PharmacologyOnLine December 30, 2015

Archives • 2015 • vol.3 • 76-83

ANTI-INFLAMMATORY EFFECT OF NICA-EM IN RODENT MODELS OF ACUTE INFLAMMATION

Areshidze, D.[1*]; Timchenko, L.[2]; Rzhepakovsky, I.[2]; Kozlova, M.[1]; Syomin, I.[1]

[1]Researching Laboratory of Experimental Biology and Biotechnology, Moscow State Regional University, Moscow, Russian Federation.
[2]Institute of Applied Biotechnology, Department of the living systems of the North Caucasus Federal University, Stavropol, Russian Federation.

[*]notbio@mgou.ru

Abstract

The conducted research is devoted to studying of influence of the tissue preparation "NICA-EM" on an organism of mammals in rodent models of acute inflammation. It is established that at application of the studied preparation on model of acute inflammation the dynamics of inflammatory reaction, characterized by change of affected hind paw volume of an animal, was less expressed in rats of the experimental group. Action of a preparation leads to decrease in rates of ESR and CRP. Hematologic and biochemical parameters also speak well for anti-inflammatory action of "NICA-EM". Results of histological and morphometric research of a spleen and a thymus testify to proliferation and migration of immunocytes, to activization of immune reactions. In a thymus of rats of experimental group in comparison with control the increase in the dimensions of thymic lobules, and also the increase in quantity of Hassal's bodies testifying to intensification of synthesis of the thymic hormones participating in process of an immunopoesis are revealed. As a result of application of a preparation there are an increase of ability of an organism to resist to alteration and also essential decrease in a level of development of inflammatory reaction of an organism. The conducted research testifies to the expressed immunomodulatory action of a preparation "NICA-EM" at an experimental model of inflammation.

Keywords. Immunomodulation, inflammation, thymus, spleen.

Introduction

The increasing loading with environmental, psychosocial, age-related, drug-associated and other factors leads to change of immune function of human and other mammals in the modern world. By that is caused the necessity of search for biologically active agents capable to support and stimulate immune functions [1-6]. Immunomodulators are meant as the substances having impact on individual mechanisms of the immune response. Thus, "immunomodulation" represents change, strengthening, suppression of parameters of cell-mediated and humoral immunity and non-specific factors of protection. Immunomodulatory effect is generally reversible. The extreme manifestations of immunomodulatory effect of biologically active agents are the immunosuppression and immunostimulation [7-13]

As the immunostimulating preparations the substances derived from tissues of live organisms are widely applied. Such biologically active tissue preparations exist in the form of extracts, emulsions, hydrolysates, products of a bacterial fermentation, supernatants [14-18]. Supernatant fluids of tissue extracts, containing biologically active agents with a low-molecular mass (< 10 kD), possess especially high biological potential, high bioavailability and comprehensibility [19]. So, the extract of a porcine placenta in phosphate-buffered saline entered into a diet of pigs considerably raises lymphocyte activation, the percentage of granulocytes and concentrations of interferon-γ in blood serum of animals and increases animal resistance to rotavirus [20]. The low-molecular peptide derived from placenta of goats promotes the immunological function of normal murine macrophages and also improves the phagocytic and clearance abilities of immunosuppressed mice [21]

