

Influence of Different Polypeptides Fractions Derived from Sus Scrofa Immune Organs on the Rats Immunological Reactivity

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Abstract: The influence of protein-peptide compounds, extracted with deuterium water as solubilizer agent from mixture of Sus scrofa thymus, spleen and lymph nodes extracts, was carried out on Wistar rats with cytostatic-induced immunodeficiency model. Intra-gastric administration of extract fraction with molecular weight more than 30 kDa, did not lead to significant changes. Thus, polypeptide compounds with molecular weight less than 30 kDa, extracted from Sus scrofa immune organs, significantly increased T-lymphocytes amount, affected macrophages system by T-helper (CD4) stimulation and activated cycle of complementary cascade. Differences between the experimental results in T-helper synthesis activation (CD4) in animals treated with the medium and low molecular weight fractions can be explained by the ability of biomolecules having a molecular weight less than 30 kDa (in particular, isolated from the spleen, which, in its cellular structure contains key regulatory factors) to change the speed of cell differentiation.

Keywords: DDW, immunodeficiency, interleukin, CD3, CD4, thymus, lymph nodes, spleen, tissue-specific bioactive compounds.

INTRODUCTION

Nowadays there has been a rapid development of a new field of knowledge (biopharmacology) due to allergic diseases prevalence in the population, it can reduce the drug therapy adverse reactions and improve remedial measures efficacy because of natural medicines application. It is known number of drugs based on animal bioactive compounds derived from various endocrine organs, so it is particularly relevant today. On this basis it become possible to create a more effective medicaments with directional action for correction diseases including immunodeficiency.

Immunomodulatory medicines endogenous origin are represented, mainly, single-agent preparation-immunoregulatory cytokines and peptides isolated from the central (thymus and bone marrow) and peripheral immune organs (spleen) predominantly young or embryos of pigs and cattle. Thus, it was developed and introduced on medicine market three generations of immunoregulatory peptides based on thymus and bone marrow. Products based on extracts containing native hormone polypeptides with a molecular weight from 1 to 5 kDa – it is "Taktivin" (Biomed, Russia), "Timalin" (Tsitomed, Russia), "Timoptin" (GNIISKLS, IES, Russia), "Timostimulin" (TP-1, Italy), "Timaktid" (MEZ,

Russia), "Vilozen" (Biopharma, Ukraine), "Tumysamin" (Institute of Clinical Bio-regulation and gerontology, Russia), and others - traditionally refers to the first generation immunomodulators [1-3]. These products are aimed for normalization and improvement of the immunity and hematopoiesis T-system functional activity, enhance cellular immune and antibody response, coagulation and anti-coagulation system, neuroendocrine regulation, reparative tissue regeneration. Among the positive properties of thymus polypeptides there were noted high action selectivity, low doses efficacy, lack of adverse effects and rapid elimination from organism. Synthetic analogues of thymus hormones, or fragments referred to the second and the third generation thymic origin immunomodulators ("Immunofan" (Bionoks, Russia), "Timogen" or "Oglyufanid" (Tsitomed, Russia), "Refnot" (Refnot Farm, Russia) [4, 5].

Content in spleen of great number thymus-dependent, thymus-independent and macrophagic immune factors, such as nonspecific serum tetrapeptide taftsin, being composed in IgG Fd-fragment, activating functional properties of macrophages and polymorphonuclear leukocytes, complement components (C3 and C4 fragments, such as C3b), factor P - caused creating a wide range of products based on spleen embryos and young cattle biologically active substances [6-11]. For example, such medicines as «Polyerga» (Merck, UK), containing

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oligopeptides; "Diasplen" (Dialek, Belarus) - deproteinized derivative; "Splenin" and "Splenoid" (Farmak, Ukraine) and dietary supplement "Peptide complex 1» (Eni-sala, Russia) - peptide complexes. These products increase T-lymphocytes content in peripheral circulation and functional activity, stimulate STF production, normalize free radical peroxidation of lipids, stimulates reparative and trophic processes [12-14].

