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ORIGINAL ARTICLES

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HISTOULTRASTRUCTURAL FEATURES OF THYMOCYTES DUE TO THE IMPACT OF THE EXPERIMENTAL GENERAL DEHYDRATION OF A MILD DEGREE

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ABSTRACT

The aim: The objective of the current study was to reveal ultrastructural changes in rats' thymocytes in experimental data in conditions of mild general dehydration.
Materials and methods: The study was conducted on 20 non-linear adult male laboratory rats weighing 150-170 g. Histological and semi-thin slides of the thymus were prepared according to the required guidelines.
Results: On average, in the cortical zone of the thymus, there was decreased cellularity by 13.4% ($p<0.001$), while in the medulla zone this indicator turned out to be unreliable - 5.5% ($p=0.19$), compared to the indicators in animals of the control group. The study showed that a slight degree of general dehydration of the body causes ultrastructural changes in the thymus and is accompanied by a cell-mediated response of the central link of immunogenesis and results in morphological changes in the thymus, which are atrophic in nature with a typical pattern of remodeling of the organ's microstructure, which corresponds to cellular aging and the associated sign of accelerated involution.
Conclusions: General dehydration of a mild degree in the experiment is accompanied by a cell-mediated response of the central link of immunogenesis and results in morphological changes in the thymus, which are atrophic in nature with a typical pattern of remodeling of the organ's microstructure, which corresponds to cellular aging and the associated sign of accelerated involution.

KEY WORDS: Rat, thymus gland, histology, ultrastructure, experiment

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INTRODUCTION

The state of water homeostasis of the organism is crucial for maintaining its structures and the appropriate level of physiological functions. Mild dehydration can lead to several side effects that can occur covertly or with little manifestation. The problem of dehydration is expected to become increasingly relevant in connection with climate change, where more and more people will face the need to prevent and overcome the effects of heat stress, the emergence of new diseases, prevention, and treatment of which will require special attention to adequate hydration [1-5].

Maintaining the optimal hydration status of the body throughout life is a prophylaxis measure to prevent the development of age-related degenerative disease and a necessary condition for the normal functioning of the immune system.Hypohydration is known to increase plasma sodium concentrations and increase the expression of Nuclear Factor of Activated T-cells 5 (NFAT5) in many tissues, including the liver, thymus, spleen, and kidneys. NFAT5, originally identified as a key transcription factor involved in maintaining cellular homeostasis in hypertensive and hyperosmotic environments, in response to hyperosmotic stress induces the generation of TH17 cells and proinflammatory macrophages, thus, contributing to the development of autoimmune and inflammatory diseases [6]. Current experimental studies show that chronic hypohydration accelerates age-related pro- inflammatory changes, which along with coagulation changes are well-known modifiers of the aging rate [7].

Based on a comparative analysis of neuroendocrine regulation in conditions of low and high-water consumption, Armstrong et al proposed a theoretical model of the differential risk of chronic diseases and reduced life expectancy associated with insufficient hydration. The model is based on a homeostatic neuroendocrine response to mild dehydration, the central mechanism of which is increased secretion of vasopressin, a known neuropeptide produced by neurons of the supraoptic and paraventricular nuclei of the hypothalamus [8]. Vasopressin is found in various tissues of the body, including

the thymus and, according to recent data, is an important immunoregulatory peptide for innate and adaptive immunity, the effect of which is associated with the ability to stimulate the hypothalamic-pituitary-adrenal axis and prolactin production [9,10]. Vasopressin stimulates the release of glucocorticoids, which increase the concentration of serum Glucocorticoid- inducible Kinase 1 (SGK1), the expression of which is increased due to dehydration- activated transcription of NFAT5. Increased SGK1 activity promotes the development of a number of diseases, including modeling immune responses (SGK1 is involved in regulating inflammation, activating CD4 + helper T cells that produce proinflammatory cytokines) and promoting the development of fibrous and calcified tissue under decompensated cellular stress associated, for example, with the depletion of the energy potential of cells [11,12].