Methanolic extract of marine sponge Xestospongia testudinaria demonstrates a marked antiinflammatory and immunomodulation effects at rodent model of carrageenan-induced edema, which is showed in particular in decrease of level of inflammatory cytokines, decrease of capillarity congestion and inflammatory cells infiltrate in damaged tissues [22]. Parental supplementation with fish oil emulsion increases total liver and lung macrophage number and phagocytosis in rats and also has an immunomodulatory effect on lipid-modulated generation of human leukocytes in postoperative trauma [23]. Bovine lactoferrin (bLF) shows anti-infective, anti-cancer, and anti-inflammatory effects. BLF administration in influenza virus infected mice reduced the lung consolidation score and the number of infiltrating leukocytes in bronchoalveolar lavage fluid. It is showed that bovine lactoferrin activates the transcription of important immune-related genes in the small intestine, and such transcriptional activation may promote a systemic immunity [24]. Embryonic tissues are one of the most promising sources for producing the biostimulating preparations, and the most promising source of immunomodulators are avian eggs and developing avian embryos [25-30]. Based on the data described above, we, in turn, assumed potential immunomodulatory and antinflammatory effect of a tissue preparation of an embryonic origin "NICA-EM", made of embryonic tissues of chickens, on organism of mammals on model of an experimental acute inflammation at subplantar introduction of carrageenan. Characteristics of the substances which are a part of preparation “NICA-EM” allowed us to expect its positive influence on immunity and a condition of an organism as a whole. The preparation "NICA-EM" is made of natural raw materials of an embryonic origin, contains a wide range of biologically active agents: organic acids, including a significant amount of free DNA and RNA, vitamins, enzymes, hormones, macro- and microelements, i.e. the natural components inherent for live cell or tissue. Earlier it was shown by us the certain influence of preparation on morphofunctional condition of a liver of rats and the expressed homeostasis-stabilizing effect at norm and at experimental non-alcoholic steatohepatitis [31]. In essence, "NICA-EM" constitutes a tissue preparation with the low-molecular nanostructured activated composition which properties are reached due to a complex of the manipulations directed on activation of biochemical processes in embryonic tissues that allows to increase the level of content of biologically active agents in a substratum, including due to formation of biogenous stimulators. The main feature of technology of this biologically active product is use of method of high pressure homogenization (HPH). The way of production of the preparation "NICA-EM" passed patent search and is presented to Federal Service for Intellectual Property (Rospatent), the request No. 2014139637 of 30.09.2014. The multiplex chemical composition and structural features of this preparation allow to expect its positive influence on mechanisms of regulation of hormonal, metabolic and probably the immune status, and also can cause anti-inflammatory and homeostatic effects. Basing on above-described data, we conducted research on influence of a preparation "NICA-EM" on an organism of mammals

at an experimental acute inflammation.

Matherials and methods

Animals

Male Wistar Albino rats of body weights ranging from 170 g to 200 g were used in the study. The rats were group-housed in polypropylene cages with no more than four animals per cage. They were maintained under standard laboratory conditions with natural dark-light cycle and were allowed free access to standard pellet diet and tap water *ad libitum*. All the experiments were carried out using three groups, each containing 20 animals. All the animal experiments were performed according to the compliance with the EC Directive 86/609/EEC and with the Russian law regulating experiments on animals.

Carrageenan-Induced Hind Paw Edema in Rats

Acute inflammation was produced by injecting 0.1 ml of carrageenan (1% in saline) locally into the plantar aponeurosis of the right hind paw of the rats [32,33]. First group served as intact control, where no inflammation was induced. This group was used for evaluation of biochemical parameters. At rats of the control group the carrageenan-induced paw edema was modelled. At animals of experimental group the carrageenan-induced paw edema was also modelled, but at the same time these rats were subdermally injected with preparation "NICA-EM" in a dose of 30 mg per 1 kg of body weight. Injections were made twice, for 14 and in 7 days prior to modeling of an inflammation.

The paw volume up to the ankle joint was measured using a digital plethysmometer (Ugo Basile, 7140 Comerio, Varese, Italy) at the beginning of experiment, in 1th, 2nd, 3rd, 4th, 8th and 24th hours after induction of edema. After each measurement the change of paw volume in each group in comparison with initial was defined. The increase of paw volume was calculated by formula P=(O-I)/I*100%, where P – a paw volume increase, I – paw volume before introduction of a carrageenan, O – paw volume after introduction of a carrageenan. Inflammation inhibition rate was calculated by formula R=100%-((O-I/I*O) /(O-I/I*K))*100%, where R - inflammation inhibition rate, O – paw volume in experimental group, K - paw volume in control group, I – paw volume before introduction of a carrageenan. After the end of experiment animals were sacrificed in carbon dioxide chamber.

