

INFLUENCE OF THE TISSUE PREPARATION "NICA-EM" ON MORPHOFUNCTIONAL CONDITION OF A LIVER OF RATS AT NORM AND AT EXPERIMENTAL NON-ALCOHOLIC STEATOHEPATITIS.

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Abstract

The conducted research is devoted to influence of a tissue preparation "NICA-EM" on a morphofunctional condition of a liver of mammals at norm and at an experimental steatohepatitis. Results of research testify that application of a preparation at norm causes to intensification of a number of synthetic processes in a liver, thus, without alteration of morphological integrity of organ. At an experimental non-alcoholic steatohepatitis the preparation provides an expressed hepatoprotective and a metabolism-stabilizing effect. Application of a preparation conducts to preservation of morphological integrity of a liver, normalizes levels of mitotic, necrotic and binuclear cells indexes, increases intensity of apoptosis, also normalizes the level of the content of the main metabolites, characterizing a functional condition of a liver, in blood serum. Thus, it is possible to draw a conclusion on effective influence of a preparation "NICA-EM" on metabolic processes in an organism of a mammal and on its ability to correction of changes in a liver at a serious pathological state – non-alcoholic steatohepatitis.

Keywords: liver, non-alcoholic steatohepatitis, tissue therapy, chicken embryo, biogenic stimulants.

Introduction

Preparations on the basis of tissues of animals and plants find application in various branches of biology, medicine and veterinary science, beginning with ethnopharmacological practices and finishing with modern high-tech industries of pharmacology and biomedicine. Each society of the world has the traditions of application of natural bioactive agents which are now being actively studied, developed and supplemented for improvement of health care [1-5]. Modern technologies allow to improve process of receiving biologically active agents from natural raw materials, to specify the active components of preparations and mechanisms and targets of their action [6-7].

Biologically active preparations are produced from tissues of a wide range of species of plants and animals. Biological activity is peculiar for extractions from organisms of insects, mollusks, worms, vertebrates, and also vegetative and generative organs of plants [8-12]. The whole organisms, or immunocompetent organs, liver, skin, muscles, placenta and chorion, blood serum and whole blood, antlers, fragments of tissue of an embryo of various species or whole embryo completely can be feedstock for receiving biologically active agents [13-14]. One of the most promising sources for receiving the biostimulating preparations is the chicken embryo [15-18].

Biologically active preparations exist in the form of extracts, infusions, decoctions, hydrolysates, products of a bacterial fermentation, homogenates, cellular and subcellular suspensions, supernatants. 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 [21]. Effect of bioactive preparations of natural origin is caused by that they contain the natural composites similar with living cell or tissue constructs and carry out stimulation in process of functional inquiry of an organism. They are a source of the major nutrients, bioactive peptides, growth factors, and also the vitamin and mineral nutrition for an organism. The biogenous stimulators containing in natural bioactive preparations have impact on the main parties of a metabolism that is expressed in changes of metabolic conversion and energy processes of an organism [20-22]. One of bases of efficiency of the preparations of natural origin are so called biogenous stimulators and adaptogens [23-25]. These concepts are meant as group of biologically active agents of non-uniform chemical structure. It is usual to carry to them the

low-molecular organic compounds containing in living cells, in particular, organic acids (dicarboxylic and tricarboxylic acids, RNA, DNA etc.), humin substances, phospholipids, vitamins, microelements and low molecular weight peptides [26].

Besides traditional use of various extraction from living tissues, there is a special area of their application known under the name "tissue therapy". This concept includes a complex of methods allowing not only to extract biologically active agents accumulating in cells of animals and plants in process of vital activity but also to raise their contents and biochemical activity, using certain properties of living tissues. Increase of biological activity, bioavailability and improvement of pharmacological properties may be attained with various methods, but the essence of all methods consists in the stress-producing impact on a fragment of a living tissue or the whole organism inducing it to adaptation reaction - production of a complex of biologically active agents possessing a range of stimulating and adaptational effects. The continuous hypothermia, influence of chemicals and/or weak ionizing radiation, laser or ultra-violet irradiation, etc. can act as the stressor causing similar reaction [27-28].

