

The Effect of the Biologically Complex of a Medical Leech Active Substances on the Immunosuppressive State of Rats

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Abstract

The course of all infectious diseases largely depends on immunity. An organism with a weak immune system gives up faster before diseases. Also, the number of immunodeficiencies with various etiologies has increased. Therefore, scientists are looking for various methods of dealing with these diseases. One such method is hirudotherapy using medicinal leeches, which has many therapeutic effects. As a result, the study of animals with a chemically immunosuppressive state has become relevant. The experiment was carried out on 45 mature male nonlinear rats with a body weight of 360-370 g. To simulate immunosuppression in experimental groups of animals, we used "Endoxan". It was administered intraperitoneally to mature rats after the formation of 3 study groups: 1) control - without intervention; 2) "Endoxan" at a dose of 100 mg / kg; 3) "Endoxan" at a dose of 100 mg / kg + water-salt extract of *Hirudo verbana*. After 3 weeks, the animals were measured for weight and body length, and were decapitated. Blood was taken with the addition of an anticoagulant. Then the total number of erythrocytes and leukocytes, the relative blood leukocyte formula, the mass and morphology of the thymus, the weight of the spleen and liver were examined. As a result, in the second and third groups, all indicators decreased, which indicates the immunosuppressive effect of the drug. After the introduction of the water-salt extract in the third group, the indicators increased (in some respects, even more) in comparison with the control group.

Keywords: medical leeches, infectious diseases, immunosuppressive, hirudotherapy

1. Introduction

A significant number of infectious and non-infectious diseases has grown in the world. The most common infectious diseases include: influenza, tonsillitis, pneumonia, tuberculosis, rabies, herpes, hepatitis, salmonellosis, dysentery, acquired immunodeficiency syndrome, as well as the number of coronavirus infections has increased significantly. All these diseases can occur at different times of the year, especially during the cold season. The most common non-communicable diseases include hypertension, coronary heart disease, heart attack, stroke, malignant neoplasms, diabetes, chronic obstructive pulmonary disease, and bronchial asthma. The course of all these infectious and non-infectious diseases is largely dependent on the immune system. Diseases of various etiologies often develop with weak immunity. Therefore, scientists are looking for various methods of dealing with these diseases with natural and synthetic methods (Pejin *et al.*, 2014; Tešanović *et al.*, 2017; Pejin *et al.*, 2019; Zouaghi *et al.*, 2021; Girgis *et al.*, 2021; Rawung *et al.*, 2021). And they are also looking for different ways to stimulate the immune system, because it is known, as we described earlier in our work, that the balanced immune system is already a great chance for recovery.

One of these methods is naturo-therapeutic - the use of medicinal leeches. Many scientists have already proven that their biologically active substances have a positive

effect on the links of immunity: they normalize the cytokine profile, increase local and systemic immunity, reduce cytolysis and the absolute number of neutrophils, increase the content of lysosomal cationic proteins in neutrophils, and stimulate lymphopoiesis (Deniskina, 2003; Spitsina, 2005; Borovaya, 2008; Zolonyi *et al.*, 2010) and negatively affect various infections caused by different pathogens: *Escherichia coli* MG1655, *Botrytis cinerea*, *Verticillium Lateriticum*, *Candida guillermoudii*, *Shizosaccharomyces pombe*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* (Yudina *et al.*, 2012; Pavlova *et al.*, 2015) and others. This mainly depends on the ingestion of a medicinal leech substance - destabilase. Among the world's major diseases, their positive effect on cancer cells and cancer therapy has been proven now, which may be due to the effect on them of biologically active substances of eglin, which prevent division and destroy cancer pain and cancer cells (Kalender *et al.*, 2010; Shakouri *et al.*, 2018), *Mycobacterium tuberculosis* (Ojo *et al.*, 2018). Their action has also been proven in various diseases of a non-infectious nature. Among them: treatment of chronic dermatoses (Karadag *et al.*, 2011; Zhulebina, 2017); psoriasis, in which the antielastase substance plays an important role (Kumar, 2012; Senchukova, 2012); in the treatment of heart failure (Kuznetsova, 2008), radiculopathy (Belyakov, 2008), arthritis (Mikhalsen, 2003; Abduvaliev, 2017). Factor Xa inhibitors are anticoagulants, which include antistasin and gilant, which have the same effects. Also, a trypsin inhibitor is involved