Hydration parameters affect the morphofunctional features of regional lymph nodes, determining the variations for their structure and functional specialization [13].

Adequate hydration of the body is necessary to maintain adequate mucociliary clearance of the respiratory system. According to the latest experimental, clinical, and epidemiological data, chronic suboptimal hydration a fewweeks before the infection is associated with an increased risk of death from COVID-19. Regarding the mechanism, the hypothesis is that suboptimal hydration-related increase of Angiotensin Converting Enzyme 2 (ACE2) receptors in the lungsincreases the likelihood of infection, as ACE2 is a known site of coronavirus intrusion into alveolocytes [14,15]

Given this, further studies of the effects of hypo- and dehydration of various degrees on the immune system, in particular on the morphology of the thymus as a central organ of immunogenesis, will contribute to a better understanding of the structural basis of adaptive mechanisms and pathological processes in water deficiency.

THE AIM

The aim of this study was to establish the features of ultrastructural changesin thymocytes in conditions of mild general dehydration of the organism in the experiment.

MATERIALS AND METHODS

The study was conducted on 20 non-linear adult male laboratory rats weighing 150-170 g. The animals were kept in standard vivarium conditions in accordance with the international principles of the European Convention "About the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes" (Strasbourg, 1986) and " General ethical principles of experiments on animals", adopted by the First National Congress on Bioethics (Kyiv, 2001). 10 animals of the experimental group were fed by dry granulated combined feed with complete restriction of water intake. Thus, on the 3rd day of the experiment, the animals were put into a state of mild general dehydration, which was ascertained by the loss of body weight and thymus weight. 10 animals of the control group were on the usual drinking regime (had free access to water) and food ration. The animals were removed from the experiment by euthanasia with carbon dioxide. The thymus was selected for research.

Histological preparations were made according to the generally accepted method using a sled microtome MS-2 (Ukraine). Hematoxylin-eosin staining was used. To conduct an electron microscopic study, parts of the thymus with a size of 1mm^3 were separated, which were first treated for 24 hours in glutaraldehyde according to Karnovsky, then kept in 1% osmium tetroxide according to Palade for 1 hour [1]. After that, the samples were dehydrated in ethanol of increasing concentration, followed by pouring the material into a mixture of epoxy resins (epon-araldite). Polymerization was carried out for 36 hours at a temperature of 600°C. Semi-thin sections with a thickness of 1–2 μm and ultrathin sections with a thickness of 0.5–1 μm were made on an ultramicrotome UMTP-6m (Ukraine). Semi-thin sections were stained with 1% methylene blue in 1% sodium tetraborate. Ultrathin sections were contrasted with a Reynolds solution of uranyl acetate and lead citrate. Visual evaluation of electron micrographs was carried out on a PEM- 125K electron microscope (Ukraine) at an increasing voltage of 75 kV.

The study of semi-thin sections was carried out on an Olympus light microscope (Japan) with photographic documentation of the morphological picture by a video camera Baumer/optronicTyp: CX05c (USA).

On semi-thin sections in the cortical and medulla of the thymus, the absolutenumber of lymphoid cells in the field of view corresponding to an area of 0.009 mm^2 (magnification x1000) was determined using the program "ImageJ" (USA).

For conducting an immunohistochemical study, sections with a thickness of 5×10^{-6} m were made, which were subjected to standard deparaffinization and dehydration in xylene and alcohols of increasing concentration. Unmasking of antigens was carried out in a water bath "VB-4" (Ukraine) at a temperature of 97- 980C. The antigen-antibody reaction was visualized using the "UltraVision Quanto Detection System HRP DAB Chromogen" detection system (USA), which

Table I. Dynamics of the parameters changes of the male rats thymus of in control and experimental groups (M±SD)

Parameter, units of measurement	A group of animals	
	Control	Experimental (3 days)
Thymus weight, mg	298,76 ± 22,84	286,85 ± 35,50 p = 0,68
Quantity of the lymphoid cells in the cortical zone of the thymus, visual field 0,009 mm^2	253,46 ± 20,72	219,83 ± 16,11 p<0.001
Quantity of the lymphoid cells in the medulla zone, visual field 0,009 mm^2	133,83 ± 11,72	126,48 ± 12,24 p=0.19

included blocking of endogenous peroxidase activity with hydrogen peroxide, blocking of non-specific background staining using "Ultra V block", amplification of the "Primary Antibody Amplifier" reaction Quanto" and final visualization with diaminobenzidine (DAB) with additional staining of nuclei with Mayer's hematoxylin.