Presence of the substances developed as a result of stress-producing influence at tissue preparation brings it to more considerable (in comparison with other methods of phytotherapy and zootherapy) increase of resistance of an organism, decreases intensity of inflammatory process, intensifies specific physiological functions of cells and tissues of an organism, stimulates growth and productivity [29].

Experiments show that extractions from living tissues quite often are effective at smoldering pathological processes of various nature - inflammatory, degenerative, atrophic, tumoral, etc., as they activates immune and regenerative functions of an organism. Being formerly only an element of traditional practices, in the modern world the natural products pharmacology shows the efficiency at eye and skin diseases, diseases of bones and joints, in treatment of burn injuries, in oncology, at treatment of diabetes and Alzheimer's disease [30-34]. Similar preparations also show the properties allowing to draw a conclusion on their applicability as adaptogens at high physical activities at athletes and manual workers and also at high psychoemotional loadings [35-40].

Derivatives of plant and animal tissues are widely applied also in veterinary science where are necessary not only large volumes and high efficiency of the applied substances, but also potential safety for the end user. Phyto – and zootherapeutic agents

find application at infectious and noncontagious diseases of farm animals, at rankling wounds, ulcers, some other diseases of skin, lungs, bones, etc. It is also important to note use of animal and plant products as stimulators at sagination of young growth and whitebaits, for increase of wool efficiency of sheep and dairy efficiency of cows, for increase of fertility and that is especially important, for decrease of mortality among young growth and breeding animals, especially for decrease in postnatal mortality [41-45]. Speaking about targets of action of preparations of tissue origin it is impossible to ignore one of the most important organs of providing a homeostasis – a liver. The role of the central link of a metabolism which is carried out by a liver causes its vulnerability to such threats as bacterial and viral infections, environment toxins, adverse factors of a diet, medicine drugs and products of their metabolism. One of presently frequent complications caused by high load of a liver is so-called non-alcoholic fatty hepatitis or non-alcoholic steatohepatitis (NASH).

Non-alcoholic steatohepatitis – the serious illness of a liver which is characterized with a fatty infiltration of liver (steatosis), leading to a considerable oxidative stress and a hepatocellular inflammation. In the absence of treatment or at incorrect therapy non-alcoholic steatohepatitis involves development of cirrhosis and a hepatocellular carcinoma [46]. Manifestations of NASH are histologically similar to an alcoholic liver disease, however this disease is observed at the people who aren't taking considerable quantities of alcohol.

Non-alcoholic steatohepatitis is believed now to be one of the most common explanations for abnormal liver chemistries in adults [47]. According to several authors, non-alcoholic steatohepatitis is the hepatic manifestation of metabolic syndrome [48-51]. Risk factors for NASH include high-fat diet, obesity, type II diabetes, hyperlipidemia, total parenteral nutrition, and the use of certain drugs, including antibiotics [52-54]. Therapy of non-alcoholic steatohepatitis may be proceeding in various ways. Pharmacological correction includes application of antioxidants, cytoprotective drugs and resolvents. Besides, extremely important part of correction of this state is normalization of metabolic processes, in particular a lipid metabolism [55-56].

In therapy of non-alcoholic steatohepatitis are quite often applied preparations of natural origin possessing antioxidant, anti-obesity, hepatoprotective effects, and also used as a diet source of necessary nutrients, vitamins, supplements and polyunsaturated fats [57].

Based on the data described above, we, in turn, assumed potential adaptogenic, hepatoprotective and a metabolism-stabilizing effect of a tissue preparation of an embryonic origin "NICA-EM", received from embryonic tissues of chickens, on liver of mammals at norm and on model of an experimental non-alcoholic steatohepatitis. Characteristics of the substances which are a part of this preparation allowed us to expect it positive influence on tissues of a liver 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 living cell or tissue (Table 1).