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in the treatment of these diseases. In case of endometriosis, uterine myoma, chronic inflammation of the appendages, the main active ingredient is bdelins that relieve inflammation (Dobrynina *et al.*, 2005, Sorokina, 2008). In the treatment of thrombophlebitis (Tashiro *et al.*, 2016), varicose veins and other vascular diseases (Porshinsky *et al.*, 2011; Nikar and Alam, 2011; Prakash *et al.*, 2013; Makhova, 2015), plastic and reconstructive surgery (Hackenberger and Janis, 2019) hirudin and kalin play an important role. Male and female infertility (Lyalina, 2016; Stokoz and Bystritskaya, 2016) and male priapism (Sayed, 2017) are treated with destabilase. In experiments on rats: an increase in body mass and organs, hemoglobin (Babayi *et al.*, 2018), improved liver performance (Praise *et al.*, 2019), improves wound healing (Kaveh *et al.*, 2014). Hirudological influence in veterinary medicine is used in various diseases of cats, dogs, horses (Popov *et al.*, 2008; Canpolat and Sağlam, 2004; Sobczak and Kantyka, 2014; Rychapova, 2017), for example, for the treatment of mastitis and increase reproductive capacity in cows (Kondratieva *et al.*, 2015; Glazunova and Anodina, 2013). And this is only a large part of the complex therapeutic effects of a medical leech *Hirudo verbana*. As a result of its enormous positive effect in many pathological conditions, which mainly arise due to a violation of the immune system, it has become urgent to analyze animals with an artificial immunosuppressive state under the influence of a complex of biologically active substances obtained from a medicinal leech.

2. Material and methods

The research was performed in the laboratory of cell and organism biotechnology of Zaporizhzhya National University. All experimental research with animals was carried out in compliance with the Law of Ukraine - On the Protection of Animals from Abuse, the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes. National Health and Medical Research Council of Australia, 'Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition' (National Health and Medical Research Council; Canberra, 2013). The experiment was conducted on 45 male non-linear adult rats weighing 360-370 g. "Endoxan" was used to simulate immunosuppression in experimental groups of animals, in the form of a powder for injection of 200 mg/kg, manufactured by Baxter Oncology GmbH (Germany), which was prepared in saline. This drug was used because a conventional chemical model can cause prolonged immunodeficiency in animals without significant destructive effects on cells and tissues. Saline was made from NaCl powder with a final concentration of 0.9%.

It was administered intraperitoneally to sexually mature male rats after the formation of 3 research groups of 15 animals each: 1) control-without intervention; 2) "Endoxan" in a dose of 100 mg / kg; 3) "Endoxan" at a dose of 100 mg / kg + water-salt extract of medicinal leech *Hirudo verbana*. The extract was administered on the third day with a final concentration of water-salt extract of 5 µg / g in animals (1 time in 3 days). The total amount of injection of the extract 5 times. After 3 weeks, the animals, after measuring body weight and length, were decapitated using the method of dislocation of the cervical vertebrae.

Blood was taken with the addition of the anticoagulant - 2% heparin (9/1). The total number of erythrocytes and white blood cells, the relative amount of blood leukocytes, the weight and morphology of the thymus, and the weight of the spleen and liver were then examined.

