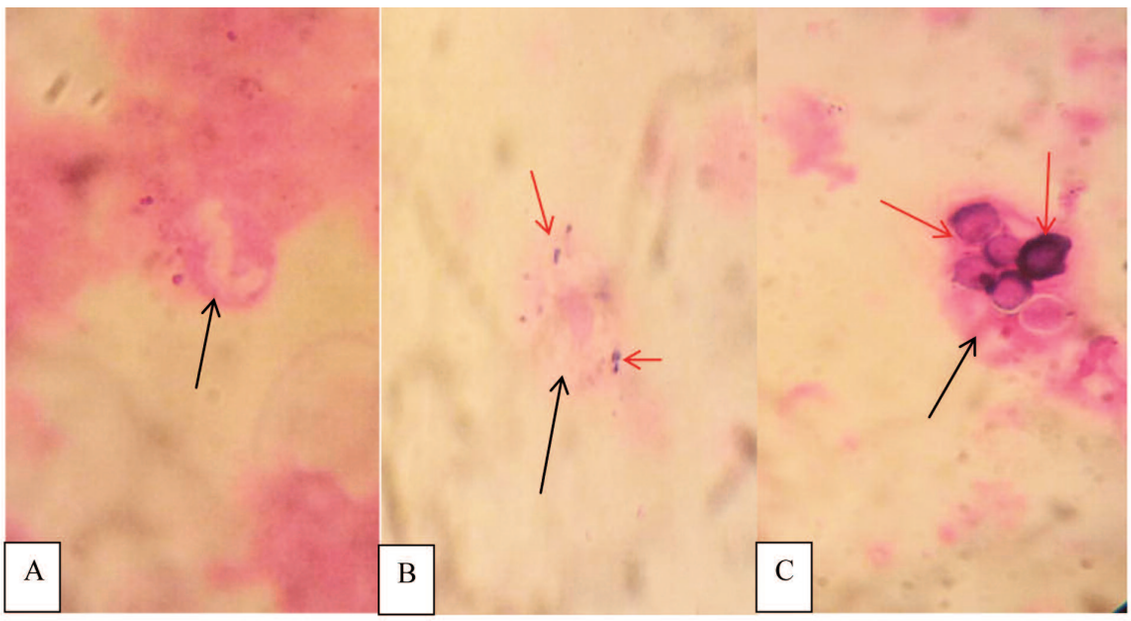

Figure 2. Nitroblue tetrazole test: A – not active, B – spontaneous test, activated neutrophil, C – stress test, activated neutrophil. Black arrows indicate neutrophil, red arrows indicate nitro-blue tetrazole granules

parasite substances on leukopoiesis and erythropoiesis. It should also be noted that the leukocyte blood count of the experimental group of females and offspring was balanced and did not statistically differ from the control group (Tab. 1).

The immunostimulating effect of the substances of the saliva of the parasite was also confirmed by the analysis of indicators of the phagocytic and metabolic activity of neutrophils in the blood of the host (females and offspring) in the table 1, figures 1 and 2.

When studying the phagocytic activity in the experimental group of females after pregnancy and in their offspring on the 60th day, there was a significant increase in the phagocytic index and phagocytic number (Tab. 1) ($P \leq 0.05$) and a slight increase in these indicators on the 30th day compared to the control group of animals. When studying metabolic activity in females and offspring: all indicators significantly increase in both spontaneous and stress tests (Tab. 1) ($P \leq 0.05$).

Histological analysis of the central organ of the immune system, the thymus, showed in experimental female rats and their offspring, both in the cortical and medullar substances, an increase in the number of cells per unit area (Fig. 3–8), the predominance of the cortical substance of the thymus over medullar which indicates the indirect

