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Changes in blood and inner organs of immunodeficient rats induced by complex of natural peptides and proteins with immunostimulating activity

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ABSTRACT

The results of complex extract (Sus Scrofa thymus, spleen and lymph nodes) and its fractions (molecular weight: <5 kDa; 5-30 kDa; > 30 kDa) study on alterations in morphology, blood biochemical status, and antioxidant capacity of Wistar rats with cyclophosphamide immunodeficiency model are present in this article. The significant difference noted in fractions <5 kDa and fractions >30 kDa that was a minimal tendency to recovery after immunosuppression. Thus, positive effect was in fraction 5-30 kDa, it was revealed protein metabolism normalization leads to stable increase in rat weight, main renal stabilization background and hepatic markers, corresponding to decrease in pathological changes in liver and kidneys. Also it was detected significant increase in antioxidant blood serum activity and decrease in signal amplification in ESR spectroscopy. Further investigation advisability of immune pig organs extracts fraction will be devoted to fraction 5-30 kDa, which demonstrated maximum therapeutic effect.

Keywords: DDW, deuterium, immune system, biochemistry, antioxidant capacity.

1. INTRODUCTION

Nowadays, there is an increase in number of diseases that develop because of impaired T- or B-cells reactions. Impaired immune response is considered as a result of prenatal or postnatal exposure to environmental factors, nutritional factors or pharmacological substances (Obernikhin et al., 2014; Yaglova et al., 2013). Immunocorrective therapy is currently ineffective, since there are no exact data on both causes of their occurrence, and on key elements that take an active part in response processes and reactions. Thus, various new research methods are used. However, one of the most important areas for studying immunocorrectors properties is an adequate and reproducible immunodeficiency model reconstruction, in which primary immune response main symptoms reproduction to bacterial, viral and other infections is possible. At the moment, modeling is widely used to immune system evaluate responses and malfunctions using chemical factors as medicinal immunosuppressants such as cyclophosphamide, batridene, heparin, antilymphocytic globulin and physical factors, including low-frequency ultrasound or hard x-ray irradiation (Colvin, 1999; Berge et al., 1984; Berge et al., 1994). In this case, cyclophosphane in biological properties and action mechanism is a unique drug, since it is "transport" form that acquires biological activity only when ingested.

Recent publications show that while recreating cyclophosphamide immunosuppression model, o single high (near-lethal) dose toxic drug effect is mainly assessed. It is established that exposure negative manifestations are visualized as deterioration in animal's external well-being, weight loss, drowsiness and lethargy, cystitis appearance, impaired digestive function and hemopoiesis suppression. Conjunctivitis appearance and small hemorrhages, and sometimes necrosis of the skin, are also shown. Deep and persistent changes in all hematopoiesis germs in rats and mice were detected only when cyclophosphamide was administered in toxic and subtoxic doses. In this case, the lymphopoiesis is most sensitive to drug, animals have decrease in lymphocyte content in parallel with number of leukocytes. Granulocytes count in blood decreases insignificantly at simulation beginning, and then it increases greatly. Thrombocytopoiesis and erythropoiesis shifts are unimportant.

Particular interesting changes in animals were observed in the internal organs to which cyclophosphamide was administered. In animals, there is development of destructive and dystrophic changes in liver with serous inflammation appearance and micro necrosis foci, which are accompanied by reversible reticuloendothelial cells activity inhibition. These pathological shifts are explained by cyclophosphamide activation in the liver. It has been revealed that under microsomal liver enzymes action hydroxycyclophosphamide metabolite is formed, which under phosphotases action is converted into active substance - phosphamidiprit. Formed metabolites act on rapidly proliferating cells growth. The drug also has toxic effect on kidneys, changing their function and water-electrolyte metabolism. As clinical symptoms, it was noted decreased diuresis, dysuria and hematuria, toxic nephrosis emergence. It was also revealed that cyclophosphamide large doses cause thymus involution and decrease in number of lymphoid elements in spleen and lymph nodes, which leads to immunological reactions inhibition (Fedulova et al., 2017). Cyclophosphane, which has a degrading effect on normal cells, also causes lung function disruption, erythrocyte and leukocyte production inhibition in bone marrow, reduces oxygen transport by erythrocytes, disrupts nucleic acids functioning, B and T cells inhibits antibodies production in serum (Fedulova et al., 2019). In addition, noted drug toxic effect on oxidation-reduction processes state: blood aldolase and peroxidase activity decreases, aspartate aminotransferase activity increases. Alanine aminotransferase activity does not change significantly.