Statistical processing of quantitative data was carried out using the Statistica v.10 program (StatSoft Inc., USA). Descriptive analysis of each sample was performed with calculation of Mean (M) and Standard Deviation (SD). The non- parametric Mann-Whitney U-test was used to assess the differences between the two samples according to the studied indicators. A difference of p≤0.05 was considered significant.

RESULTS

According to the organometric study data, at the time of the introduction ofthe experiment, no reliable reduction of the thymus was found in the research groupanimals, which can be explained by specific compensatory processes aimed at maintaining the degree of hydration of the organ under conditions of generaldehydration.

According to the results of the histomorphometric analysis, in the animals that were in a state of dehydration, compared to the animals of the control group, no significant changes were found in the ratio of the cortex and medulla zones of the thymus (cortex:medulla ratio), which in both groups of animals was close to 2:1. However, mainly in the cortical zone of the gland, a decrease in the density of thymocytes is observed during dehydration. On average, in the cortical zone of the thymus, there was decreased cellularity by 13.4% (p<0.001), while in the medulla zone this indicator turned out to be unreliable - 5.5% (p=0.19), compared to the indicators in animals of the control group (Table I).

The cortical zone of the animals' thymus in a state of dehydration is formed mainly by thymocytes, among which there are cells with signs of karyopyknosis and compaction of the cytoplasm, epithelial reticulocytes with large light nuclei and long processes of the cytoplasm, and macrophages (Fig. 1A). The medulla zone of the thymus is formed by sufficiently differentiated lymphocytes, contains epithelial reticulocytes and Hassal's bodies in a small amount. Some of the lymphocytes of the cortical and medullary zones of the rodents' thymus in a state of dehydration have relatively large nuclei and a narrow cytoplasm. The ratio of CD3+ thymocytes of both zones of the thymus, in the presence of morphological signs of focal delymphatization, indicates more in favor of comparatively compensated thymopoiesis (Fig. 1B).

The subcapsular zone of an organ in a state of dehydration is expanded and formed by epithelial reticulocytes, lymphoblasts, prethymocytes, and also contains macrophages in a small amount.

Nuclei of lymphoblasts are typically round or oval in shape, large, with one or two nucleoli. Heterochromatin is located in the form of small pits mainly on the periphery of the nucleus, it is quantitatively inferior to euchromatin. The contours of the karyolemma are of increased osmiophility, loose, with multiple intussusceptions and protrusions, sometimes with areas of lysis.

The perinuclear space is well defined, in some cells it has condensed cytoplasm or is significantly expanded and filled with structureless amorphous masses. In the cytoplasm of individual lymphoblasts, there are vacuoles with electron-transparent contents and areas of cytolysis in the form of lightening. The Golgi complex is surrounded by multiple vesicles. Mitochondria in small numbers, increased in size, have an electron-bright matrix, and sometimes reduced disoriented cristae (Fig. 2). Figures of mitosis of different stages were found for individual prethymocytes.

On the background of a decrease in the thymocytes number in the animals' thymus in conditions of dehydration, an increase in the number of small lymphocytes is observed, compared to such data in animals of the

