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Original papers

The impact of fetal load of *Hirudo verbana* saline extract antigens on morphometrical, hematological and immunological parameters of rats in the early stages of post-embryonic development

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ABSTRACT. The aim of the study was to determine the immunostimulatory effect of antigens from saline extract of the medicinal leech on the morphometric parameters of the body and major immunogenic organs of rats. Wild-type female rats were administered medicinal leech saline extract antigens intraperitoneally two weeks before and two weeks after mating (in total four injections). The offspring were examined, as were the mothers after feeding the offspring, at 1, 15, 30, 60 days after birth. The morphometric parameters of the animals were recorded. The animals were then decapitated under anesthesia and subjected to the following tests: morphometric parameters of immunogenic organs (spleen and thymus), total white blood cell count, red blood cell count, hemoglobin, color index and leukocyte formula. In addition, the absorption activity of neutrophils was examined: phagocyte index, phagocyte number, phagocytic capacity of the blood, the number of active phagocytes. From day 1, the medicinal leech antigens were associated with increased numbers of white blood cells and red blood cells, elevated hemoglobin level, increased absorption activity of neutrophils; however, the colour index remained in the normal range. These changes indicate that the antigens found in the saliva of medicinal leeches have an immune-stimulating influence on leucopoiesis, erythropoiesis and morphogenesis.

Key words: medicinal leech, immunology, hematology, rat

Introduction

The medicinal leech (ML) is an obligatory haemophage [1], which evolved from ectoparasitic to mutualistic symbiosis with aquatic vertebrates, mainly amphibians, and terrestrial mammals, most commonly grazing ungulates. During feeding, it inserts more than 100 biologically active substances (BAS) present in its saliva into the host; these have a homeostatic effect on all physiological systems [2], and so the leech is widely used in medicine and veterinary medicine.

The majority of the therapeutic effects are indirectly dependent on the state of the immune system [3], which helps control and regulate morphogenesis. Recent studies have cast new light on the structure of the receptors on immune cells, which demonstrate the close relationship between innate and adaptive immunity, as well as on the mechanisms behind the regulation of histological reactions [4]. Several studies have shown hirudotherapy to have positive effects on body weight, milk yield, quantity and body weight of offspring, as well as on changes in the morphological and biochemical composition of the blood of sheep and cattle [5].

The ML saliva stimulates phagocytic activity of neutrophils (PhAN) and anti-complementary secret activity [1]. The original researches dedicated to studying the influence of HT on functional activity of macrophages in diseases accompanied by secondary immunodeficiency states (for example, emotional burnout syndrome) showed that HT provides activation of pre-lowered phagocytic indexes monocyte / macrophages to the lower limit

of normal [6]. Optimal and cytotoxic concentrations antigens (AG) of saline extract ML for phagocytic neutrophil reaction and lymphocyte reactivity in humans have been established [7].

Hirudotherapy plays an important role in the treatment of osteoarthritis and rheumatoid arthritis [8], hypertension, cerebrovascular disease [9], psoriasis [10] and many others. Medicinal leeches are often used to eliminate venous congestion during transplantation [11–16]. In goats, hirudotherapy is accompanied by the migrational redistribution of blood lymphocytes, with their temporal accumulation around the locations where the ML feeds, as well as an increase of neutrophil phagocytic activity [17]. Aim of this work was to examine the morphometric parameters of the body and immunogenic organs of female wild-type rats and their offspring, as well as the hematological and immunological parameters of their blood, under the influence of biologically-active substances obtained from the medicinal leech. We first investigate the metered effect of the biologically active substances of the salt extract from the bodies of medical leech (*Hirudo verbana*) (Carena, 1820) on the immune state of the animal in different periods of post-embryonic ontogenesis. Salt extract was input only to male rats. Their studied supplements were not subjected to repeated administration of the substance.