Determination of hematological and immunological parameters was performed by standard methods (Higgins, 2017). Organ weights were measured on analytical scales. Removed lymphoid organs after weighing were fixed in 10% formalin solution in a glass-darkened glassware, stored at room temperature for 3 days before histological examination. Next, the thymus was poured into paraffin blocks using standard histological techniques, from which 6 micron thick sections were made. Serial sections were made using a Thermo Scientific HM 325 microtome and stained with hematoxylin-eosin according to the standard procedure (Zolotarev *et al.*, 2013). Morphometric and cytological studies were performed directly on histological specimens using a Carl Zeiss Primo Star microscope. The micrographs were prepared using a PrimoStar iLED microscope and Axio CamERc5s (ZEISS, Germany), which were analyzed using the ZEISS ZEN 2011 microscopy program.

Statistical data processing was performed using the computer program SPSS v.21.0. (IBM SPSS Statistics., USA). The selected parameters indicated in the table below have the following notation: X - sample mean, SE - standard error of the mean. The significance of differences between the mean values was evaluated by the Student criterion after checking the normal distribution. Differences were considered significant at $p < 0.05$.

3. Results and discussion

In the second group of animals, which were injected only with the cytotoxic drug "Endoxan", a significant decrease in the total number of leukocytes was observed as compared to the control group of animals (Table 1). In the analysis of the third group, which was administered five times more water-salt extract, there was a significant increase in the total number of leukocytes in comparison with the control group table 1.

Table 1. The state of the indicators of blood, body mass and organs

Indicators	Group of animals		
	I Control	II Endoxan in the dose 100 mg / kg	III Endoxan at a dose of 100 mg / kg + water-salt extract of 5 µg / g <i>Hirudo verbana</i>
The total number of leukocytes ($\times 10^9$)	11,9±0,49	9,5±0,37*	16,6±0,66*
The total number of erythrocytes ($\times 10^{12}$)	7,7±0,36	3,4±0,19*	9,5±0,45*
Body weight (g)	373,5±15,4	342±13,2*	366±14,2
Body length (cm)	22±0,9	21,5±0,7	23,3±0,8
Thymus weight (mg)	119±4,2	89±3,5*	211±5,1*
Spleen weight (mg)	1213±50,1	1103±44,3*	1227±48,6
Liver weight (g)	17,8±0,73	13±0,56*	17,3±0,68

Note: * - $p < 0,05$ in comparison with the control group

The decrease in the number of leukocytes in the second group may be associated with a decrease in the total number of lymphocytes. We see this by analyzing the leukocyte blood count. An increase in neutrophils occurs due to a decrease in lymphocytes. It is also known fact that "Endoxan" inhibits the lymphoid row of cells to a greater extent. In the third group, all types of leukocytes were equated to the control group with a slight increase in lymphocytes due to a decrease in neutrophils. This dynamic indicates the stimulation of cell proliferation from the complex influence of biologically active substances of the water-salt extract. Analysis of the total number of erythrocytes showed their significant decrease in the second group and their significant increase in the third group as compared to the control group accelerated cell proliferation. When analyzing body weight, there was a significant decrease in the second group compared to the control of table 1. When analyzing the thymus weight,

growth in the third group and a decrease in the second compared with the control group of animals, table 1. A decrease in weight in the second group can indirectly confirm the suppression of lymphoid cells, and in the third, on the contrary, increased proliferation. The study of the weight of the liver and spleen showed a significant decrease in the second group compared with the control. The weight of the spleen and liver of the third group within the control group table 1. An increase in blood counts, body weight and the studied organs in the third group compared with the control indicates a compensating and then immune-stimulating effect of the water-salt extract of the medicinal leech. The complex of biologically active substances corrected the lymphotoxic effect of endoxan. This is evidenced by a slight increase in lymphocytes in the leukocyte blood count, even in comparison with the control table 2.