In connection with above, toxic state leveling after cyclophosphamide injection is achieved by medication with standard antitoxic agents, in combination with anti-allergic and anti-inflammatory agents. It was shown that water with lower deuterium content accelerates pathogens removal from infection chronic foci and contributes to reducing carbon tetrachloride toxic effects (Basov et al., 2018). In addition, water with low deuterium content affects various organs and tissues metabolic activity (Goncharuk et al., 2018;

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Dzhimak et al., 2018; Zlatska et al., 2018), including with typical pathological processes complex correction (Yavari et al., 2019; Dzhimak et al., 2017; Syroeshkin et al., 2018). Therefore, its use in conjunction with immunomodulating peptides for immunosuppressive states correction is biology and veterinary science actual task (Kotenkova et al., 2018; Chernukha et al., 2018).

Aim of this work was to study fractions effect of thymus, spleen and mesenteric lymph nodes on morphological indexes and blood biochemical status of laboratory rats with cyclophosphamide immunodeficiency model.

2. MATERIALS AND METHODS

The subjects were Sus scrofa thymus, spleen and mesenteric lymph nodes extracts mixtures based on 0.9% sodium chloride solutions of distilled water (DW, D/H = 150 ppm) and water with modified isotopic composition (WMIC, D / H = 40 ppm) (Fedulova et al., 2018) of Deuterium concentration determination in water was carried out with a JEOL JNM-ECA 400MHz pulsed NMR spectrometer (Dzhimak et al., 2015). Study was carried out on adult clinically healthy sexually naive male Wistar rats spf-category (n = 60) average weight (400 ± 20 g, group spread ± 8 g), obtained from the CGD ICG SB RAS (Novosibirsk). Rats were held in individually ventilated system cells (VENT II fan unit and rack-type cells Bio.AS type III (EHRET, Germany)) at optimum microclimate in each cell (temperature ((22 ± 3) ° C), humidity ((50-60)%), lighting 12/12.

After adaptation for 20 days part of animals (n = 50) reproduced immunodeficiency model (IDM) [19], remaining part of animals (n = 10) stayed without treatment (intact animals; 6 group). In 12 days after the simulation, groups (n = 10) were randomly formed:

1 group (Control) - control, with uncorrectable immunodeficiency, which was intragastrically treated with purified water in a volume of 2 ml/ kg;

2 groups (Complex) - immunodeficiency correction by intragastric treatment of complex on the basis of WMIC in dose 0.05 g protein/ kg rat weight;

3 group (Fr <5 kDa) - immunodeficiency correction by intragastric treatment of fractions up to 5 kDa of complex extract based on WMIC in dose 0.005 g protein/ kg rat weight

4 group (Fr 5-30 kDa) - immunodeficiency correction by intragastric treatment of extract fractions 5-30 kDa complex extract based on WMIC in dose 0.015 g protein/ kg rat weight;

5 group (Fr> 30 kDa) - immunodeficiency correction by intragastric introduction of extract fractions over 30 kDa complex extract based on WMIC in dose 0.03 g protein/ kg rat weight;

6 group (Intact) - intact rats not exposed to exposure and under similar conditions of maintenance.