Materials and Methods

The study was performed in the Laboratory of Cellular and Organismal Biotechnology, Zaporizhzhya National University. In total, 45 female wild-type rats and 225 offspring were included in the study. The adult female wild-type rats were administered saline extract of antigens obtained from the medicinal leech *Hirudo verbana* (Carena, 1820); the injections were administered intraperitoneally two weeks before and two weeks after mating (four injections in total).

The antigens were obtained from medical leeches by the Frolov method [18]. Briefly, medicinal leeches were selected, dry, fragmented and ground. Following this, the tissue was subjected to extraction and filtration. The tissue was added to saline buffer at the ratio of 1:10 and placed in the refrigerator where the antigens were extracted in the buffered saline solution. After that the buffered saline was subjected to centrifugation at cold and further it sterilized. The amounts of extracted antigens was measured using the by the Lowry standard method.

While being injected, the rats were immobilized using an immobilizing device [19]. For this purpose, the animals were divided into three groups: the first group were injected with 0.5 ml medicinal leech antigens (3 μg/g animal weight), the second group received no injection, the third group (control) was administered 0.5 ml of 9% saline solution. The offspring, and the mothers after feeding offspring were studied after 1, 15, 30 and 60 days.

All experimental studies were performed in accordance with the international principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, according to the Ukraine Legislation dated 21.02.2006 number 3447-IV “On the protection of animals from cruelty” and in accordance with ethical norms and rules of work with laboratory animals. The animals were kept in vivarium conditions on a standard diet in individual cells.

The body weight, body length, tail length, chest circumference (CC) and abdominal circumference (AC) of the rats was measured. Following this, all animals were decapitated under anaesthesia. An autopsy was performed to determine the weight, width and length of the immunogenic organs (i.e. the spleen and thymus); haematological parameters (erythrocyte count, hemoglobin, color index, number of leukocytes, leukocyte formula) were determined, and the absorption activity of neutrophils was evaluated: phagocyte index (PHI), phagocyte number (PHN), phagocytic capacity of the blood, the number of active phagocytes.

Examinations were performed on rats 1, 15, 30 and 60 days after birth. Days 1–5 correspond to the neonatal period, days 6–21 to the nursing period, days 22–50 to the onset of puberty, and day 60 corresponds to puberty [20]. In the current experiment, the experimental group was compared with the control group.

Such laboratory parameters as the total amount of leukocytes, red blood cells, hemoglobin, color index and leukocyte formula were determined by standard methods. Evaluation of indicators of innate cellular immunity was investigated using the tests, characterizing the activity of neutrophils. Phagocytic activity of neutrophils was assessed in the dough with yeast. Calculated the PHI and PHN. Stimulated with 1% of yeast suspension.

Statistical analysis of the results was performed

Table 1. Changes in morphometric parameters of the body of female rats and their offspring, M ± m

Morphometric parameters bodies of females and their offspring						
Day	Group of animals	Body weight (mg)	Body length (cm)	Tail length (cm)	Chest circumference (cm)	Abdominal circumference (cm)
1	Control	6.2±0.25	5.0±0.2	1.7±0.1	4.43±0.18	4.75±0.19
	Experience	6.33±0.25	5.2±0.2*	1.8±0.1	4.46±0.18	4.75±0.19
15	Control	20.6±0.8	8.35±0.3	4.9±0.2	6.76±0.27	7.15±0.29
	Experience	24±0.95*	8.5±0.34	4.6±0.2	7.51±0.3*	7.7±0.3
30	Control	57.5±2.3	13±0.51	8.9±0.4	8.48±0.34	9.84±0.39
	Experience	78.5±3.1*	13.5±0.5	11±0.4*	9.16±0.37*	11.1±0.44*
60	Control	143±5.7	16±0.7	14±0.57	12.1±0.48	14.2±0.57
	Experience	153±6.1	16.5±0.7	13±0.5*	11.3±0.45*	14.2±0.57
Female	Control	201±8.4	19.2±0.4	17±0.3	15.1±0.3	15.4±0.4
	Experience	217±9.3	19.7±0.4	17±0.2	14.7±0.2	16.3±0.4*

* Figures that differ significantly from the control ($p \leq 0.05$)

by calculating the arithmetic mean, the arithmetic mean error, standard deviation using software SPSS v.21,0 and Microsoft Office Excel 2010. Probability differences between the average values estimated by Student's criterion. Differences considered significant at $p \leq 0.05$ [21].