In essence "NICA-EM" constitutes a tissue preparation which properties are reached due to a complex of the manipulations directed on activation of biochemical processes in embryonic tissues that allows to increase in a substratum the level of content of biologically active agents, including due to formation of biogenous stimulators. The main feature of technology of this biological product is use of method of high pressure homogenization (HPH). The used technologies characterize a preparation as the low-molecular nanostructured activated composition. The way of receiving a preparation of "NICA-EM" passed patent search and is presented to Federal Service for Intellectual Property (Rospatent), the request No. 2014139637 of 30.09.2014.

The complex 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.

Materials and methods

Animals

Male Wistar Albino rats of body weights ranging from 170 g to 200 g were used in the study. The animals were fed with standard pellet diet and water ad libitum. They were maintained in controlled environment (12:12 h light/dark cycle) and temperature (30±2°C). 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.

Modeling of NASH

The Group III (n=20) and Group IV (n=20) consisted

of the rats who were on the special hyper high-calorie hepatogenous diet sated with animal fats, within 60 days. We used the fatty loading with melted beef fat, which made about 20% of the general structure of a diet, increasing the general caloric content of a diet. In addition, from the 30th day of research tetracycline in a dose of 30 mg per 1 kg of body weight was injected intraperitoneally once daily for 10 days.

Treatment Design

Experiment on modeling and treatment of non-alcoholic steatohepatitis was carried out within 60 days. The 80 animals (Male Wistar Albino rats) were randomized and divided into four groups.

Group I, including 20 animals, served as intact control.

Animals of the Group II (n=20) were hypodermically injected with "NICA-EM" in a dose of 0,1 ml per 1 kg of body weight for 15th, 30th and 45th days of research.

At animals of the Group III (n=20) an experimental NASH by the above described technique was modelled.

At rats of the Group IV (n=20) experimental non-alcoholic steatohepatitis was also modelled, but to them was hypodermically injected "NICA-EM" in a dose of 0,1 ml per 1 kg of body weight for 15th, 30th and 45th days of research.

On the 61st day of research animals were sacrificed in carbon dioxide chamber.

Biochemical analyses

Level of glucose, total protein, albumin, creatinine, triglycerides, cholesterol, total bilirubin, of an alanineaminotransferase (ALT), aspartataminotransferase (AST) and alkaline phosphatase in serum of blood were investigated by means of the biochemical StatFax3300 analyzer (USA) with sets of Spinreact firm (Spain).

Histopathological analysis

Small portions of liver 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, including determination of mitotic, apoptotic, necrotic and binuclear cells indexes in liver. At hematoxylin and eosin stained sections were determined mitotic and necrotic cells. At sections stained by methylene blue-azure II with after-staining by fuchsine were determined apoptotic cells. Visualization was

performed using a light microscope Nikon Eclipse 80i at 900 × magnification. Study was made at 5 fields of view on each section.

Apoptotic index was calculated by the formula:

$$AI=N_a/N\times 100\%,$$

where N_a - the number of apoptotic cells; N - total number of cells in the test population.

The mitotic index was calculated by the formula:

$$MI=N_m/N\times 100\%,$$

where N_m - number of mitosis; N - total number of cells in the test population.

Necrotizing index calculated by the formula:

$$NI=N_r/N\times 100\%,$$

where N_r - number of necrotic cells; N - total number of cells in the test population.

Binuclear hepatocytes index (relative number of amitotic cells) was calculated by the formula:

$$BI=N_b/N\times 100\%,$$

where N_b – number of binuclear cells, N - total number of cells in the test population. All studies was made for 10 fields of view on each section.

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 deviation unless noted as different. Difference between the control and experimental groups was analyzed using Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

At the analysis of microslides of a liver it is established that the structure of a liver of rats of the Group I accords with norm. In a liver of rats of the Group II at the morphological analysis it is also noted absence of essential differences from norm. In addition, in a part of hepatocytes we note moderate proteinaceous graininess which in the absence of pathological changes in organ is the evidence of enhancement of protein synthesis in this organ.