Samples administration *per os* (complex extract, fractions with different Mw) was performed daily for 26 days. For health status assessment it was daily recorded clinical status and behavior of the animals. Weighed rats were performed every 3 days on electronic technical scales (Ohaus, Adventurer Pro, USA) with accuracy (± 0.1) g. During experiment, rats ration (ad libitum) consisted of complete feed (Assortiment-Agro, Russia) and drinking water, obtained on water installation EMD Millipore RiOs™ 50 (Merc Millipore, Germany) and mineralized by mineral salts addition (314-382 mg/l: hydrocarbons – 144-180, sulfate <1 chloride – 60-76, calcium - 6, magnesium - 3, sodium – 50-58, potassium 50-58), temperature (10-12) °C. On the 26st day of experiment, rats were euthanized in euthanasion camera (VETtech) according to Directive 2010/63/EU of European Parliament and Council of European Union for protection of animals used for scientific purposes. Stunned animals bloods were taken from right ventricle of heart. Hematologic parameters (whole blood erythrocytes, hemoglobin, hematocrit, platelets) were determined on veterinary hematological analyzer Abacus junior vet 2.7 (Diatron Messtechnik GmbH, Austria) using Diatron reagents. For biochemical studies, blood serum was separated by centrifugation at 1300g for 5 minutes. The analyzed indexes were examined on automatic biochemical analyzer BioChem FC 360 (HTI, USA) using high technology (USA) reagents: total protein was identified by standard biuret blue method at wavelength 540 nm; albumin – by bromocresol green at 630 nm; Total bilirubin - by DMSO method at 560 nm; creatinine - by Jaffe method at 510 nm; gamma-glutamyltransferase (GGT) – by kinetic method at 405 nm; aspartate aminotransferase (AsAt), alanine aminotransferase (AlAt) – by IFCC kinetic method at 340 nm; lactate dehydrogenase (LDH) by Wacker/Tris modified kinetic method at 340 nm.

Antioxidative activity (AOA) of blood serum was identified by measuring optical density on a Biochem SA analyzer at 600 nm. Phosphate buffer (pH = 7.4) 0.5 ml was successively introduced into chemical tubes, 2,6-dichlorophenolindophenol solution 0.15 ml and ferrous sulfate solution 0.15 ml were added and mixed rapidly to determine D_{op} and D_k. Immediately after mixing, blood plasma 20 µl was added to D_{op} tube, and distilled water20 µl was added to D_k tube, mixed and incubated at 37°C for 5 minutes. Then optical density was measured. The same reagents were introduced into the tube, but instead of ferrous sulphate solution distilled water 0.15 ml was added to determine D_{max}. The inhibition constants (K_i, ml × min^{-1}), the difference in the oxidation rate constants of 2,6-dichlorophenolindophenol in the control (K_k) and in the experiment (K_{op}) versus the serum concentration in the mixture was determined to calculate AOA:

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$K_i = (K_k - K_{op})/C$,

where $K_k = 0,2*(\ln D_{max} - \ln(D_{max} - D_k)$;

$K_{op} = 0,2*(\ln D_{max} - \ln(D_{max} - D_{op})$;

C - serum concentration in incubation mixture, mg/l.

Animal's autopsies were carried out according to a standard procedure. Liver, kidney, spleen, heart and thymus of all animals were properly separated from all adjacent tissues, washed in sterile physiological saline and weighed on electronic technical scale (Ohaus, Adventurer Pro, USA). Internal organs relative coefficients were determined by calculation. ESR spectroscopy was performed on basis of REC of TsKP "DSSN" Kuban State University using JES FA 300 ("JEOL", Japan) spectrometer at 24°C in the X-band (microwave power was 1 mW, microwave radiation frequency was 9144 MHz, high-frequency modulation amplitude was 0.1 mT). Investigated samples of esophagus and diaphragm were preliminarily subjected to lyophilization in drier LC-1000 ("Prointeh", Russia), before direct ESR spectroscopy, samples were weighed to an accuracy of 0.01 mg ("Ohaus" scales, PRC). Sample ESR signal was measured in sample whose mass in resonator zone was 0.03 g (in quartz with diameter 5 mm), ESR signal integrated intensity was calculated by double numerical integration. This procedure was performed by comparing obtained signal with standard sample ESR signal of 2,2,6,6-tetramethylpiperidin-1-yl oxidanyl (TEMPOL), containing paramagnetic centers in count $6.4 \cdot 10^{-7}$ mol, and paramagnetic centers concentration determination in each of samples studied.

