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Histomorphometric Changes in The Spleen of Rats Under the Systematic Exposure TO +G_X-Acceleration.

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ABSTRACT

The present experimental study is a continuation of the complex evaluating of the organism's reactivity to the gravitational overloads, and especially, immune organs to evaluate age-related histomorphometric features of the structure of the rat's spleen under the systematic and repeated exposure to +G_x-acceleration of 9G as well as to determine a possible corrective potential of *Arginine glutamate*, a pharmaceutical commercially available drug product, consisting of L-arginine and L-glutamic acid. Experiment was carried out on male Wistar rats (144 animals) using experimental centrifuge. Changes in spleen were evaluated using histomorphometric method. It was concluded that gravitational overloads alter the relationship of the structural components of the spleen, and especially its white pulp component. The most pronounced changes can be seen in 2-months old rats, especially following 10-day exposure. Use of *Arginine glutamate* in experiments with all three age groups of rats exposed to +G_x-acceleration led to partial lowering of morphological and functional disturbances in the spleen and some immune correction in 10-day experiments, but not in 30-day experiment.

Keywords: histomorphometric, arginine glutamate, +G_x-acceleration

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INTRODUCTION

An evolution of all the living organisms is inextricably associated to the gravity factor, which determines the weight of the physical body. So, ontogenetic development of all the organ systems (i.e. musculoskeletal system, cardiovascular, nervous, immune and other) is directly affected by the physical effects of gravitational fields [1-3]. However, the role of the gravitational forces on the organism systems is studied extremely insufficiently, because of constant both intensity and direction of gravitation during phylogenesis and ontogenesis. On the other hand, recent scientific and technological progress, coupled with the development of high-speed and highly maneuverable aircrafts, poses necessity to assess the influence of the above-threshold acceleration on different systems and organs of the organism. Until recently, space flight has been limited to government-sponsored astronauts, cosmonauts, and several citizens who could afford the multimillion dollar cost. However, we are now embarking on a new era in which many people will have the opportunity to travel into space. So, the emergence of the commercial human space flight industry will significantly affects the field of aerospace medicine [4]. Additionally, modern science and technology raises the problem of the study of adaptation to different environmental factors. Area of the particular interest is the long-term impact of acceleration and space flight factors on the human and animal organisms (acceleration, vibration, weightlessness, etc), along with development of the activities to enhance resistance of the organism to extreme conditions, including hypergravity. Different direction accelerations may occur in various periods of the flight and may pose a threat to the life and activities of the astronauts.

The reaction of the organism to environmental stimuli largely depends on the morphological and functional state of one of the most important system, namely the immune system, within the tight interactions with nervous and endocrine systems. Although there were published some articles devoted to the systemic exposure of acceleration [5-7], there are almost no data about age reactivity of the immune system to the systematic gravitational overloads. However, authors previously published some experimental data regarding effects of G_x-acceleration of the structural components of the thymus gland [8]. It was shown that thymus of the mature rats under systematic exposure to $+G_x$ acceleration is characterized by the marked structural transformation with the events of accidental involution and microcirculatory disturbances caused by the systematic action of gravitational forces. Such changes were considered as potentiated by the age-related involution. Proliferation of the connective tissue structures, replacement of the parenchyma by the adipose tissue, and degeneration of the epithelial component were observed. Histoarchitecture of the thymus was characterized by typical stress-induced changes, similar to changes revealed at the experiments with glucocorticoids administration [9-11]. Providing the above mentioned data, the present experimental study is a continuation of the complex evaluating of the organism's reactivity to the gravitational overloads, and especially, immune organs. Taking into account changes in the thymus, the purpose of the present study is to evaluate age-related histomorphometric features of the structure of the rat's spleen under the systematic and repeated exposure to $+G_x$-acceleration of 9G. The choice of the spleen as an object of the research study is dictated by the uniqueness of this organ in terms of its structural and functional characteristics. The clear structural and functional separation of T- and B-dependent zones, as well as wide variety of cellular composition and functional capabilities of antigen-dependent differentiation of lymphocytes [12] allows evaluating transformations of the spleen under the exposure of gravitational overloads using histomorphometric methods.