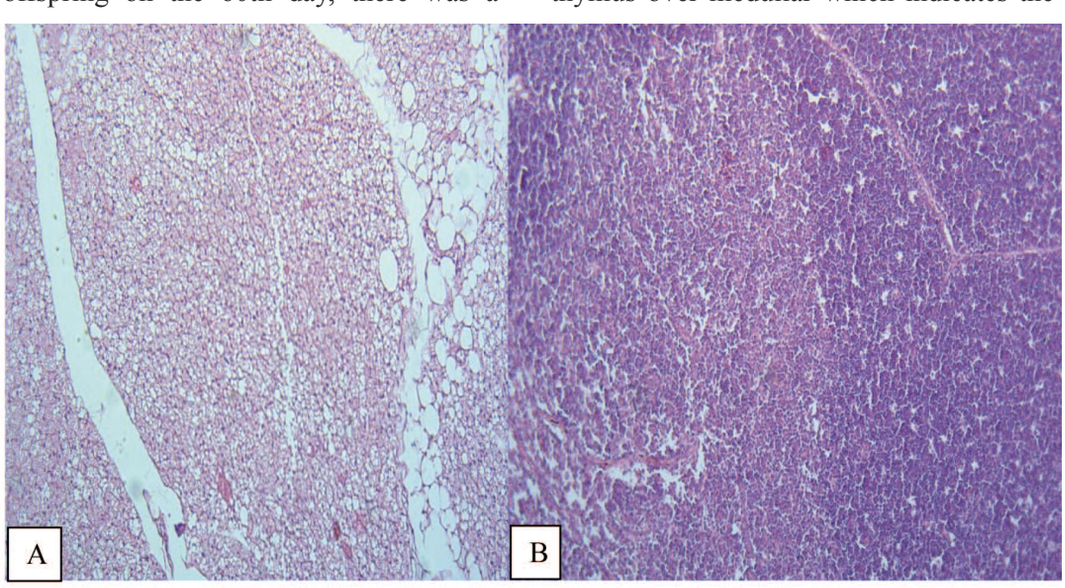

Figure 3. Thymus of female rats: A – control, B – experiment

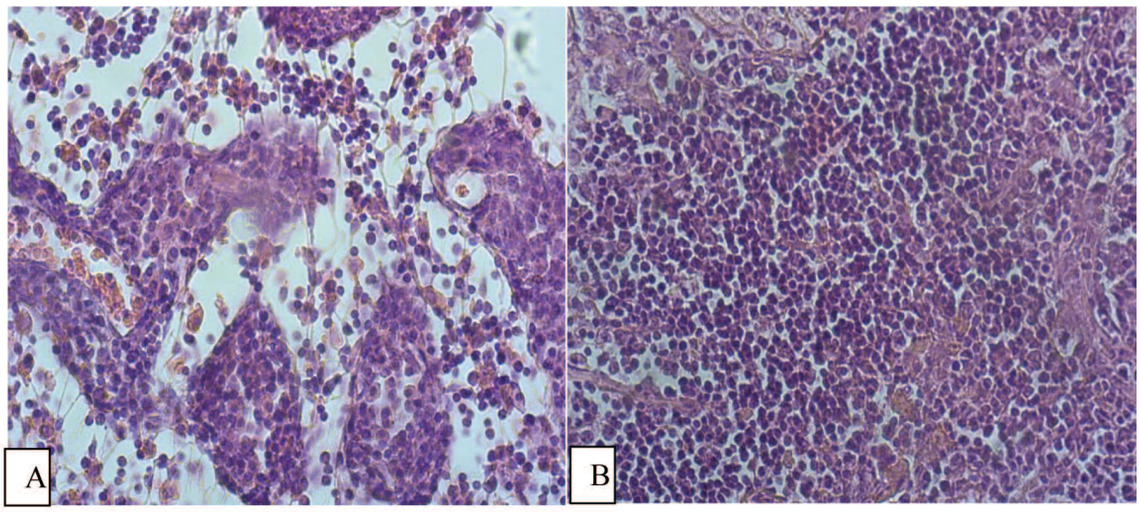

Figure 4. Thymus of female rats: A – control, B – experiment

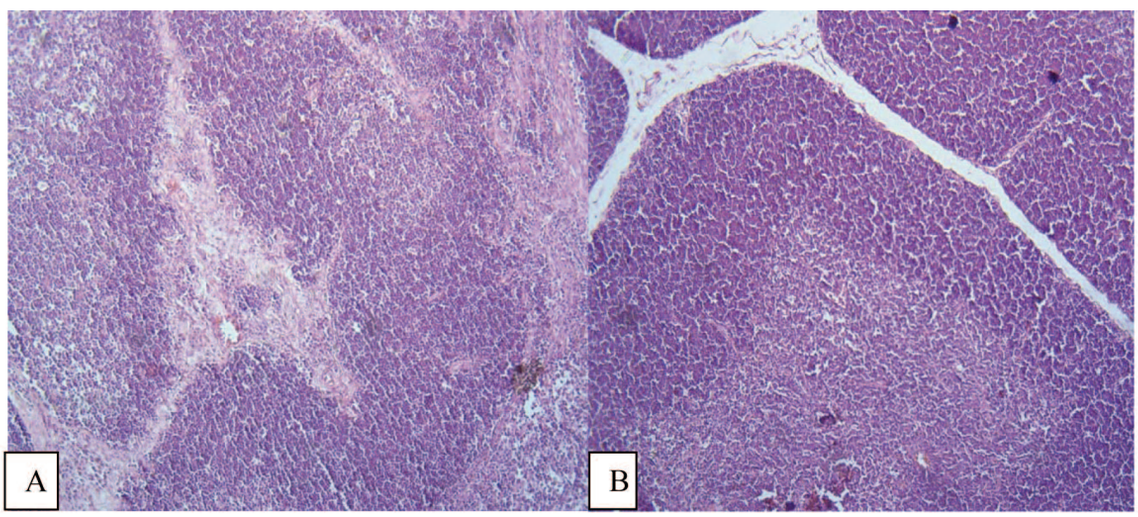

Figure 5. Thymus of offspring 30 days: A – control, B – experiment

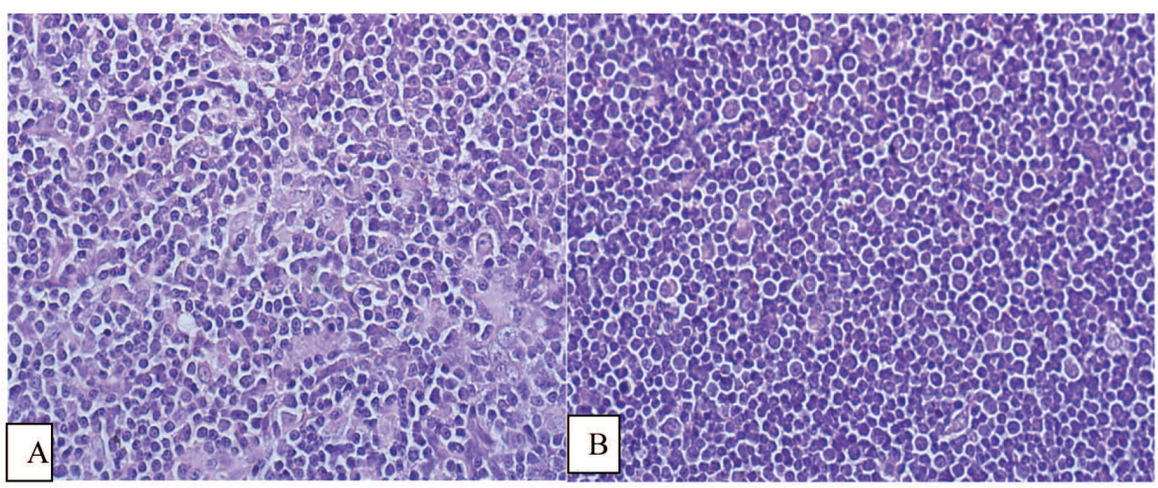

Figure 6. Thymus of offspring 30 days: A – control, B – experiment

stimulating effect of the saliva of the hematophagous parasite *H. verbana* on the thymus. In control female rats, the thymus turns into involution, which characterizes the age physiological changes of the animal. A study of the progeny cortex showed an increase in the number of cells per unit area (400 μm^2) by 30 (31.20%) and 60 (35.65%) days. When examining the substance of the medulla, the number of cells per unit area (400 μm^2) increases by 30 (3.44%) days.