The microscopic analysis also revealed that histologic structure of a liver of rats of the Group III underwent the considerable changes expressed in increase of the area of the necrotizing sites (14-17% of total area of the studied sites of body) surrounded with lymphocytic-macrophagal infiltrate, violation of a frame structure of segments of a liver. The trabecularity of a liver is not expressed, hepatocytes settled down randomly, sinusoids aren't traced. Expansion of large portal paths due to growth of a stroma and filling with its inflammatory infiltrate was noted. Some portal paths were with necrotic changes of vessels. Walls of such vessels were poorly differed and in certain cases were broken. The destruction of biliary channels accompanied with the big centers of inflammatory infiltrate was also observed.

In periportal and central zones of liver lobules sinusoidal capillaries were dilated, the central veins are abundantly filled with blood. Round of bilious channels the insignificant mononuclear infiltration was noted. Fatty degeneration of hepatocytes was of the form of an atomized and globular vacuolization. It was also revealed the moderate intralobular infiltration of a liver parenchyma and a hypertrophy of hepatocytes.

At microscopy of a liver of rats of the Group IV it wasn't revealed any essential pathomorphological changes in organ. At the same time it should be noted that in large part of cells the moderate acidophilic stippling was revealed. But, however, the necrotizing cells, vacuolated hepatocytes or cells with other signs of dystrophic changes were not revealed at a significant amount, that allowed us to refer an emergence of such granularity to an initial stage of cloudy degeneration which, as we know, at the initial stage of development is a reversible process (Figure 1).

At the analysis of the studied indexes (apoptotic, necrotic, mitotic and binuclear hepatocytes indexes) the essential decrease in mitotic and apoptotic activity at reduction of quantity of binuclear cells and increase of the necrotic index is established at the group with untreated NASH (Group III). Thus, in a liver of rats of other groups the studied parameters don't differ authentically from parameters of intact animals (Table 2), except for the level of apoptotic activity in a liver of animals of the experimental Group IV.

Results of biochemical researches testify to considerable changes of level of the studied substances in blood of rats with an experimental non-alcoholic steatohepatitis (Table 3).

Thus, in blood of animals of the Group III we noted

an increase of level of albumin at unauthentic increase in level of the total protein. At the same time, for rats of the experimental Group IV the increase in level of triglycerides and alkaline phosphatase is noted.

Discussion

The conducted research testifies that the tissue preparation "NICA-EM" at application according to the scheme described by us have a certain influence on a morphofunctional condition of a liver of white rats at a condition of non-alcoholic steatohepatitis, while in a healthy organism an application of the preparation leads to intensification of a number of biochemical processes in a liver, without affecting morphological integrity of the organ.

In particular, by results of the morphological analysis of a liver of the healthy animals treating with "NICA-EM" by us it is established that the liver structure in this case doesn't differ from norm. At research of microslides of a liver of animals of this group (Group II) it wasn't revealed the expressed influence on intensity of mitotic, apoptotic or necrotic activity of hepatocytes. Also it isn't noted influence on quantities of binuclear cells in the organ. Besides, at application of "NICA-EM" for healthy animals we found signs of the intensified synthesis of proteins in hepatocytes.

As a result of biochemical researches by us it is established that application of "NICA-EM" leads to some increase of level of the total protein in blood serum of rats, and also to reliable increase of level of albumin.

The expressed effect on a morphological and functional condition of a liver of "NICK-EM" manifests at experimental model of non-alcoholic steatohepatitis. In particular, application of a preparation preserve morphological integrity of organ, significantly reducing the level of necrotic activity in comparison with organs of animals with experimental model of NASH. At the same time rats of the experimental Group IV have a higher level of apoptotic activity. It testifies that in a liver of rats with NASH at application of "NICA-EM" apoptosis, but not a necrosis represent the leading type of cellular death.

The studied biochemical parameters of blood serum of rats of experimental Group IV, excepting the level of triglycerides and alkaline phosphatase, don't differ authentically from parameters of intact animals, that shows considerable influence of "NICA-EM" on maintenance of a homeostasis of an organism at experimental NASH.