Thus target raw material source for biomolecules extraction were selected thymus, spleen and mesenteric lymph nodes of pigs aged 8-9 months. By technology production animal drugs are classified into preparations from whole organs and tissues, containing components of specialized cells and surrounding structures (connective tissue, interstitial fluid, lymph, basic substance, etc.); preparations of organ and tissue cells, including all cellular biomolecules (cytoplasm, nucleus, membranes, etc.); preparations based on cytoplasm of differentiated cells extracts ("revitalizing" cells component).

Today, modern equipment employment made possible selection of cell cytoplasm regulatory components, called cytochrome inducers, including growth factors, differentiation, different bioregulators, proteins, chalcones, cytamins, peptides, synthetic hormones precursors, neurotransmitters and others.

In medicine production for maximum preservation of biologically active substances of animal or vegetable origin the most popular is use of lyophilic drying [15] requiring optimal sample preparation in the extraction and biomaterial filtration stages. Latter can be attained due to solvent physicochemical properties updating, for example by changing the ratio of light and heavy isotopes in its composition. According to analyzed material there were chosen methods aimed to biomolecules selection from animal raw material, comprising water-salt extraction, filtration and ultrafiltration, then lyophilic drying, with pretreated test specimens in all these stages by solutions on the basis of water with modified isotopic D/H compound (WMIC) [16, 17]. Comparative study of WMIC selectivity and total extractability carried out in connection with recent publications on the water isotopic composition influence in the biological properties of proteins and peptides to change living systems adaptation properties [18-22].

Thus, aim of the study was to investigate water with modified isotopic D/H composition effect on

immunological activity of protein-peptide compounds extracted from *Sus scrofa* immune organs.

MATERIALS AND METHODS

The subjects was water-salt extract obtained from *Sus scrofa* organs (combined mixture of spleen tissue extracts; thymus tissue extracts; extracts of mesenteric lymph nodes) based on water with modified D/H composition (WMIC) and its fractions: fraction up to 5 kDa, fraction from 5 to 30 kDa and fraction over 30 kD.

Extraction

For immunocompetent polypeptides extraction it was used water with reduced deuterium concentration - 704.6 ‰, which was produced by electrolysis with drying of obtained electrolysis gas, electrolysis gases conversion in water and water vapor condensation [23]. Deuterium concentration control in obtained water was determined on pulsed NMR spectrometer JEOL JNM-ECA 400MHz in "Kuban State University" [24, 25].

Extract preparation consisted in organs separation from related tissues, grinding to a particle size of 3 ± 1 mm, extraction physiological solutions WMIC (Hydromoduls 1: 5, speed 400 rev / min for 3 hours) at 4°C in laboratory dispersing system (Laboteks, Russia). After extraction finished it was centrifuged in a centrifuge CM-6M (ELMI, Latvia) at 3500 rev / min for 5 min, supernatant was collected.

Fractionation

Extract's fractionation of protein-peptide complexes was performed by step ultrafiltration on Vladisart unit (Vlidisart, Russia), pressure $P = 2.5$ bar, polyethersulfone modules with plastic fittings and tanks VivaFlow 200 (Sartorius, Germany). As a result, extract fractions were obtained with molecular weight in the range of: less than 5kDa, from 5 to 30 kDa and over 30 kDa.

In Vivo Research

Study was carried out on adult clinically healthy sexually naive male Wistar rats spf-category ($n = 90$) 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 ($n = 80$) reproduced immunodeficiency model (IDM), remaining part of animals ($n = 10$) stayed

without treatment (intact animals; 6 group). Immunodeficiency model was implemented by intraperitoneal injections of Cyclophosphan (Russia) dosed as 75 mg/kg three times every 72 hours.

Immunosuppression development dynamics was assessed by modelling completion (day 12) by estimating of peripheral blood parameters taken from tail vein.