Results and Discussion

All investigated morphometric and cytological indices in females who were administered antiglobulins salt extract of ML intraperitoneally in pre-embryonic and embryonic period of development and in their offspring at all stages of early ontogenesis had more unidirectional changes to their increase compared with the control (Tables 1–4). But the amplitude of these changes was not the same. Physical development is a dynamic growth process in different periods of ontogenesis. Key indicators of physical development are body weight, body length, abdominal circumference and chest. Overall indicators of physical development reflect the functional state of the body and they are important for the assessment of health [22].

Females that have been derived from the experiment at day 60 was noted slight tendency to increase body weight (Table 1). While most of morphometric parameters of the central (thymus) and peripheral (spleen) organs weight increased statistically significantly (Table 2).

These data suggest that the stimulating effect of the antigenic loading of the biologically active substances of the medicinal leech on pre-embryonic and embryonic periods contributed to the further development on the central and peripheral divisions of the immune system of female rats.

Morphometric reactions in the immune system in females were further manifested by a significant increase in the number of leukocytes in peripheral blood in experimental rats up to 11.7 ± 0.5 at 6.9 ± 0.2 in the control group of animals $p \leq 0.05$ (Table 3).

Comparative analysis of blood leukocyte formula in experimental groups of female rats revealed no systematic differences. These data indicate about homeostatic development of innate and adaptive parts of immune system of examined rats under the influence of ML's BAS.

In the experimental group of females also significantly increased rates of the number of erythrocytes (6.4 ± 0.3 at 6 ± 0.3 in the control), hemoglobin (191.0 ± 10.3 at 146.0 ± 3.0 in control, $p \leq 0.05$) and colour performance indicator which dependent on these two features (Table 4). Such contrast enhancement of erythropoiesis can be explained by the increased need for metabolism, as a consequence of the stimulation of the general morphogenesis of tissues and organs in the experimental group of animals.

Table 2. Changes in morphometric parameters of immunogenic organs of female rats and their offspring, M ± m

Day	Group of animals	Morphometric immunogenic organs of females and their offspring					
		Thymus			Spleen		
		Weight (mg)	Length (cm)	Width (cm)	Weight (mg)	Length (cm)	Width (cm)
1	Control	15.5±0.6	0.47±0.02	0.35±0.01	22.0±0.9	1.32±0.05	0.22±0.01
	Experience	17.2±0.69*	0.44±0.02	0.33±0.01	21.0±0.84	1.1±0.04*	0.7±0.03*
15	Control	88.6±3.5	0.83±0.03	0.73±0.03	72.6±2.9	1.6±0.06	0.36±0.01
	Experience	83.6±3.3	0.9±0.04*	0.82±0.03*	78.9±3.1*	1.68±0.06	0.3±0.01
30	Control	251±10	1.44±0.06	1.24±0.05	140±5.6	2.14±0.08	0.48±0.02
	Experience	306±12.2*	1.4±0.06	1.43±0.06*	239±9.5*	2.13±0.08	0.67±0.03*
60	Control	460±18.4	1.77±0.07	1.63±0.06	522±20.9	3.13±0.12	0.8±0.03
	Experience	407±16.3*	1.6±0.06*	1.8±0.07*	549±22	3.6±0.1*	0.77±0.03
Female	Control	268.4±11.4	1.45±0.03	1.2±0.03	465±23.3	3.2±0.09	0.7±0.03
	Experience	345±17.1*	1.64±0.1*	1.5±0.08*	566±53.4*	3.58±0.09*	0.91±0.04*