The experiment was approved by Bioethics Commission of The V.M. Gorbatov Federal Research Center for Food Systems of Russian Academy of Sciences (Protocol No. 2/2017). Statistical processing was carried out using the software "STATISTICA 10.0", ANOVA variational analysis in combination with Duncan test. Significance level is assumed equal to 0.05. The results are presented as M ± SE.

3. RESULTS

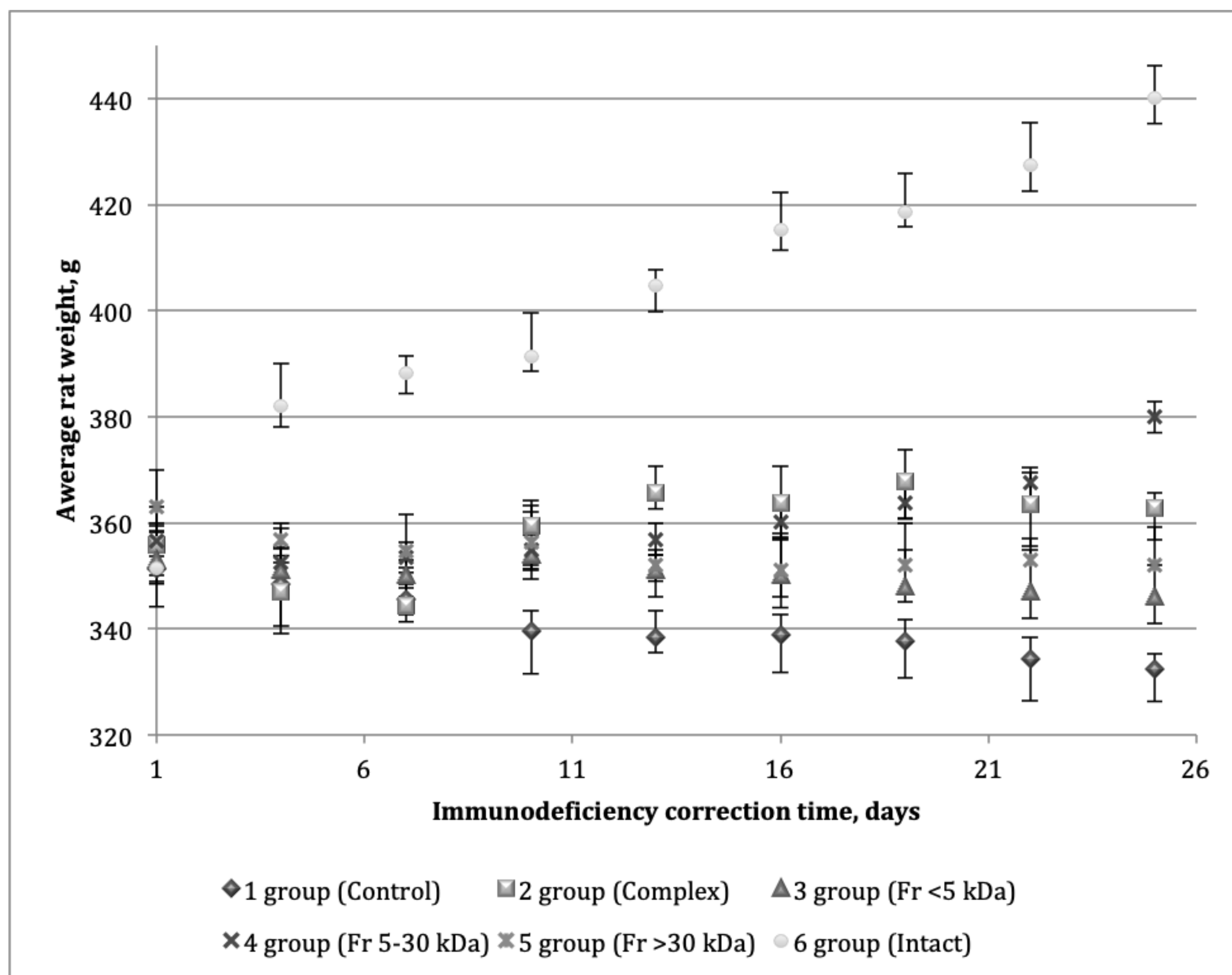

Figure 1 Laboratory animals body weight dynamics during the study.

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Intact rats (6th group) steadily gained weight throughout study, adding an average of 3.6 grams per day (Figure 1). By experiment end, animals of this group increased their body weight from initial to 20 %. While immunodeficiency modelling; stable weight loss was observed in all experimental rats. After model completion, control group animals (1) continued to reduce weight, losing an average of 0.8 g per day. Within 26 days 1st group animals average weight increased insignificantly (up to 6.0 %).

In 2nd group animals there was a decrease in average weight during the first 7 days of immunodeficiency correcting (by 5.3 g); further observation showed stable increase in animals weight with slight decrease (by 0.9 g) on experiment 16th day. Animals average weight increased by 1.9g during correction period for 26 days, average of 0.3 g per day. 3rd group rats also reduced weight. Average weight of this group rats decreased by 2.1 g during immunodeficiency correction time, with average value of 0.3 g per day.