Rats with immunodeficiency model were randomly divided into groups on 12th day (after modeling):

Group 1 – control group, immunodeficiency without treatment;

Group 2 - immunodeficiency treatment by *per os* administration of unfractionated extract based WMIC;

Group 3 - immunodeficiency treatment by *per os* administration of extract fractions up to 5 kDa based WMIC;

Group 4 - immunodeficiency treatment by *per os* administration of extract fractions from 5 to 30 kDa based WMIC;

Group 5 - immunodeficiency treatment by *per os* administration of extract fractions above 30 kD based WMIC;

Group 6 - intact animals.

Samples administration *per os* (complex extract, fractions with different Mw) was performed daily in dose (20 ± 0.2) g/l of protein content; 1st group animals administrated water *per os* in equivalent volume. For health status assessment it was daily recorded clinical status and behavior of the animals.

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 28st 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.

Blood samples for hematology, biochemical and immunoassays were taken from right ventricle. Blood

sampling for hematology research was carried out in tubes with EDTA as anticoagulant. Analysis was performed using automatic analyzer Abacus junior vet 2.7 (Diatron Messtechnik GmbH, Austria) with HTI kits (USA). Immunophenotyping was carried out on automatic flow cytometer Guava easyCyte (MerchMillipore, France), using species-specific monoclonal antibodies CD3 +, CD4 +, CD19 +, CD20 +, immunoregulatory index was calculated as the ratio of CD3 to CD4. Immunoassay were carried out on microplate reader ImmunoChem 2100 (HTI, USA) with microplate Thermo-Shaker Immunochem-2200 (HTI, USA) and microplate washer Immunochem-2600 (HTI, USA) according to ELISA tests (Cusabio, China) – IgM, IgG, Il-2, Il-4, Il-6, complement components C5, C3, C4, C1q, circulating immune complex (CIC).

Approval for experiment was obtained from the Bioethics Committee of the V.M. Gorbатов All-Russian Meat Research Institute (Protocol №2). Software “STATISTICA 10.0” was used. The results are presented as Mean \pm SE. Significance of difference was tested using analysis of variance using one-way ANOVA test in conjunction Duncan's test. A probability of 0.05 was chosen as the significant level. Results are presented as M \pm SE.

RESULTS AND DISCUSSION

Hematological analysis on the 12th day of animals with IDM (1-5 groups) revealed significant lymphocytes and monocytes reduction (up to 21 % and to 33 %), due to relative granulocytes content increase (up to 57 %), red blood cells and platelets (up to 12 % and to 23 %) in relation to intact animals. This leukocytes component displacement indicated immunological crisis, confirming approximation of immunodeficiency model.

In vivo experiment results revealed that on 31th day of experiment control group rats (1) observed: hematological parameters analysis (Figure 1) showed significant reduction of lymphocytes and relative content (by 40.0 %), while granulocytes percentage increasing (up to 48.3 %); lymphocytes phenotyping revealed CD4 percentage significant reduction (in 2 times), partially offset by CD3 subpopulation content increase (by 15.9 %), CD3 / CD4 was 2.69 (more than 2 times higher than intact animals index) (Table 1) in relation to intact animals (6).

Immunoassays analysis revealed in rats serum (Group 1) significant reduction of IgG and IgM (by 13 %, $p < 0,05$), Il-2 and Il-6 (by 21 % and 14%),