Thus, the conducted research allows to claim that

tissue preparation "NICA-EM" intensifies protein synthesis function of an intact liver of mammals, without causing others morphofunctional changes at this major link of providing a homeostasis. At experimental NASH of "NICA-EM" provides the expressed hepatoprotective effect which is shown in normalization of level of a number of important metabolites, and also in preservation of morphological integrity the organ.

As non-alcoholic steatohepatitis is a multifactorial metabolic disease, normalization of metabolism and providing of reasonably balanced nutritional care is an essential factor of prevention and correction of this life-threatening condition [58]. The reason of NASH may be as at different-reason deviations in lipid metabolism, as well as at the violation of a mineral exchange (in particular, copper metabolism) having a certain influence on lipid balance [59]. Official and dietary remedies applied at correction of NASH and at its prophylaxis serve to prevention of development of a steatosis and its consequences – hepatic lipid peroxidation, necroinflammation, fibrosis, etc., and also provide additional intake of the elements involved in structurally functional and metabolic processes in organism (60-62]. The preparation "NICA-EM", which is made of biologically activated tissues of a chicken embryo and constitutes a complex aggregate of organic and inorganic substances, at application against the modelled non-alcoholic steatohepatitis showed reliable improvements of a morphological condition of a liver, normalization of level of its main metabolites and the expressed homeostasis-stabilizing effect. Thus, it is possible to draw a conclusion on efficiency of influence of this tissue preparation on metabolic processes in an organism of a mammal and on its ability to correction of a condition of a liver at serious pathological state - non-alcoholic steatohepatitis .

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Conflict of interest

The authors declare that there is no conflict of

interests regarding the publication of this paper.

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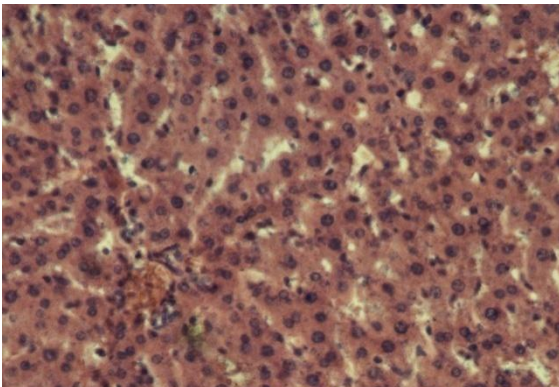


Figure 1. Liver of intact rat (Group I). Staining with H&E, $\times 400$.

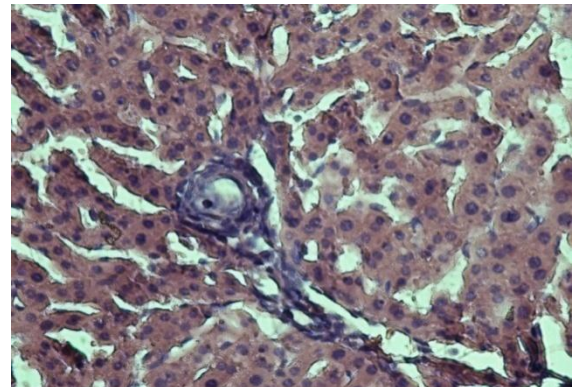


Figure 2. Liver of rat of Group II. Staining with H&E, $\times 400$.

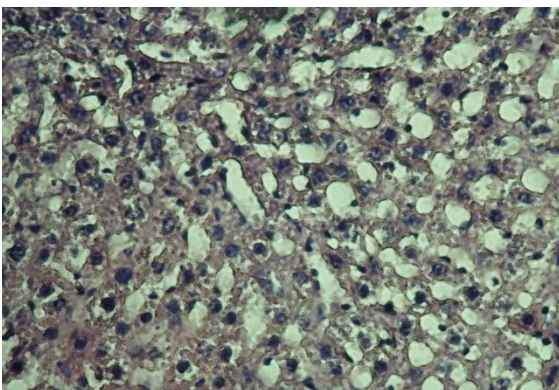


Figure 3. Liver of rat of Group III. Staining with H&E, $\times 400$.