On the other hand, one of the important aspects is studying the methods of the protection against G-force accelerations, because pilots of the supersonic aircrafts are exposed to intensive and long-term gravitational accelerations about $+G_x/-G_x$ of 9 to 12. However, preventive methods used in modern aerospace medicine do not have sufficient effect, as proven by relatively high morbidity values among pilots [13-15]. So, the secondary task is to determine a possible corrective potential of *Arginine glutamate*, a pharmaceutical commercially available drug product, consisting of L-arginine and L-glutamic acid. The expected effect of this pharmaceutical corrector directed against systematic exposure to $+G_x$ acceleration seem to be implemented by its membrane stabilizing and antioxidant activities [16, 17].

MATERIAL AND METHODS

This experimental research study was carried out on male Wistar rats. Total number of experimental animals was 144 rats. Rats of three age periods were used: 2-months old rats (initial body weight is 120 to 130 g), 6-months old rats (initial body weight is 200 to 220 g) and 12-months old rats (initial body weight is 260 to

280 g). Using of these age periods need to evaluate age-related changes in spleen structure and reactivity under the exposure to $+G_x$ acceleration in juvenile, mature and pre-senile periods of ontogenesis. All the animals were divided into four groups (2 control and 2 experimental) taken from a closed colony. Animals of the 1st experimental group (E1) were subjected to $+G_x$ acceleration of 9G for 10 minutes per day for 10 or 30 consecutive days. Rats of the 2nd experimental group (E2) also were exposed to the similar hypergravity on the background of concomitant administration of *Arginine glutamate* at a dose of 100 mg per kg of body weight. Rats of the 1st control group (C1) were not exposed to acceleration, but they were in the same type of container, placed on the outer surface of the centrifuge. Rats of the 2nd control group (C2) also were not subjected to $+G_x$, but they were administered sterile saline in an equivalent dose 30 minutes before onset of the experiment. Group C1 was used to compare with group E1 results, and C2 results were compared to E2. $+G_x$ acceleration (direction from back to chest) was modeled using the experimental centrifuge C-2/500 with a 0.5 m radius and with a working range from 1 to 50G. The magnitude of the modeled $+G_x$ acceleration was 9G. Gradual onset rate and gradual decline rate were 1.4 to 1.6G/sec and 0.6 to 0.8G/sec, respectively. Experimental rats were subjected to centrifuging on a daily basis (for 10 or 30 consecutive days) at the same time within 10 minutes. Model was implemented by means of three intervals, each lasting 3 minutes with an interval of 30 seconds between. Such parameters of the experiment were chosen according to the routine hypergravity exposure during high-speed and highly maneuverable flights [4].

Arginine glutamate was chosen due to its antioxidant properties, ability to inhibit lipid peroxidation, and to maintain systemic and local hemodynamic parameters due to the nitric oxide formation from L-arginine [18].

Animals were sacrificed using excess of anaesthesia on the next day after the last centrifuge run (following 10 or 30 days for acute and chronic experiment, respectively) and the spleen was removed, measured and weighed. Entire experiment was conducted in accordance with current bioethical standards. Spleen was subsequently fixed in 10% buffered formalin solution over a period of 2 to 3 days. Following fixation, the tissues were passed through alcohols, xylol and paraffin per laboratory standards according to GLP. Each spleen specimen was sectioned serially at 3-4 µm. Sections used for histological observations were stained by the hematoxylin and eosin technique, as well as using van Gison and azure-II-eosin methods. Detailed histological structure was studied using Olympus CX31 (Olympus, Japan) cytomorphometric complex hardware. Obtained images were processed using freeware ImageJ [19] to calculate relative areas of red pulp, white pulp and connective tissue stroma components of the spleen. Within the white pulp relative areas of lymphatic follicle (mostly, B-dependent zone) and periarteriolar lymphocyte sheath (mostly, T-dependent zone) were calculated.

Data distribution was normal (according to the results of Kolmogorov-Smirnov test [20]). Thus, parametric statistical methods were used such as arithmetic mean and standard error of mean (SEM) ($M \pm m$). Obtained quantitative data were statistically processed using Student's t-test ($p < 0.05$).