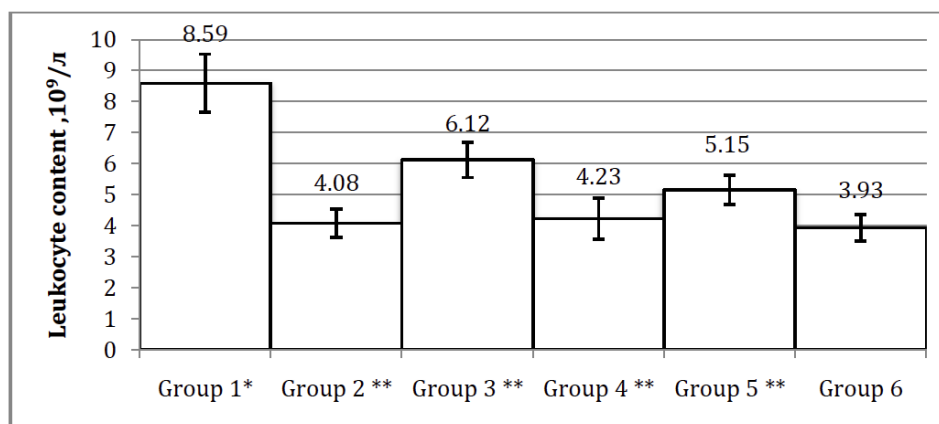


Figure 1: Leukocyte content in laboratory animals blood after experiment.

* - significant difference from intact (6) group ($P < 0,05$).

** - significant difference from control (1) group ($P < 0,05$).

complement component C1q (up to 26 %) and C4 (by 20 %), while unreliable Il-4 and CIC (by 4 % and 3 %); extension of C5 and C3 content (up to 9 % and 2 % ($p > 0,05$) - Table 2).

In experimental groups rats depending on studied extract fractions it has been shown identical changes of analyzed indicators, in varying severity, relative to control animals (1).

Hematological analysis revealed increase of lymphocytes and monocytes relative content by 18 % and 27 % ($p < 0,05$) for 2nd group (treated with complex extract); up to 13 % and 12 % ($p < 0,05$) for 3rd group (treated with fraction less than 5 kDa); up to 22 % and 31 % ($p < 0,05$) for 4th group (fraction Mm 5-30 kDa) and up to 5% for 5th group (treated with fraction more than 5 kDa), with granulocytes percentage reducing (by 20 % - in rats 2nd and 4th groups, by 7 % - in rats 3rd and 5th groups) relative to control animals (1).

Lymphocytes phenotyping showed CD3 and CD4 content significant increase: for 2nd group by 24 % and

40 %, for 3rd group by 11 % and 16 %, for 4th group by 20 % and 40.3 %, for the 5th group by 5 % and 4 % relative to control animals (1).

Immunoregulatory index CD3/CD4 decreased for 2nd group by 21 %; for 3rd group – by 13 %, for 4th group - by 11 %; for 5th group - by 3 %, relative to control animals (1).

Immunoassays analysis revealed in serum of rats 2nd, 3rd and 4th groups significantly increase IgG content by 10 %, 5 % and 16 %, respectively; IgM content by 16 %, 8 %, 24 %, respectively; Il-2 and Il-6 increased up to 10 % relative to the group 1. Il-4 and CIC content were not significantly changed in rats of 2nd, 3rd and 4th groups in relation to intact (6) and control (1) groups.

Components complement analysis in 2nd groups rats revealed increase of content C1q up to 10 % and C4 up to 13 % while decrease content of C5 by 6 % and C3 by 4 %. In group 3 rats it was marked C1q content increase by 9 %, at the same time C4 content

Table 1: Cytometric Analysis of Rats Blood after Experiment

Relative content of	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
lymphocytes, %	49,67±2,61*	61,98±4,57**	55,13±3,32**	60,55±4,24**	52,00±3,11	72,96±8,29
granulocytes, %	49,54±5,63*	36,97±7,17**	45,97±4,78	39,48±5,21**	46,12±4,78	25,63±8,67
monocytes, %	0,84±0,05*	0,96±0,19**	0,95±0,08**	1,09±0,21**	0,86±0,10	1,41±0,21
CD3, %	37,12±3,98	49,24±4,15**	41,9±4,42	46,25±3,57**	38,86±2,34	31,20±4,03
CD4, %	13,81±4,45*	23,12±3,42**	16,4±2,73	19,38±2,47**	14,33±1,77	27,15±2,94
Immunoregulatory index CD3/CD4	2,69±0,03	2,13±0,02	2,33±0,02	2,39±0,01	2,62±0,02	1,24±0,02

* - significant difference from intact (6) group ($P < 0,05$).