* Figures that differ significantly from the control ($p \leq 0.05$)

The active morphogenetic reaction of tissues and organs took place in the offspring of the studied female rats. From the first day there was a tendency to increasing all morphometric parameters of the body (Table 1). However, statistically significant boundary it was reached at the body length. At 15 days all common indicators also increased reaching significance in body weight and chest circumference At 30 days, when the formation of the body of rats is completed, a sufficient increase in morphometric changes in the experimental group of the offspring was noted for body weight, tail length, chest circumference and abdominal circumference. At the early puberty after 60 days we observed the growth of the body in the experimental group achieving significance at the length of the tail and CC. As well as in experimental female rats, an increase in general indicators of anatomical body of their offspring also accompanied by a positive increase in immune organs (Table 2). So, under the

Table 3. Total number of leukocytes and blood leukocyte formula of female rats and their offspring, M ± m

Day	Group of animals	Leucocytes/l ($\times 10^9$)	Leucocytes are a formula of blood, %					
			Neutrophils			Lymphocytes	Monocytes	Eosinophils
			Band neutrophils	Segmented	The total percentage			
1	Control	8.0±1.1	37±4.8	17±3.8	54.2±4.98	39.9±5	5.9±2.4	0
	Experience	8.9±0.9	28.5±4.5	18.4±3.9	46.9±5	50.2±5*	2.26±1.5	0.64±0.08*
15	Control	4.4±0.3	3.8±1.91	9.4±2.92	13.2±3.38	83.2±3.8	3.4±1.81	0.2±0.04*
	Experience	6.0±0.8*	6.73±2.5	14.6±3.5	21.33±4.1*	75.7±4.3	2.7±1.6	0
30	Control	5.3±0.7	3.6±1.86	2.66±1.6	6.26±2.42	89±3.13*	4.66±2.1	0
	Experience	4.6±0.3	8.3±2.7*	6.75±2.5	15±3.57*	79.7±4.0	5.25±2.2	0.5±0.07*
60	Control	5.5±0.3	4.66±2.1	5.66±2.3	10.32±3.04	87±3.36*	2.68±1.6	0
	Experience	7.2±0.7*	13.5±3.4*	7.83±2.7	21.33±4.1*	74±4.39	3.83±1.9	0.8±0.09*
Female	Control	6.9±0.2	7.7±2.66	18.7±3.9	26.4±4.41	71.8±4.5	1.35±1.2	0.5±0.07
	Experience	11.7±0.5*	6±2.37	17.7±3.8	23.7±4.25	72.1±4.5	3.8±1.91	0.4±0.06

* Figures that differ significantly from the control ($p \leq 0.05$)

Table 4. Content of red blood cells, hemoglobin and color index in female rats and their offspring, M ± m

Indicators	Group of animals	Day				
		1	15	30	60	Female
Erythrocytes/l ($\times 10^{12}$)	Control	1.7±0.43	1.3±0.2	2.8±0.4	4.5±0.3	6.0±0.3
	Experience	2.1±0.28	2.0±0.3*	3.8±0.4*	5.2±0.4	6.4±0.3
Hemoglobin, g/l	Control	73.3±3.7	80.6±1.9	101.2±3.0	132.4±3.9	146.0±3.0
	Experience	106.1±2.9*	107.4±2.8*	110.6±7.8	146.6±9.5*	191.0±10.3*
Color indicator	Control	1.98±0.19	2.04±0.26*	1.21±0.14	0.95±0.09	0.74±0.03
	Experience	1.72±0.28	0.97±0.19	0.98±0.17	0.86±0.07	0.91±0.08*

* Figures that differ significantly from the control ($p \leq 0.05$)

influence of biologically active substances of medical leech antigens compared with the control on the first day significantly increased the weight of the thymus and spleen's width. At 15 days determined statistically significant increase of three factors: the length and width of the thymus, and spleen weight. At 30 days determined weight and width of the thymus, weight and width of the spleen. At 60 days determined growth of 5 indicators, and a statistically significant increase in weight, width and length of the thymus and spleen length.