In 4th group rats, decrease in average weight while first 4 treatment days (by 4.0 g), further, up to 26th day, stable increase in weight was noted, while average weight increased by 6 % while treatment, average of 0.9 g per day. 5 experimental group animals weight decreased from 0.45 to 0.4 g per day during treatment, slight increase in rats weight (up to 2 g) was observed on immunodeficiency correction 10th and 19th days. Rats average weight decreased by 5.7 g. While analyzing 1st group control animals hematological parameters with respect to intact animals (6th group), decrease in erythrocytes to 14 % was established with an increase in hemoglobin to 15 % and decrease in platelets by 13 %. 2, 3, 4 and 5 experimental animals groups showed an increase in erythrocyte concentration against the background of decrease in hemoglobin concentration in comparison with 1 group (control rats): in 2nd group - by 8.8 % and up to 15 %, in 3rd group - by 7.9 % and up to 6 %, in 4th group - by 7.6 % and by 9.8 %, in 5th group - to 2 % and by 12.6 %. There was increase in number of platelets in experimental animals relative to control: in groups 2 and 5, up to 8%, in group 3 by 5.5%, in group 4 by 10.9% (Table 1).

Table 1 Some hematological indexes in rats blood on 26th experiment day.

Indexes	1 group	2 group	3 group	4 group	5 group	6 group
Erythrocytes, 10^{12}/l	7.44±0.82	8.10±0.89	8.03±0.84	8.01±0.69	7.57±0.74	8.61±0.95
Hemoglobin, g/l	166.36±18.30*	141.83±15.71	156.92±15.89	149.73±17.26	145.35±16.41	140.62±15.47
Hematocrit, %	43.26±4.76	43.32±4.64	42.69±4.78	42.29±4.70	44.35±4.65	41.89±4.62
Platelets, 10^9/l	851.43±102.17	927.67±139.15	901.02±83.56	956.34±99.12	928.45±105.20	977.81±97.78
* - Significant difference from intact group (P<0.05).						

When assessing control rats (1st group) biochemical parameters in comparison with intact animals (6th group), a significant increase in total protein and creatinine content to 18 % (Fig. 2A, B), GGT - by 13 %, significant decrease in bilirubin - by 6 %, albumin - by 7 %, activity of AsAt by 13 %, AlAt by 8 % and LDH by 11 % (Table 2).