Biochemical analysis

Levels of total protein and albumin in serum of blood were investigated by means of the biochemical StatFax3300 analyzer (USA) with sets of Spinreact firm (Spain).

To check the inflammatory stimulus induced by the intervention the blood levels of C-reactive protein (CRP) were assayed. The levels of CRP were measured by the use of commercial ELISA (eBioscience (Bender MedSystems)).

Hematological analysis

Hematological analysis was performed using the hematological analyzer Abacus junior vet (Diatron, Austria). The examined parameters included red blood cells (RBC), hematocrit (HCT), hemoglobin, white blood cells (WBC), lymphocytes, neutrophils, monocytes, eosinophils and basophiles.

The Westergren method was used for the measurement of ESR

Histopathological analysis

Thymus and spleen were taken and fixed in 10% formaldehyde. After several treatments for dehydration in alcohol, sections having 5μm thickness were cut. Sections were subjected to stain with hematoxylin and eosin, and then the histopathological analysis was carried.

Morphometric Studies

By means of image analyzer "ImageScope" («Leica Mycrosystems Gmbh», Austria) at hematoxylin and eosin stained sections the area of segments of thymus and area of white pulp of a spleen were defined. A percentage ratio of cortical and medullary substances of a thymus and quantity of Hassal's bodies were also defined [34]. All calculations were carried out on photographs of a histologic specimens made from 10 various fields of vision with calculation of average value.

Statistical analysis

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 11.0 packed program. Data were presented as mean ± standard. Difference between the control and experimental groups was analyzed using Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

In an hour after subplantar introduction of a carrageenan at rats of control and experimental groups was observed the expressed edema of the

affected paw, which volume significantly increased in comparison with initial volume by 47.0±4.2% and 29.5±2.1% respectively. At animals of intact group paw volume practically didn't change. Thus, inhibition of an inflammation in the first hour after introduction of carrageenan under the influence of a preparation "NICA-EM" made 37.23%. In two hours change of paw volume at animals of control and experimental groups made respectively 55.8±4.3% and 44.3±3.2% (Figure 1). Inhibition of an inflammation under the influence of a preparation of "NICA-EM" made 20.61%. The analysis of change of paw volume of rats of control and experimental groups within the next 6 hours didn't reveal reliable distinctions. However in 24 hours after the beginning of experiment reliable change of paw volume in experimental group in comparison with control is revealed and made 29.7±3.6%, at the same time in control the value of this parameter made 42.90±4.10. Thus the coefficient of inhibition of an inflammation made 30.77%. The assessment of change of paw volume at development of carrageenan-induced edema at white rats treated with the full course of use of a preparation "NICA-EM" testifies to considerable decrease in initial level of inflammatory reaction within the first two hours, then inflammatory reaction authentically doesn't differ from control. However in 24 hours the level of inflammatory reaction characterized by change of paw volume is much lower at white rats of experimental group. Such dynamics of inflammatory reaction can testify that the immune system after introduction of a preparation "NICA-EM" is in a certain balanced state and readiness to resist adequately to alteration and thus to reduce a level of development of inflammatory reaction. Results of research of hematologic parameters of experimental animals are presented in Table 1 and confirm the above-stated conclusion. The analysis of results testifies that in 24 hours in group of animals previously injected with a preparation "NICA-EM" the parameter of ESR decreased almost twice in comparison with control that can testify to decrease in inflammatory reaction. Thus considering that the quantity of erythrocytes in all groups does not differ considerably, this parameter testifies to the expressed decrease in concentration of inflammatory proteins in blood serum. At the analysis of cellular composition of blood in experimental group the expressed decrease in quantity of leukocytes more than twice to 7.55±0.88 10^9/l is revealed. Decrease in quantity of granulocytes (neutrophils, eosinophils, basophiles) to 1.66±0.14 10^9/l and the cells relating to MID (monocytes, eosinophils, basophils) to 0.83±0.09 10^9/l is especially indicative because these types of cells are considered effector at acute inflammatory reaction. Decrease of absolute count of leukocytes in experimental group against even rather large paw volume and rather high ESR can testify about already subacute course of inflammatory reaction. By results of biochemical researches it is established that in blood of rats of both groups the increase of the C-reactive protein level is noted which is more expressed in control group (Table 2). Thus, in plasma of rats of experimental group the content of the total protein is higher. At the same time the content of albumin in both groups is higher, than in group of intact animals, but doesn't differ authentically among themselves. In our opinion, the increase of content of total protein against almost constant level of albumin testifies to activation of humoral mechanisms of immunity. This assumption is confirmed by results of morphological researches. In a spleen of rats of experimental group in distinction from control a large number of follicles with well-marked light centers and also a large number of leukocytes in a red pulp are revealed. Results of morphometric researches testify to increase in the area of a white pulp of a spleen from 20.91±6.84% in intact to 26.33±4.31% in control and to 43.80±2.90 at experimental rats (Figure 2). Thus we observed an intensive infiltration of a parenchyma of a spleen with cells of white blood, in particular macrophages and plasmatic cells, at rats experimental group. At the same time we noted an increase in the area of segments of a thymus from 550.80±19.56 μm^2 in organs of intact animals to 561.0±15.1 μm^2 in thymus of rats of control group and to 642.88±21.51 μm^2 in a thymus of rats of experimental group. In a thymus of rats of experimental group, in comparison with control, the arterial hyperemia and lymphoid infiltrates around vessels, and also well expressed border between cortical and medullary substances are found (Figure 3).