RESULTS

The spleen of 2-months old rats following 10-day exposure to $+G_x$-acceleration was characterized by the significant increase of the white pulp (on 63.56% [$p<0.05$]) due to reduction of red pulp (on 18.40% [$p<0.05$]) compared to the control (C1). Relative area of the connective tissue components did not differ significantly from the control. In turn, white pulp in experimental spleen was characterized by reduction of lymphoid follicles (on 13.95% [$p<0.05$]) and increase in periarteriolar lymphocyte sheath on 11.77% [$p<0.05$] compared to control. Decreasing of relative area of lymphoid follicles was primary due to less pronounced germinal centers (on 30.37% [$p<0.05$] compared to control). In experiments using *Arginine glutamate*, redistribution of the spleen structural components had the same character as in control group (C2), as well as in the 1_{st} experimental group, but was less pronounced and characterized by the tendency to be similar to the control group. The white pulp was characterized by an increase in the relative area of lymphoid follicles and decrease of periarteriolar lymphocyte sheath area (see Table). It should be noted that distribution of structural zones in the lymphoid follicles was characterized by a significant increase in the proportion of germinal center (on 70.13% [$p<0.05$]) and a marked decrease in the marginal zone area (on 15.61% [$p<0.05$]) compared to the control.

Increase of the hypergravity exposure up to 30 sessions also resulted in significant increase in the relative area of the white pulp on the background of the reduced red pulp (see Table). At the same time area of the connective tissue components (capsule, trabeculae, etc.) was significantly increased (on 20.65% [$p<0.05$]) compared to the control. White pulp was characterized by increase in number of lymphoid follicles without germinal centers, resulting in shift of the proportion between periarteriolar lymphocyte sheath and lymphoid follicles to the last one. Parallel to this, as well as following 10-days exposure, area of germinal centers within lymphoid follicles was decreased compared to the control (on 28.01% [$p<0.05$]). Rats subjected to 30-days exposure to $+G_x$-acceleration on the background of *Arginine glutamate* use were characterized by ratios of red / white pulp that are opposite to the results of hypergravitational exposure without correction. These transformations were characterized by a significant decrease in white pulp area with an increase in red pulp area. At the same time, percentage area of connective tissue components was remained at the level of the hypergravity without correction. Values of relative areas of periarteriolar lymphocyte sheath and lymphoid follicles within the white pulp did not differ from the control (see Table). However, area of germinal centers in lymphoid follicles compared to control was characterized by more significant decrease (on 40.46% [$p<0.05$]) than in experiments without correction.

Spleen of 6-months old rats following 10-day exposure to gravitational overloads was characterized by a slight but significant increase in area of white pulp and connective tissue components on the background of decrease of red pulp compared to control. The ratio of lymphoid follicles and periarteriolar lymphocyte sheaths within the white pulp remained at the level of the control values (see Table). Also, relative area of germinal centers in lymphoid follicles was decreased by 14.98% [$P<0.05$]. In the group with correction, redistribution of the white and red pulp was shifted to the red pulp and connective tissue components (10.37% and 11.36%, respectively [both $P<0.05$]) with the parallel decrease in white pulp (by 21.02% [$P<0.05$]) compared to control. White pulp was present by less area of lymphoid follicles and more area of periarteriolar lymphocyte sheaths (see Table). At the same time, redistribution of the structural zones within lymphoid follicles was non-significant compared to control. After 30-day exposure values of relative areas of white pulp, red pulp and connective tissue stroma components were actually matched control (see Table). However, white pulp was characterized by marked decline in the area of lymphoid follicles (by 16.42% [$P<0.05$]) and increase in periarteriolar lymphocyte sheaths area (by 11.58% [$P<0.05$]) compared to control. Also, total area of the marginal zone within lymphoid follicles was increased significantly (by 14.10% [$P<0.05$]). The same exposure on the background of *Arginine glutamate* use resulted in redistribution of white and red pulp towards decrease in white pulp relative area (by 13.95% [$P<0.05$]) compared to the control. Lymphoid tissue was characterized by less pronounced changes from the control (such as decrease in lymphoid follicles and increase in periarteriolar lymphocyte sheaths) compared to the experimental group without correction (see Table). Values of relative areas of the functional zones within lymphoid follicles were close to the control.