Table 2 Biochemical analysis results in rats blood serum on 26th experiment day

Indexes	Animal groups					
	1 (Control)	2 (Complex)	3 (Fr <5 kDa)	4 (Fr5-30 kDa)	5 (Fr >30 kDa)	6 (Intact)
Albumin, g/l	40.85±1.03*	43.87±1.18	44.69±1.27**	45.01±1.23**	42.52±2.26	44.11±1.89
Bilirubin, mlmol/l	2.49±0.18	2.73±0.20	2.77±0.19	2.71±0.23	2.58±0.21	2.64±0.16
AsAt, E/l	89.18±5.02*	97.09±7.91	96.38±6.24	99.13±5.10	91.01±6.32	101.12±6.22
LDH, E/l	430.07±16.15*	464.77±19.72**	466.2±10.41**	465.23±10.56**	426.82±9.39	476.30±12.89
GGT, E/l	1.84±0.13*	1.67±0.12**	1.98±0.09	2.04±0.10	1.75±0.14	1.60±0.25
AlAt, E/l	41.53±2.98	45.26±3.73	44.58±2.05	46.27±3.12	43.15±2.34	45.29±2.30
* - Significant difference from intact group (P<0.05). **- Significant difference from control group (P<0.05).						

In experimental 2-5 groups, relative to control animals, increase in total protein content by 6 % and bilirubin by 8 % for 2nd and 4th groups rats (Fr 5-30 kDa) was revealed; by 5 % and 10 % for 3rd group rats (Fr <5 kDa) and by 5 % and 4 % for 5th group rats (Fr> 30 kDa). Also, in comparison with 1st group animals indexes, increase in AsAt and AlAt activity was found (for 2nd and 4th groups rats by 9 % and 8 %, for 3rd group rats by 9 %, for 5th group rats by 3 % and 4 %). It should be noted that in relation to control group animals indexes, an increase in GGT activity was observed in groups 3 and 4 animals by 7 % and 8 %, respectively; in

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groups 2, 3 and 4 animals, LDH activity increased to 8 %. At the same time, GGT activity in 2nd and 5th groups rats, relative to control animals, decreased by 9 % and 5 % (figure 2).