As a result of morphological researches of a thymus of rats it is established that application of a preparation "NICA-EM" at a carrageeenan-induced inflammation leads to significant increase in a percentage of cortical substance and increase in a cortical/medullary ratio in a thymus (Table 3). Besides, in a thymus of experimental rats the count of Hassal's bodies per 1 mm^2 increases.

Discussion and conclusion

As a result of the conducted research it is established that dynamics of inflammatory reaction which is

characterized by change of paw volume of an animal was less expressed in rats of experimental group. Such dynamics testifies that application of the preparation "NICA-EM" according to the scheme used by us brings immune system of rats to more balanced state in comparison with control. As a result there are an increase of ability of an organism to resist to alteration and significant reducing of a level of inflammatory reaction development in an organism. The considerable decrease in ESR in experimental group also argues for this. Taking into account almost unaltered quantity of erythrocytes, it speaks well for decrease in concentration of inflammatory proteins in blood serum. The hematologic and biochemical parameters described above also speak well for the subacute course of inflammatory process in experimental group. The results of histological and morphometric researches of a spleen and thymus testify to proliferation and migration of immunocytes, testifying, in turn, to activization of immune reactions. In a thymus of rats of experimental group in comparison with control the increase in the dimensions of thymic lobules, and also the increase in quantity of Hassal's bodies testifying to intensification of synthesis of the thymic hormones participating in process of an immunopoesis are revealed. The conducted research testifies to the expressed immunomodulatory action of the preparation "NICA-EM" at an experimental model of acute inflammation. It is possible to assume that the expressed immunomodulatory effect of the preparation "NICA-EM" is based on its ability to correction of the impaired parameters of immune system at all levels: humoral, cell-mediated and nonspecific.

Acknowledgments

The study was conducted under Task number 2014/2016 on the implementation of public works in the field of scientific activities of the base portion of the state task of the Ministry of Education and Science of the Russian Federation. Financial support of research was carried out by the Ministry of Education and Science of the Russian Federation, within performance of a basic unit of the state task (2014/2016), and by Moscow State Regional University.

Conflict of interest

There are no conflicts of interest.