Table – Percentage (%) areas of the structural components of the spleen of rats under the systemic exposure to $+G_x$-acceleration (M ± m)

Groups	Red pulp, %	White pulp, %	Capsule and trabeculae, %	Lymphoid follicles, % in white pulp	Periarteriolar lymphocyte sheath, % in white pulp
2-months old rats (n=48)					
10 days					
I (A)	59.64±0.15 *	34.46±0.15 *	5.89±0.09	39.38±0.15 *	60.62±0.15 *
II (A + AG)	68.04±0.15 *	25.89±0.12 *	6.07±0.09 *	52.41±0.12 *	47.59±0.12 *
III (C1)	73.10±0.11	21.07±0.09	5.83±0.07	45.76±0.09	54.24±0.09
IV (C2)	71.79±0.24	22.50±0.19	5.71±0.10	47.62±0.19	52.38±0.19
30 days					
I (A)	57.68±0.15 *	35.71±0.12 *	6.61±0.06 *	44.00±0.12 *	56.00±0.12 *
II (A + AG)	72.14±0.15 *	21.43±0.09 *	6.43±0.06 *	41.67±0.09	58.33±0.09
III (C1)	70.71±0.11	23.81±0.11	5.48±0.07	40.00±0.11	60.00±0.11
IV (C2)	67.50±0.24	27.14±0.24	5.36±0.10	42.11±0.24	57.89±0.24
6-months old rats (n=48)					
10 days					
I (A)	59.29±0.13 *	34.05±0.11 *	6.67±0.04 *	41.96±0.11	58.04±0.11
II (A + AG)	67.40±0.12 *	26.23±0.14 *	6.36±0.05 *	32.67±0.09 *	67.33±0.14 *
III (C1)	61.96±0.17	31.79±0.15	6.25±0.06	42.13±0.15	57.87±0.15

IV (C2)	61.07±0.24	33.21±0.24	5.71±0.10	40.86±0.24	59.14±0.24
30 days					
I (A)	64.11±0.17	28.93±0.15	6.96±0.06	34.57±0.12 *	65.43±0.15 *
II (A + AG)	67.50±0.17 *	26.43±0.12 *	6.07±0.06 *	42.57±0.12 *	57.43±0.12 *
III (C1)	63.93±0.20	28.93±0.17	7.14±0.09	41.36±0.17	58.64±0.17
IV (C2)	62.50±0.24	30.71±0.29	6.79±0.14	45.35±0.29	54.65±0.29
12-months old rats (n=48)					
10 days					
I (A)	65.71±0.13 *	28.33±0.13 *	5.95±0.04 *	38.24±0.13 *	61.76±0.11
II (A + AG)	68.78±0.16 *	24.49±0.16 *	6.73±0.06 *	43.33±0.16 *	56.67±0.13 *
III (C1)	71.07±0.11	22.62±0.13	6.31±0.04	37.89±0.07	62.11±0.13
IV (C2)	70.36±0.24	23.21±0.14	6.43±0.10	40.00±0.14	60.00±0.14
30 days					
I (A)	67.62±0.16 *	25.24±0.13 *	7.14±0.07 *	36.32±0.09 *	63.68±0.13 *
II (A + AG)	71.95±0.14 *	21.56±0.12 *	6.49±0.07 *	24.70±0.09 *	75.30±0.12 *
III (C1)	70.71±0.16	22.62±0.07	6.67±0.07	40.00±0.09	60.00±0.07
IV (C2)	69.64±0.19	24.64±0.14	5.71±0.10	43.48±0.14	56.52±0.14

Abbreviations: A – +Gx-acceleration; AG – *Arginine glutamate*; C1 – control group 1; C2 – control group 2; * – P <0.05 compared to the respective control.