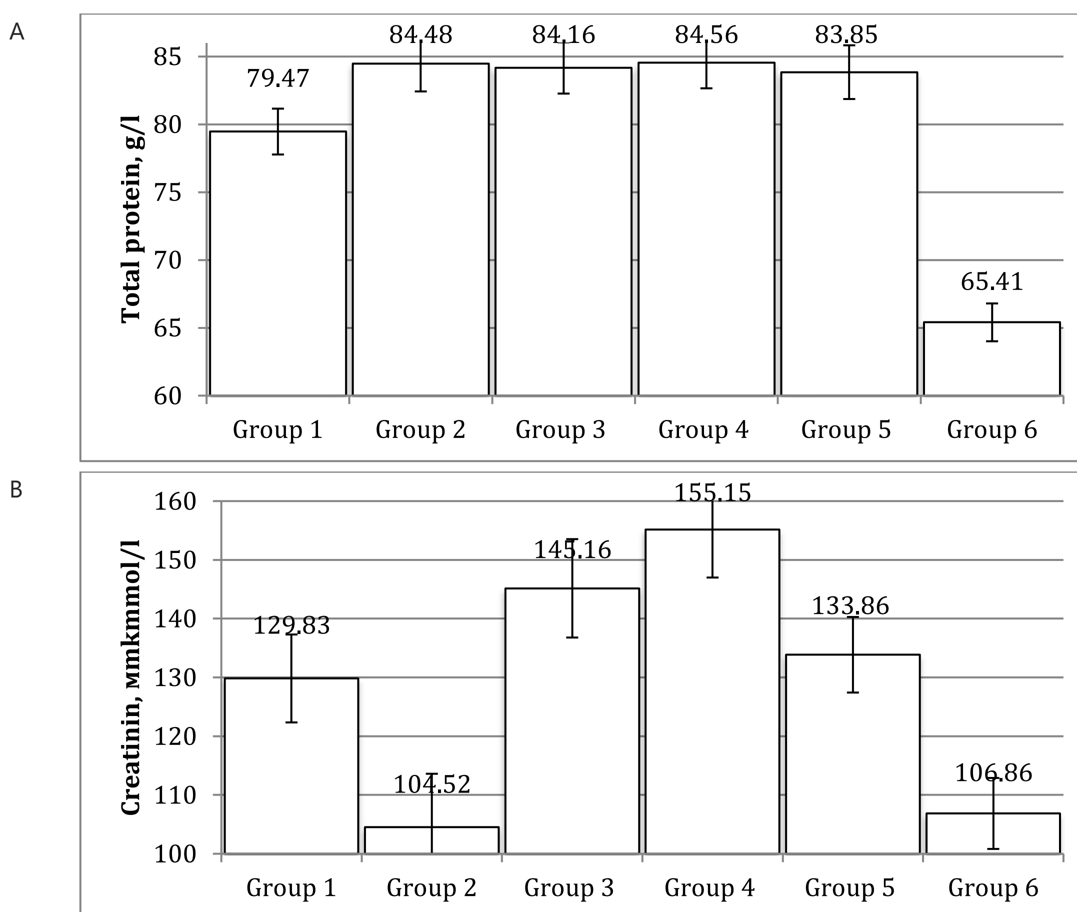

Figure 2 Total protein (A) and creatinine (B) in rats blood serum on 26th experiment day.

Antioxidant capacity study of rats blood serum in control group revealed decrease in AOA by 32.5 % compared to intact rats. In this case, extract and fractions from 5 to 30 kD and fractions of less than 5 kDa test samples treatment promoted an increase in blood serum AOA, relative to 1st group animals values: in 2nd group rats - by 10.2 %; 3rd group - up to 9 %; 4th group - by more than 20 %. 5th group rats blood serum AOA did not change significantly (Table 3).

Table 3 Antioxidant properties of rats blood serum.

Index	1 group	2 group	3 group	4 group	5 group	6 group
AOA, min-1*10^{-3}	0.805±0.017*	0.887±0.015*,**	0.876±0.019**	0.992±0.013**	0.812±0.015	1.193±0.012
* - Significant difference from intact group (P<0.05). **- Significant difference from control group (P<0.05).						

In ESR spectroscopy data analysis, greatest signal amplification was found in rats in group 1 by 24 % compared to group 6, which indicates an increase in free radical oxidation reactions and oxidative stress development while modeling cyclophosphamide immunodeficiency modelling. In groups 2-5 rats, increase in free radical oxidation intensity was less pronounced than in animals in group 1 (by 6-14 %). All this justifies advisability of using drugs that increase AOA in complex therapy for these experimental groups. Intact animals' examination (6th group) did not reveal any changes: coat was white, shiny, clean, the skin and visible mucous membranes were pale, shiny. Internal organs violations are not noted - thymus, spleen, liver, kidneys, heart were normal. While examining 1 and 3 groups rats, depletion was noted, yellow hair, untidy, dry skin, porphyrin in the nasal sinuses and eyes. Changes in immune organs were revealed: thymus was visually reduced, flabby, yellowish; spleen was enlarged, flabby, with a rough uneven capsule. Kidneys were enlarged, flabby, with poorly discernible cortical layer, while in 70 % cystic formations up to 2 mm in diameter

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were detected. Liver was also enlarged, friable, edges are rounded, in 50 % abscesses up to 11 mm in diameter were detected (Table 4).

Table 4 Relative mass of rats internal organs.