Table 2. White blood cell condition

Group of animals	Leukocyte blood formula, %				
	Neutrophils		Lymphocytes	Monocytes	Eosinophils
	Segmentonuclear	Palichkonuclear			
I Control	8,11±0,25	3,75±0,12	87,14±1,2	0,75±0,12	0,25±0,02
II Endoxanin the dose 100 mg / kg	16,51±0,11*	15,16±0,10*	67,23±2,9*	0,81±0,06	0,29±0,03
Experiment III Endoxan at a dose of 100 mg / kg + water-salt extract of 5 µg / g <i>Hirudo verbana</i>	7,50±0,27*	2,86±0,11*	89,56±1,1*	0,80±0,23	0,28±0,05

Note: * - $p < 0,05$ in comparison with the control group

The complex of biologically active substances corrected the lymphotoxic effect of "Endoxan". This is evidenced by an insignificant increase in lymphocytes in the leukocyte blood count even in comparison with the control group table 2. Our theory stated above is confirmed by the results of a histological study of the cytological composition of the thymus gland, which were analyzed in all groups of animals 3 weeks after the start of the experiment Fig. 1.

We visually observed in the third group that the number of cells is close to the control group. This is not the case for the second group, where normal cells are replaced by adipose and connective tissue.

The devastation of cells in the thymus in the second group is associated with the action of cytostatic, as described previously. "Endoxan" primarily affects cytotoxicity on lymphocytes. Visually, the third group is no different from the control. Firstly, this may be due to

the compensatory effect of the second immune-modulating properties of leeches. In our previous experiments, we showed an immune-stimulating effect on the immune system of females and their offspring (Aminov and Frolov, 2017, 2018, 2020). But immune-deficient animals under hirudological influence were studied for the first time. Other scientists have shown that the body recovers after cytostatics when its dose is not toxic. Therefore, it can be used as a drug that can cause a measurable immune-deficiency state (Akhter *et al.*, 2008; Ukpo, 2013; Mohamed, 2014; Lee *et al.*, 2019). Therefore, we chose it to obtain an immunodeficiency model that is being corrected. The results we obtained can be useful for worldwide viewing and in future applications for increasing and modulating immunity in various diseases of an infectious and non-infectious nature. As we said above, all diseases mainly arise due to weak immunity, which is not able to cope with the disease.

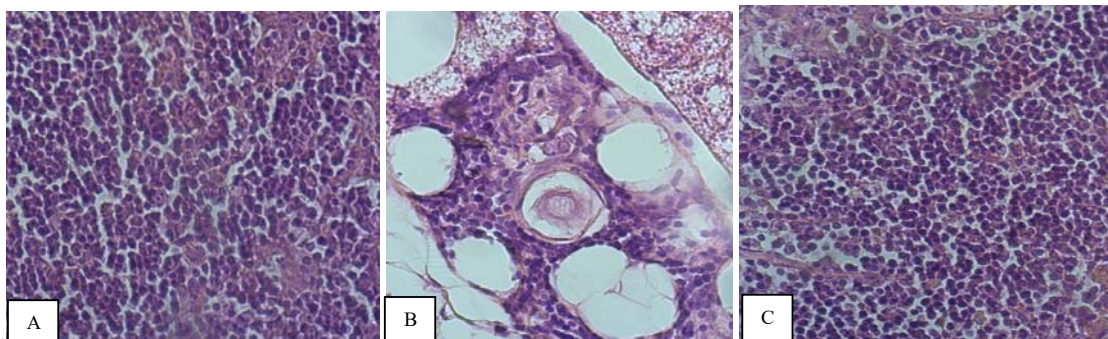


Figure 1. Morphology of animal thymus: A) control; B) endoxan in a dose of 100 mg / kg; C) endoxan at a dose of 100 mg / kg + water-salt extract of 5 µg / g *Hirudo verbana*

4. Conclusions

As a result of our research, the biologically active substances of a medicinal leech *Hirudo verbana* can modulate the immune system, which has failed, to a normal physiological state, and even more. This was manifested by an increase in hematological and immunological parameters, as well as an increase in body weight and major organs: spleen, thymus and liver, positive changes in the morphological structure of the central lymphoid organ. In the future, our research is aimed at a deeper study of this effect on immunity. So, the obtained results show the immune-modulating effect from the introduction of the extract.

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