References

1. Beisswenger, C., Bals, R., Interaction of allergic airway inflammation and innate immunity: hygiene and beyond. J Occup Med Toxicol 2007; 3(1): 1-3.
2. Gruver, A.L., Sempowski, G.D., Cytokines, leptin, and stress-induced thymic atrophy. J Leukoc Biol 2008; 84: 915–923.
3. Hirokawa, K., Utsuyama, M., Kikuchi, Y., Assessment of age-related decline of immunological function and possible methods for immunological restoration in elderly, In: Handbook on Immunosenescence. Springer Science+Business Media B.V. 2009: 1547-1570.
4. Cannizzo, F.T., Miniscalco, B., Riondato, F., et al., Effects of anabolic and therapeutic doses of dexamethasone on thymus morphology and apoptosis in veal calves. Vet Rec 2008; 163: 448–452.
5. Nakata, A. Psychosocial job stress and immunity: a systematic review. In: Psychoneuroimmunology: Methods and protocols. New York, Heidelberg, Dordrecht, London, Springer Science+Business Media, LLC 2012: 39-75.
6. Morukov, B.V., Rykova, M.P., Antropova, E.N., et al., The human system of immunity under the conditions of 105-day isolation and confinement in artificial environment. Hum Physiol 2014; 40(7): 814-821.
7. Labro, M.-T. Interference of antibacterial agents with phagocyte functions: immunomodulation or “immuno-fairy tales”? Clin Microbiol Rev 2000; 13(4): 615-650.
8. Biolatti B., Bollo E., Cannizzo F.T., et al., Effects of low-dose dexamethasone on thymus morphology and immunological parameters in veal calves. J Vet Med A 2005; 52: 202–208.
9. Gea-Banacloche, J.C. Immunomodulation. In: Principles of Molecular Medicine, 2nd Edition. Totowa, Humana Press 2006: 893-904.
10. Cannizzo, F.T., Spada, F., Benevelli, R., et al., Thymus atrophy and regeneration following dexamethasone administration to beef cattle. Vet Rec 2010; 167: 338–343.
11. Smit, E., Oberholzer, H.M., Pretorius, E., A review of immunomodulators with reference to Canova. Homeopathy 2009; 98(3): 169-176.
12. Saroj, P., Verma, M., Jha, K.K., et al., An overview on immunomodulation. J Adv Scient Res 2012; 3(1): 7-12.
13. Patil, U.S., Jaydeokar, A.V., Bandawane, D.D., Immunomodulators: a pharmacological review. Int J Pharm Pharmaceut Sci 2012; 4(1): 30-36.
14. Hecker, M., Ott, J., Sondermann, C., et al., Immunomodulation fish-oil containing lipid emulsions in murine acute respiratory distress syndrome. Crit Care 2014; 18: 1-11.
15. Jash, A., Kwon, H.-K., Sahoo, A., et al., Topical application of porcine placenta extract inhibits the progression of experimental contact hypersensitivity. J Ethnopharm 2011; 133: 654-662.
16. Wagar, L.E., Champagne, C.P., Buckley, N.D., et al., Immunomodulatory properties of fermented soy and dairy milks prepared with lactic acid bacteria. J Food Sci 2009; 74(8): 423-430.
17. Pejin, B., Stosic-Grujicic, S., Bogdanovic, G., et al., In vitro evaluation of the immunomodulatory and anticarcinogenic activity of the freshwater bryozoan Hyalinella punctata methanolic exract. Dig J Nanomater Biostruct 2013; 8(1): 187-195.
18. Lozano-Ojalvo, D., Lopez-Exposito, I., Molina, E., et al., Immunomodulatory effects of ovalbumin hydrolysates in a mouse model of food allergy. Clin Transl Allergy 2015; 5(3): 118.
19. Choi, S.-W., Moon, S.-H., Yang, H.J., et al., Antiresorptive activity of Bacillus-fermented antler extracts: inhibition of