Group	Spleen	Kidney	Liver	Heart	Thymus
1 (Control)	0.281±0.052	0.397±0.036	4.101±0.232	0.379±0.038	0.115±0.024
2 (Complex)	0.283±0.041	0.378±0.027	4.233±0.304	0.387±0.046	0.154±0.027
3 (Fr <5 kDa)	0.292±0.045	0.395±0.086	4.415±0.236	0.375±0.074	0.128±0.019
4 (Fr 5-30 kDa)	0.256±0.042	0.365±0.043	4.127±0.185	0.373±0.052	0.132±0.013
5 (Fr >30 kDa)	0.296±0.038	0.363±0.032	4.134±0.113	0.381±0.030	0.151±0.025
6 (Intact)	0.220±0.039	0.362±0.010	4.052±0.241	0.376±0.045	0.134±0.023

In 2nd group animals, similar tendency was noted, with kidneys and thymus exception: kidneys corresponded to norm (not enlarged, without cystic formations); thymus was dense, ivory, with less fat. In 4th group animals, liver enlargement was also found, in 20 % abscesses up to 6 mm in diameter were detected, thymus corresponded to norm - ivory, dense consistency, dense, cortical layer expressed, spleen without pathologies, dense, dark cherry color, capsule smooth, shiny. Analyzed organs of 5th group animals were characterized by following changes: visual increase in spleen and thymus, flabby consistency, dark color, spleen surface rough and uneven; While in 50 % of cases abscesses up to 8 mm in diameter were observed on liver surface, organ itself was a flabby consistency, dark color; on kidneys in 40 % of rats small cystic formations up to 1 mm in diameter, brownish-colored buds, flabby (Table 4) were detected.

4. DISCUSSION

Cyclophosphamide injections in toxic dose resulted in constant decrease in control animals weight due to disruption in protein metabolism, which was confirmed by marked decrease in creatinine and total protein content, disruption in excretory system function (renal and hepatic markers significantly exceed normal levels), which correlates with pathoanatomical research results and internal organs coefficients (sudden pathological changes, cystic and purulent foci in liver and kidneys), also it was shown by Lorenz et al. (2001) and Cengiz (2018). Cyclophosphamide negative effect on immune organs - thymus and spleen, as well as indicators (Wang et al., 2018), characterizing red blood cells functioning, including oxygen transport reduction, and platelets (Cengiz, 2018). Complex extract on WMIC basis showed favorable tendency to restore in 2nd group animals after cyclophosphamide injections. Fairly stable increase in rats weight with some biochemical parameters relative normalization, including indexes responsible for protein metabolism and excretory system functions, increase in total antioxidant blood serum capacity with immune system functioning partial restoration was established. In 3 (fractions less than 5 kDa) and 5 (fractions more than 30 kDa) groups minimal tendency to recovery after cyclophosphamide immunosuppression development was noted - negative changes in protein metabolism and slight increase in antioxidant activity were detected, that correlate with Jiang et al. (2020).

Earlier it was shown by Fedulova et al. (2017), that fraction with a molecular weight less than 30 kDa have a beneficial effect on the immune system. In this research the maximum therapeutic effect was observed in fraction with a molecular weight from 5 to 30 kD. In group 4 rats protein metabolism normalization indices was revealed with a stable increase in weight, against main renal stabilization background and hepatic markers, corresponding to decrease in pathological changes in liver and kidneys (Lv et al., 2019), at the same time, significant increase in antioxidant blood serum activity was detected, as in Haque et al. (2001).

5. CONCLUSION

Our research showed thatimmune pig organs extracts fraction with a molecular weight from 5 to 30 kD can reduce cyclophosphamide cytotoxic effect, including negative changes in blood and inner organs. This fraction has the best effect on the recovery of methabolic and antioxidant systems.

Authors' contributions

Elena Kotenkova– conducting an experiment, writing an article
Ekaterina Vasilevskaya – writing an article, translation of article
Ekaterina Barysheva – conducting an experiment, writing an article
Irina Chernukha – writing an article, conducting an experiment

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Andrey Lisitsyn – writing an article, translation of article

Conflict of interests

Authors declare no conflict of interest

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Data and materials availability

All data associated with this study are present in the paper.

Peer-review

External peer-review was done through double-blind method.

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