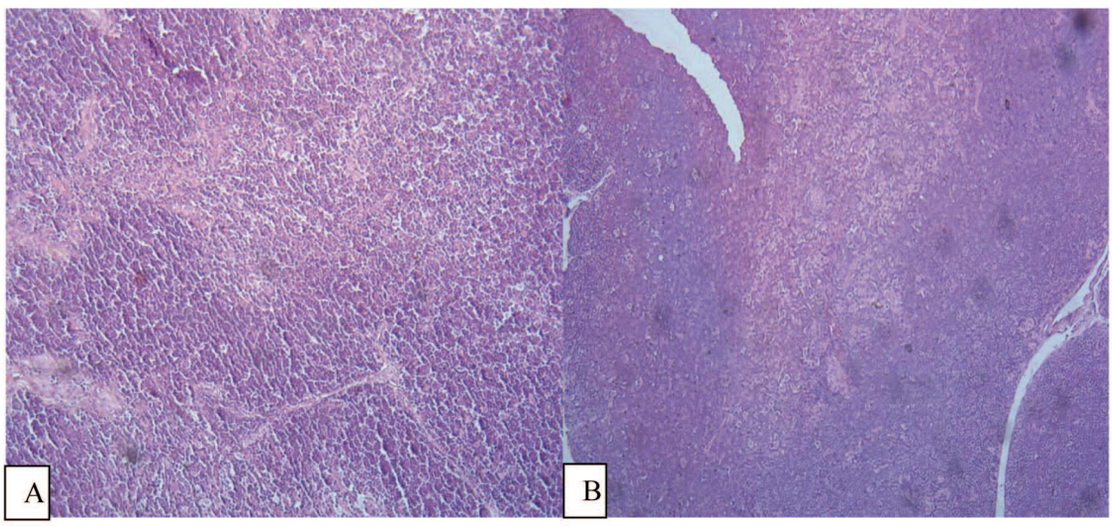

Figure 7. Thymus of offspring 60 days: A – control, B – experiment

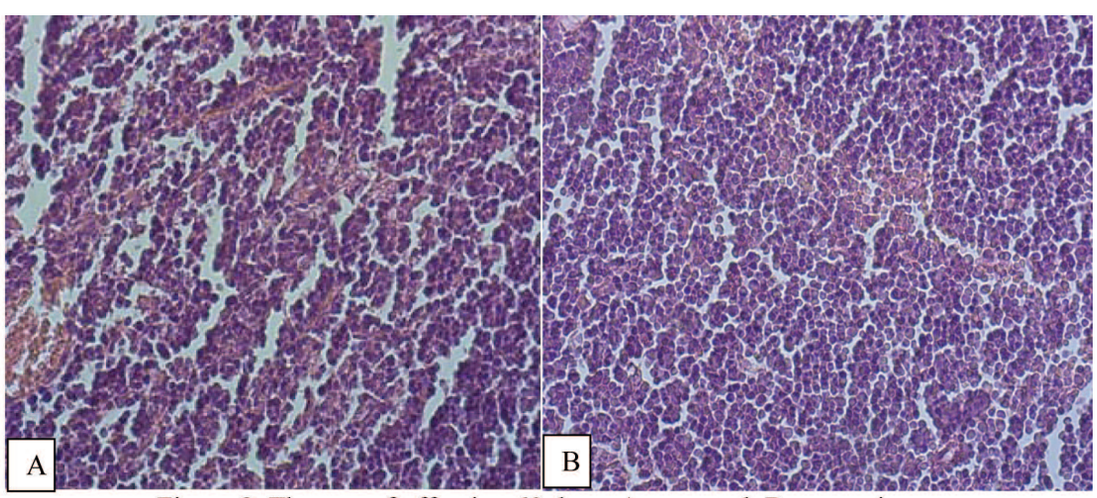

Figure 7. Thymus of offspring 60 days: A – control, B – experiment

Discussion

An increase in hematological and immunological blood parameters, the number of lymphocytes in an organ during histological analysis of the thymus of experimental females and their offspring indicates an indirect immuno-stimulating effect of the saliva substances of the hematophagous parasite *H. verbana*, which, by stimulating the immune system of male rats, is able to reflect this effect already on their offspring, and also affect the immunity of the females themselves. Perhaps the increase in immunity in the offspring of rats is associated with a decrease in sperm defect due to the influence of parasite substances, and all stages of conception and development pass effectively without defects, as a result we get healthy offspring. This is confirmed by our previous studies, in which we showed a significant increase in the number of spermatozoa, their fertility, as well as a decrease in their defects under the influence of substances of the hematophagous parasite *H. verbana* compared with the control group of animals [37]. It is also known that ejaculate in rats contains a small amount of neutrophils, which are known to play an important role in the processes of proliferation and differentiation of all cells and tissues [38–42], ejaculate also contains cytokines and many other biologically active substances that can affect normal physiological fertilization, and can also stimulate the mother's immune system and, ultimately, the immunity of the offspring. And the main role of stimulation here is already performed by the substances of the hematophagous parasite, which, as known, have a positive effect on neutrophils and the ratio of cytokines. That we can also observe in our obtained results when analyzing the phagocytic and metabolic activity of neutrophils. Scientists have also shown that neutrophils can live for 5 days [38]. Therefore, it is

possible that neutrophils in the ejaculate, as well as their decay products, which were directly influenced by the parasite's substances, can affect both the mother's immune system and the processes of conception, and at the final stage – on the immunity of the offspring itself. It should be noted that only males were directly influenced by the substances of the parasite, while females and offspring did not receive these substances directly.

It is important to note that pregnancy in the animals of the groups was the same and averaged 24 days. Also, in the experimental group, double fertilization was observed in comparison with the control group of animals. It is known that this is not a common occurrence among animals. Also, the offspring of the experimental group had a greater weight compared to the control group. Experimental females, on average, gave birth to more offspring than the control group.

Therefore, we have shown for the first time the indirect effect of the hematophagous parasite *H. verbana* on the immunity of rats.

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