osteoclast differentiation. Evid Base Compl Alternative Med 2013; 2013: 1-9.
20. Lee, K.H., Park, H.J., Seo, H.G., et al., Immune modulation effect of porcine placenta extracts in weaned the pig. J Anim Sci 2013; 91(5): 2405-2413.
21. Hua, Z., Fang, L.Q., Hong, W., et al., Effects of goat placental immunoregulatory factor on non-specific immunity of mice. Isr J Vet Med 2009; 64(3): 66-71.
22. El-Shitany, N.A., Shaala, L.A., Abbas, A.T., et al., Evaluation of the anti-inflammatory, antioxidant and immunomodulatory effects of the organic extract of the red sea marine sponge Xestospongia testudinaria against carrageenan induced rat paw inflammation. PloS One 2015; 10(9): e0138917.
23. Chen, W.-J., Yeh, S.-L., Effects of fish oil in parental nutrition. Nutrition 2003; 19(3): 275-279.
24. Yamauchi, K., Wakabayashi, H., Shin, K., et al., Bovine lactoferrin: benefits and mechanism
25. of action against infections. Biochem Cell Biol 2006; 84(3): 291-296.
26. Oh, T.H., Markelonis, G.J., Dependence of in vitro myogenesis on a trophic protein present in chicken embryo extract. Proc Natl Acad Sci Unit States Am 1980; 77(11): 6922-6925.
27. Kovacs-Nolan, J., Phillips, M., Mine, Y., Advances in the value of eggs and egg components for human health. J Agric Food Chem 2005; 53(22): 8421-8431.
27. Yang, M., Yang, C., Nau, F., et al., Immunomodulatory effects of egg white enzymatic hydrolysates containing immunodominant epitopes in a BALB/c mouse model of egg allergy. J Agric Food Chem 2009; 57(6): 2241-2248.
28. Yadgary, L., Wong, E.A., Uni, Z., Temporal transcriptome analysis of the chicken embryo yolk sac. BMC Genomics 2014; 15(1): 690.
29. Li, X., Su, Y., Sun, J., et al., Chicken embryo extracts enhance spleen lymphocyte and peritoneal macrophages function. J Ethnopharmacol 2012; 144(2): 255-260.
30. Alibardi, L., Mlitz, V., Eckhart, L., Immunolocalization of scaffoldin, a trichohyalin-like protein, in the epidermis of the chicken embryo. Anat Rec 2015; 298(2): 279-287.
31. Areshidze, D., Timchenko, L., Rzhepakovsky, I., et al., Influence of the tissue preparation "NICA-EM" on morphofunctional condition of a liver of rats at norm and at experimental non-alcoholic steatohepatitis. Pharmacologyonline 2015; 2: 108-117.
32. Winter, C.A., Risley, E.A., Nuss, G.W., Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Exp Biol Med 1962; 111: 544-547.
33. Amresh, G., Reddy, G.D., Rao, C.V., et al., Evaluation of anti-inflammatory activity of Cissampelos pareira root in rats. J Ethnopharmacol 2007; 110(3): 526-531.
34. Vascellari, M., Katia, C., Annalisa, S., et al., Evaluation of thymus morphology and serum cortisol concentration as indirect biomarkers to detect low-dose dexamethasone illegal treatment in beef cattle. BMC Vet Res 2012; 8(129): 1-12.