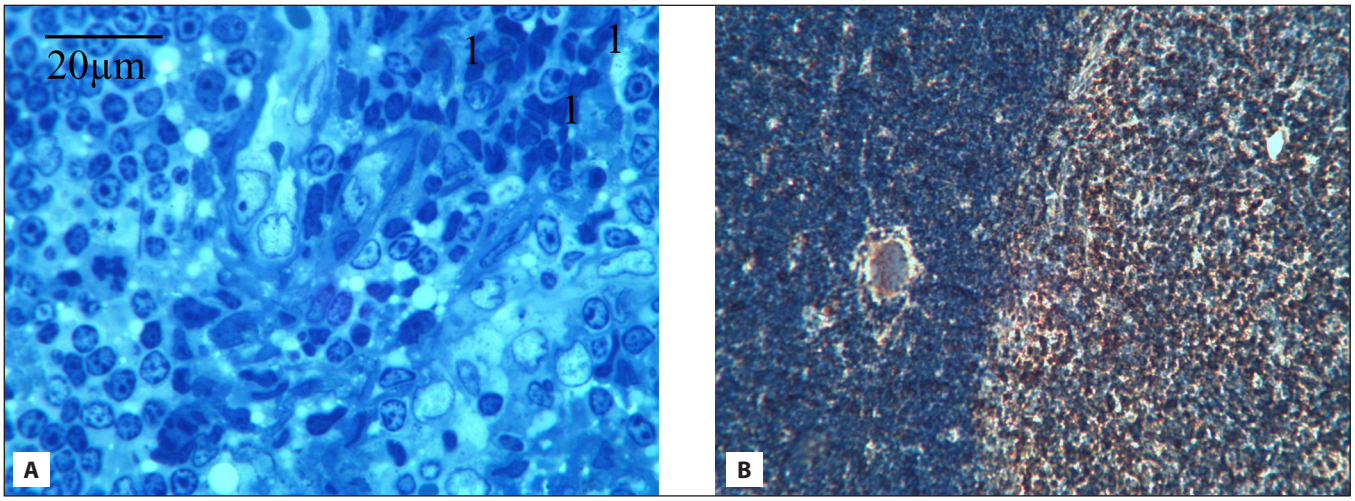

Fig. 1. A: Focal accumulation of thymocytes with signs of karyopyknosis and cytoplasmic thickening (marked 1). Semi-thin slice. Methylene blue stain. B: CD3+ thymus cortical substance. Reaction with MKAT to CD3 (P7), x200.

control group. Small lymphocytes have a nucleus with compact chromatin, an eccentrically located nucleolus, and a fairly narrow cytoplasm rim, which contains single rounded mitochondria and ribosomes (Fig. 3).

The majority of thymocytes of the cortical and medullary zones of the organ that were in a state of general dehydration of a mild degree have a typical structure. But, among them, there are cells with pronounced structural changes. Euchromatin predominates in the karyoplasm of such cells, but it is significantly less than in thymocytes of the control group animals. Heterochromatin is contoured in the form of small blocks. In some thymocytes, there is a violation of the structure of the karyolem. It has areas of lysis, which in places are replaced by vague wavy contours and intussusceptions. Areas of condensed chromatin and signs of karyolysis are present in the predominantly rounded nuclei. The perinuclear space of such cells is expanded, electron bright, sometimes filled with structureless amorphous masses. The cytoplasm has electron-illuminated areas, which can be regarded as signs of cytolysis. Such areas alternate with condensed cytoplasm, which has the appearance of a structureless mass of uneven electron density with remnants of destroyed organelles (Fig. 4).

There are thymocytes that obtain electron-dense mitochondria in their cytoplasm. Most of these organelles have a typical structure but differ in shape and size. Some of the mitochondria are damaged: the cristae are arranged irregularly, without a clearly defined orientation, some undergo reduction, the matrix is heterogeneous, and mostly electron-light. Similar ultrastructural features of mitochondria are characteristic of thymocytes, both cortical and medullary zones of the thymus (Fig. 5).