Positive changes in blood-forming organs contributed to the increase of white blood cells indicators (Table 3) and erythrocytes (Table 4) in the blood of studied rats' group. So statistically significant increase in the number of white blood cells was determined at 15 and 60 days. In leukocyte formula significant shifts occurred on 15, 30 and 60 days due to the increase of rod-and-nucleus and segmented neutrophils (Table 3). Amount of red blood cells in the experimental group increased in offspring in all terms of surveillance, but statistically significant level shift they were recorded at 15 and 30 days. In parallel with the number of erythrocytes, hemoglobin levels increased with a significant deviation from control at the first and 60 days. Noteworthy there is a clear trend towards reduction of hemoglobin in red blood cells with analysis of their colour index in the experimental group offspring. This trend can be explained as a reflection of the intensity of erythropoiesis influenced by BAS ML.

Summarizing morphogenetic and cytological differences in the experimental group of rats' offspring should be noted that the largest amplitude displacement performance deviations were

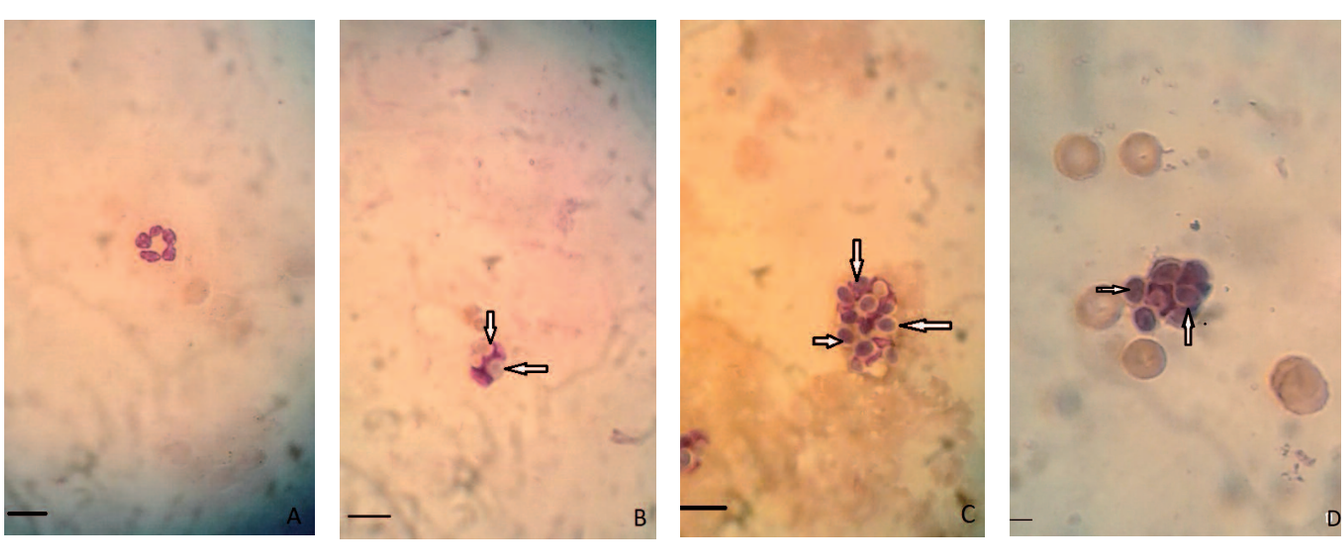

Fig. 1. Phagocytic activity of neutrophils under the influence of a saline extract of Ag of medicinal leeches: A: not activated neutrophils; B, C, D: activated neutrophils.
Light microscope (objective lens 100×, eyepiece K7×). Bars in A, B, C, D: 10 μm