** - significant difference from control (1) group ($P < 0,05$).

Table 2: Immunoassay Results in Rats Serum Blood after Experiment

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Il-2, pg/ml	281,14±9,39*	308,94±7,42**	307±5,86**	309,25±4,63**	278,33±5,6	355,87±11,89
Il-4, pg/ml	25,33±1,18	26,12±1,14	26,06±1,10	26,02±1,05	25,08±1,27	26,33±1,23
Il-6, pg/ml	29,94±1,75*	32,96±1,5**	32,42±1,29**	32,96±1,11**	29,64±1,34	34,89±2,04
IgG, mkg/ml	0,479±0,080*	0,529±0,016**	0,509±0,031**	0,570±0,018	0,494±0,013	0,532±0,022
IgM, ng/ml	0,188±0,011*	0,225±0,002**	0,204±0,012	0,248±0,013**	0,140±0,011	0,237±0,022
C1q, ng/ml	1,88±0,07*	2,11±0,05**	2,06±0,04**	2,08±0,03**	1,86±0,04	2,54±0,09
C1C, mkg/ml	0,84±0,10	0,86±0,12	0,87±0,09	0,86±0,07	0,85±0,11	0,86±0,10
C3, ng/ml	16,64±1,96	17,24±1,91	17,16±1,86	16,04±1,82	16,48±2,2	17,07±2,01
C4, ng/ml	149,81±8,34	169,88±6,64**	162,91±5,36**	169,82±4,27**	151,3±5,16	187,26±10,42
C5, ng/ml	17,21±1,78*	18,16±1,59	17,76±1,48	16,23±1,34	17,37±1,62	20,92±1,96

* - significant difference from intact (6) group (P<0,05).

** - significant difference from control (1) group (P<0,05).

was increased by 9 %, while C5 and C3 decrease by 3 %. In group 4 rats it was marked increase of content C1q up to 10 %, at the same time C4 content was increased by 13 %. Moreover, it was noted C5 content decrease by 6 % and C3 content - by 4 %.

Components complement analysis in 5th group did not showed significantly different of analyzed indicators, in relation to control animals (1): data were within the error, difference did not exceed 1.5 %.

Thus, it should be noted that resulted data about immune system of rats with IDM treated by fraction up to 30 kDa represent processes subpopulation interactions differentiation, typing and T cells functional activity formation (CD3, CD4) [26, 27], occurring during acquired immunodeficiency active phase. Polypeptide compound with molecular weight up to 30 kDa, containing in mixture of immunocompetent organs extracts, are affecting as thymic immune cells (T lymphocytes) and increasing their count, and macrophages by stimulation of T-helper (CD4) by reducing other subpopulations (suppressors, killer cells) [28-30].

CONCLUSION

Immune response in rats with cyclophosphamide-induced immunodeficiency, while treatment by extract or its fractions, accompanied by cells activation, that able to neutralize negative medicine effect by glycoproteins production increasing. It represent organisms ability to resist infectious diseases. Normalization of granulocytes relative content against increased T cells content (CD3) as well as cytokines (Il-2 and Il-4) production can be an indicator of immune

system recovery and adaptive immune response. Regulatory index displacement indicates sharp immune activation and cellular and molecular components maximum reactivity as response to cyclophosphamide cytotoxic effects. Differences between experimental results in T-helper cells (CD4) activation in animals treated with the medium and low molecular weight fraction can be explained by ability of biomolecules with molecular weight up to 30 kDa (in particular isolated from the spleen, which, in its cellular structure contains key regulatory factors) to change speed cell differentiation. Significant increase of complement components content (C1q, C4) synergistically with decreasing C5 and C3 concentration indicated cycle of complementary cascade activation, moreover C5 reduction confirm functional activity of nonspecific immune defense.

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