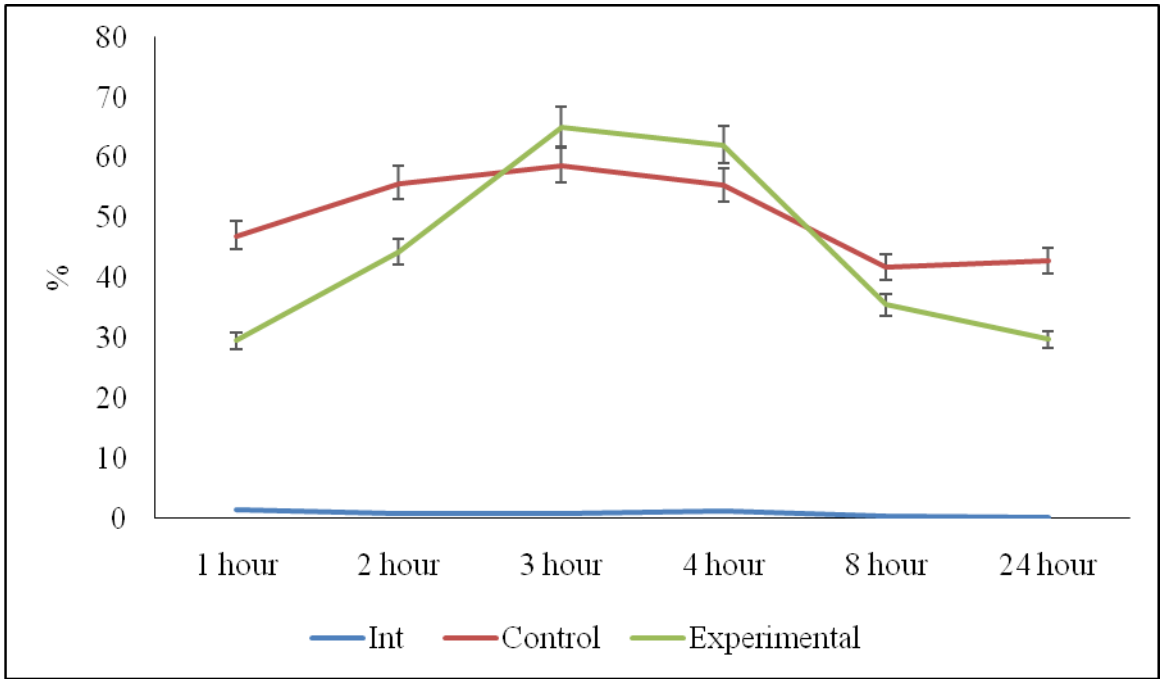

Figure 1. Dynamics of change of paw volume, % of initial volume.

Table 1. Hematological parameters of rats.

	Intact group (n=20)	Control group (n=20)	Experimental group (n=20)
WBC,10^9/l	6.4±0.61	16.20±2.80	7.55±0.88*
LYM ,10^9/l	4.2±0.12	7.67±0.49	5.05±0.64*
MID, 10^9/l	0.70±0.07	2.10±0.5	0.83±0.09*
GRA, 10^9/l	3.0±0.21	6.42±0.89	1.66±0.14*
LYM,%	53.50±2.89	54.75±4.56	66.17±1.38*
MID, %	12.50±0.47	11.91±1.02	11.45±1.05
GRA,%	35.15±2.82	33.36±4.07	22.39±1.31*
RBC, 10^{12}/l (erythrocytes)	4.71±0.18	4.86±0.18	4.66±0.17
HGB, g/l (hemoglobin)	110.5±6.81	93.92±2.94	85.31±5.38
HCT,% (hematocrit)	33.8±2.70	25.09±0.83	24.15±0.97
ESR, mm/h	8.51±0.54	22.91±4.38	13.83±1.43*

Hereinafter marked values significantly different from that of the I group (* - p≤0.05, **- p≤0.005,***- p≤0.0005).

Table 2. Effect of "NICA-EM" on biochemical parameters of blood serum.

	CRP, mg/l	**Total protein, g/l**	**Albumin, g/l**
Intact group (n=20)	2.24±0.11	73.2±2.90	33.60±3.91
Control group (n=20)	7.64±0.72	81.02±4.42	43.95±3.97
Experimental group (n=20)	3.87±0.30**	93.05±8.49	40.07±7.23