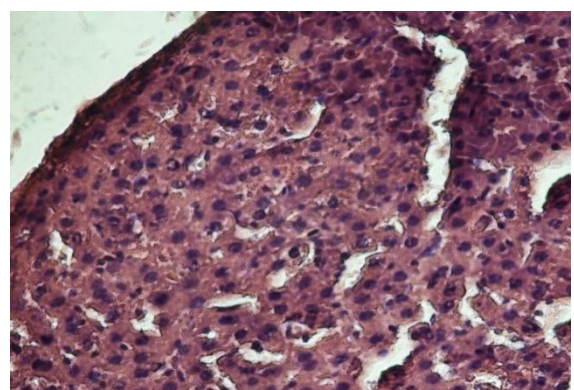


Figure 4. Liver of rat of Group IV. Staining with H&E, $\times 400$.

Table 1. Physical and chemical properties of tissue preparation "NICA-EM" received with use of HPH.

Name of parameter	Characteristic of preparation received with use of HPH
Appearance	Yellowish opalescent fluid
pH	6.0
Zeta-potential	-8.20 mV
Particle size (mean diameter, nm – quantity, %)	27.92 d nm – 12.0 % 82.31 d nm – 55 % 623.8 d nm – 32.9 %
Total protein concentration, g/l	16.4
Total DNA concentration, mg/l	3.830
Total RNA concentration, mg/l	20.00
Characteristic absorption spectrum in the wavelength range 190-840 nm	max 215 nm. 228 nm. 275 nm
Lysin, %	0.16
Methionine, %	0.07
Arginine, %	0.12
Histidine, %	0.08
Leucine, %	0.15
Isoleucine, %	0.07
Phenylalanine, %	0.11
Tyrosine, %	0.06
Valine, %	0.1
Threonine, %	0.1
Glycine, %	0.07
Asparaginic acid, %	0.2
Glutamic acid, %	0.28
Alanine, %	0.11
Serine, %	0.14
Total lipids, g/l	7.0
Glucose, g/l	5.5
Calcium, g/l	0.072
Phosphorus, g/l	0.41
Cuprum, µg/l	4.0
Manganese, µg/l	1.2

Table 2. Effect of "NICA-EM" on apoptotic, necrotic, mitotic and binuclear hepatocytes indexes in a liver of rats.

	MI,%	AI,%	NI,%	BI,%
I group (n=20)	6.41±0.37	1.87±0.22	0.70±0.09	6.59±0.38
II group (n=20)	7.15±0.41	1.69±0.31	0.93±0.20	7.41±0.25
III group (n=20)	2.41±0.36***	0.71±0.20***	14.14±2.56***	2.15±0.35***
IV group (n=20)	6.91±0.48	3.12±0.30*	1.21±0.25	7.82±0.69

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

Table 3. Effect of "NICA-EM" on biochemical parameters of blood serum.

	I group (n=20)	II group (n=20)	III group (n=20)	IV group (n=20)
Glucose, umol/L	6.58±0.52	7.28±0.44	11.84±1.10***	7.61±1.1
Total protein, g/L	75.24±5.23	83.99±5.88	76.52±4.48	74.59±4.16
Albumin, g/L	32.52±2.86	41.88±3.44*	29.94±2.63	37.80±2.86
Creatinine, umol/L	66.60±7.11	69.88±8.12	68.20±4.80	68.0±7.80
Triglycerides, mmol/l	1.21±0.10	1.19±0.24	3.63±0.38***	1.42±0.36*
Cholesterol, mmol/l	2.40±0.21	2.61±0.51	4.88±0.43***	2.92±0.42
Total bilirubin, umol/L	1.24±0.31	1.26±0.30	1.30±0.28	1.19±0.28
AST, U/L	153.81±14.60	168.55±8.77	324.90±28.40***	200.85±18.88
ALT, U/L	123.37±12.87	140.0±12.10	528.10±56.02***	183.20±20.50
Alkaline phosphatase, U/L	941.0±59.60	1081.56±138.50	1688.0±200.89***	1255.0±140.7*

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