Histological analysis of spleen in age group of 12-months old rats exposured to 10-day $+G_x$-acceleration showed an increase in white pulp area (by 25.26% [P<0.05]) on a background of a slight decrease in red pulp and connective tissue stoma compared to control. Lymphoid follicles and periarteriolar lymphocyte sheaths areas within the white pulp were practically unchanged in comparison with the control data (see Table). However, there was marked increase in the relative area of germinal centers area (by 16.98% [P<0.05]) in lymphoid follicles. In experiments with the correction changes from the control in functional zones of lymphoid follicles were mostly opposite to the results in the group of hypergravitational exposure without correction, but were less pronounced. Zones within the white pulp were redistributed in favor of a moderate increase in lymphoid follicles area (see Table). Finally, morphometric analysis of rat's spleen after 30-day duration of the experiment showed an increase in the relative area of white pulp (by 11.58% [P<0.05]) and connective tissue components (by 7.14% [P<0.05]) compared to control. At the same time, there was a redistribution of the white pulp zones towards decreasing the proportion of lymphoid follicles while increasing periarteriolar lymphocyte sheaths area (see Table). The total area of lymphoid follicle was characterized by a significant decrease in the relative area of germinal centers (by 30.32% [P<0.05]). In experiments with *Arginine glutamate* ratio of white and red pulp was characterized by a decrease in the relative area of white pulp (by 12.52% [P<0.05]) on the background of a slight increase in red pulp and connective tissue elements. Lymphoid tissue, compared to the respective control, was characterized by a marked decrease in the relative area of lymphoid follicles with an increase in periarteriolar lymphocyte sheaths compared to hypergravitational exposure without correction (see Table). Parameters of the functional zones within lymphoid follicles reliably corresponded to control values.

DISCUSSION AND CONCLUSION

Analyzing the obtained results, it can be concluded that repetitive gravitational overloads alter the relationship of the structural components of the spleen, and especially its white pulp component. The most pronounced changes can be seen in 2-months old rats, especially following 10-day exposure. An increase in relative area of lymphoid parenchyma tissue and its T-dependent zones is apparently provided by an active migration of lymphocytes from the thymus. Also, it indicates on a severe stress reaction to systemic hypergravity effect. On the other hand, increase in white pulp and relative area of lymphoid follicles following 30-day exposure due to the appearance of follicles without germinal centers can be regarded as a compensatory response of lymphoid tissue with increasing multiplicity of stress exposure. 6-months old rats were the most stable to the systematic $+G_x$-acceleration influence. In both terms of observations, changes in the structural components of the spleen were less pronounced and much less different from the control, indicating the formation of persistent adaptation mechanisms. Lymphoid tissue reaction in 12-months old rats was more pronounced than in 6-months old rats, which seems to be explained by the increasing role of the spleen in maintaining the immune status against the background of an age involution of the thymus. With increasing exposure duration up to 30 sessions, signs of immunosuppression were clearly defined, as evidenced by a decrease in white pulp and lymphoid follicles relative areas, including decrease in area of

germinal centers. Taking into account scanty published data, obtained results are in some correlation with recent experimental researches carried out on male C57/b16 mice aged 19-20 weeks after 30-day-long space flight [21]. Grigorenko DY et al. concluded that after a spaceflight, as compared with ground-based experiment, the changes of cell composition were less expressed in periarteriolar lymphocyte sheaths than in the in the germinal centers of lymphoid nodules. Also, it was shown that periarteriolar lymphocyte sheaths are more stable morphological zones, while the germinal centers of lymphoid nodules in the spleen are specific "target zones", most sensitive to a variety of factors of a space flight [22].

Use of *Arginine glutamate* in experiments with all three age groups of rats after 10-day exposure to $+G_x$-acceleration led to partial lowering of morphological and functional disturbances in the spleen and some immune correction that was manifested by approximation of relative indicators to the control data. Longer use of *Arginine glutamate* in experiments with 30-day exposure resulted in a decrease in most of the studied parameters below control data. Regardless of rat's age, inhibition of lymphoid tissue in the form of white pulp reducing, decline in the percentage areas of lymphoid follicles and its germinal centers was observed, indicating a decrease in lymphocyte migration from the bloodstream into areas of white pulp and suppression of lymphocytopoiesis. Also, spleen was characterized by increased amount of connective tissue components, which is a manifestation of compensatory and adaptive changes in the connective tissue and walls of the blood vessels. Exactly, such changes may be regarded as a morphological manifestation of increasing hypoxia and injury of the loose connective tissue stroma on a background of repeated hypergravity exposure. Thus, based on the results of present study, use of *Arginine glutamate* for the correction of structural and functional changes in spleen resulted from systematic effects of gravitational accelerations, can be justified only for 10 days. Longer use of *Arginine glutamate* (30 days) may aggravate the morphological and functional state of the spleen and thereby reduce the body's resistance to stress.

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