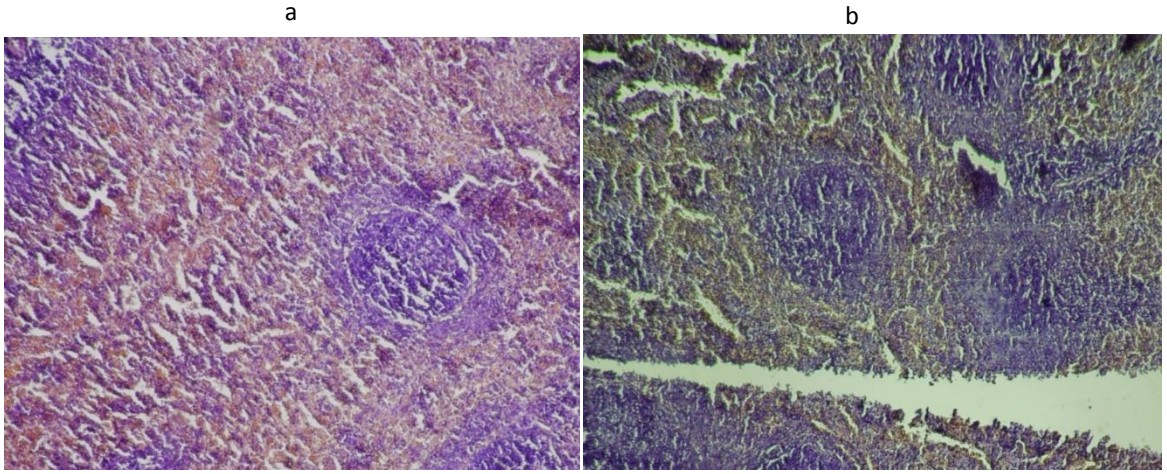

Figure 2. Spleen of rats of control (a) and experimental (b) groups. H&E, × 100

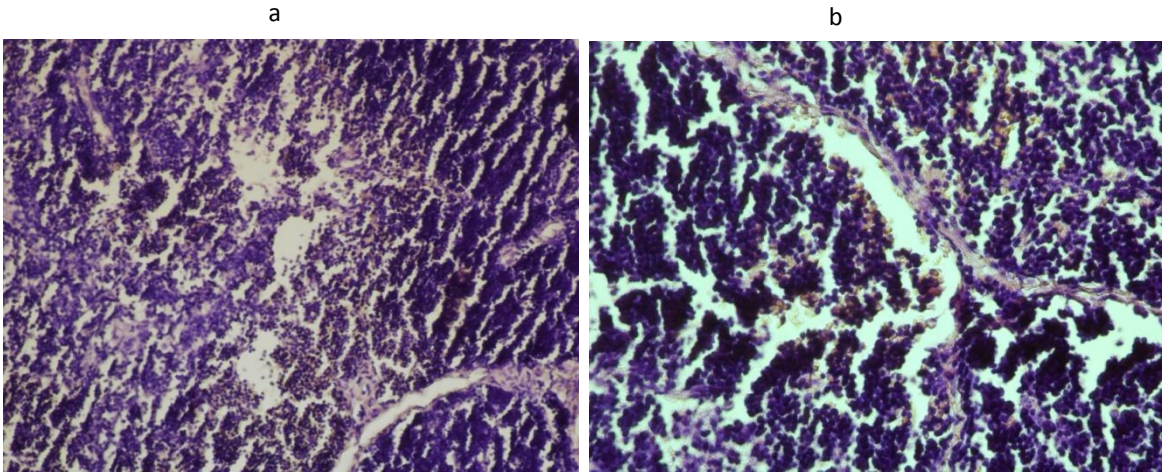

Figure 3. Thymus of rats of control (a) and experimental (b) groups. H&E, × 100.

Table 3. Morphometric parameters of thymus of rats.

	Intact group (n=20)	Control group (n=20)	Experimental group (n=20)
Percentage of cortical substance,%	34.8±2.89	37.60±3.11	41.4±2.75*
Percentage of medullary substance,%	22.2±1.72	23.40±1.89	20.60±1.39
Percentage of cortico-medullary region,%	23.70±1.82	22.51±2.10	18.50±1.22*
Percentage of capsule and trabecules,%	20.34±1.22	17.49±3.22	19.50±1.90
Cortical-medullary ratio	1.59±0.12	1.60±0.10	1.95±0.15**
Count of Hassal's bodies per 1 mm^2	3.47±0.25	4.80±0.29	7.81±0.33***