Table 5. Immunological parameters of blood of female rats and their offspring, M ± m

Indicators	Group of animals	Day				
		1	15	30	60	Female
PHI (%)	Control	32±4.78	64±4.8	39.0±4.88	60.78±4.88	37.5±4.84
	Experience	45±4.9*	66±4.74	39.4±4.89	65.84±4.74	46.48±5
Number of active phagocytes ($\times 10^9$)	Control	26±0.13	2.8±0.14	2.1±0.1	2.7±0.13	2.6±0.13
	Experience	4±0.2*	4±0.2*	1.8±0.2	4.7±0.23*	5.4±0.27*
PHN	Control	2.6±0.1*	3.35±0.17	1.98±0.1	3.02±0.15	3.0±0.15*
	Experience	1.84±0.1	5.4±0.27*	2.8±0.14*	3.21±0.16	2.26±0.11
Phagocytic capacity of blood ($\times 10^9$)	Control	20.8±1.0*	14.7±0.73	10.5±0.52	13.6± 0.68	20.8±1.04
	Experience	16.4±0.82	32.3±1.6*	13.1±0.6*	23.1±1.15*	26.4±1.32*

*Figures that differ significantly from the control ($p \leq 0.05$); PHI: phagocytic index; PHN: phagocytic number.

observed at 30 and 60 days, and during early sexual maturity when there is an active morphogenesis and final completion of the subsequent differentiation of cells in tissues.

The absorption activity of neutrophils in animals under the influence of antigens medicinal leeches has shown the increase of PHI in all study periods, a statistically significant at first day. The number of active phagocytes increased statistically all the time. (Table 5 and Fig. 1).

Phagocytic index shows the mean number of engulfed bacteria by a phagocyting cell. PHN increases from 15 days, a statistically significant increase is recorded in 15 and 30 days, statistically significant decrease is noticed from first day and in females. Phagocytic capacity of blood increases statistically with 15 days (Table 2). These data coincide with the literature data about the increase of phagocytic activity of neutrophils under the influence of a BAS of medicinal leeches. Neutrophils are the first line of protection against penetration into the body a variety of bacteria, fungi and protozoa. Therefore, the increase in absorptive activity may indicate increased functional-protective ability of cells to phagocytosis.

Positive changes in immunological parameters indicate the immunostimulatory effect of antigens medicinal leeches on leucopoiesis and on the first line of defence against entry into the body a variety of bacteria, fungi and protozoa. These data coincide with the literature data, the impact of medicinal leeches on the of phagocytic activity of neutrophils and peripheral blood leukocytes. Thus, as has been revealed that the secret of salivary glands of medicinal leeches stimulates FAN [1,23,24]. The original study [6] examining the impact of hirudotherapy on functional activity of macrophages in diseases accompanied by secondary immunodeficiency states (for example, a syndrome of emotional burnout) showed that hirudotherapy provides activation of pre-reduced phagocytic indexes monocytes/macrophages to the bottom limit of normal and enhance the functional activity of macrophage phagocytic system, but compared to research these scientists first studied antigen dose effect of salt extract on immunological processes of female rats born to background exposure antigens medicinal leech.

Conclusions

The data indicate a modulating effect of antigens of ML's BAS on morphogenetic processes that are mainly mediated by immune cells, common manifestation of which is an improvement of physical parameters of the body of experimental rats. At the tissue level, this effect reported by stimulation of myeloid and lymphoid tissues and resulting in increase in number of red blood cells, white blood cells, thymus and spleen size, absorptive activity of neutrophils. Our results coincide with the literature of the morphogenetic function of the immune system, which aims to regulate proliferation [6,25] and differentiation of cells of all tissues. Therefore, data that we found may have a perspective to study the effect of antigens of ML's BAS on the physical development of animals at all stages of ontogeny, including pre-embryonic, embryonic and early postembryonic stage.

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