Along with the morphological features described above, there are signs of cell apoptosis. Thymocytes often have pyknotically altered nuclei of an irregular shape due to chromatin aggregation into electron-dense pits. The karyolem of such cells mostly has uneven, discontinuous contours and a large number of bay-like intussusceptions. The perinuclear space is expanded, and a thin electron-light rim surrounds the compacted nucleus. Cytoplasm also undergoes densification or cytolysis: in many cases it is vacuolated or filled with fine granular material forming clusters of rounded and polygonal shapes. Mitochondria have a lighted matrix containing dense osmiophilic inclusions or multilamellar bodies, cristae and the inner membrane of organelles are often destroyed. The nuclei of apoptotically changed epithelial reticulocytes have deep intussusceptions and constrictions, which are also found in the nuclei of apoptotic thymocytes. However, the latter is characterized by an extremely high degree of chromatin condensation. At the same time, supercondensed chromatin forms clusters in the form of characteristic crescents, or completely fills the karyoplasm. In the latter case, the nucleus of an apoptotic thymocyte is practically not identified against the background of a narrow border of electron-dense cytoplasm, while the thymocyte itself takes on the appearance of an "ink spot". Apoptotic bodies containing separate fragments of nuclei preserve the characteristic structural organization of chromatin (Fig. 2).

DISCUSSION

This study showed that a slight degree of general dehydration of the body causes ultrastructural changes in the thymus, corresponding to the picture of the accidental transformation of the organ.

According to our data, the influence of a mild degree of general dehydration did not affect the cortex:

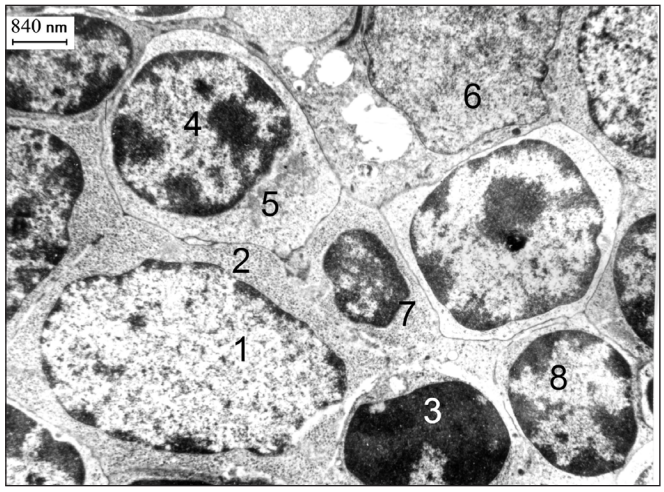

Fig. 2. Ultrastructural organization of the cortical substance of the rat's thymus. General dehydration, 3rd day. Electronic photography. Magnification: ×5000.
Designation: 1 – lymphoblast nucleus; 2 – lymphoblast cytoplasm; 3 – apoptotic thymocyte; 4 – thymocyte nucleus; 5 – narrow rim of thymocyte cytoplasm; 6 – vacuoles; 7- small lymphocyte.

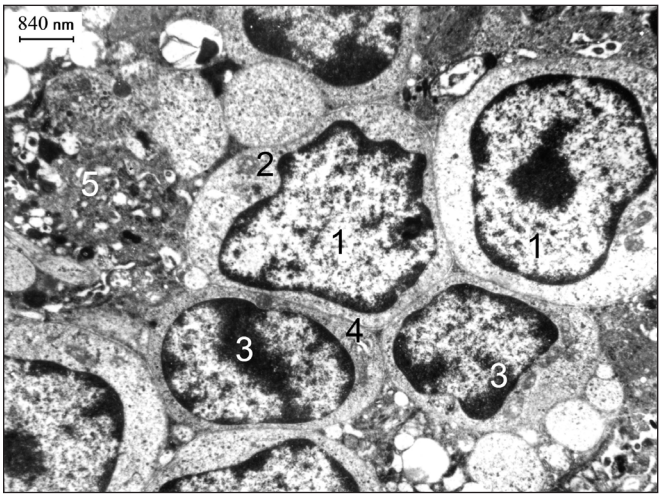

Fig. 3. Ultrastructural organization of the cortical substance of the rat thymus. General dehydration, 3rd day. Electronic photography. Magnification: ×5000.
Designation: 1 – deformed nucleus of a lymphoblast; 2 – lymphoblast mitochondria; 3 – thymocyte nucleus; 4 - thymocyte mitochondria; 5 - cellular detritus.

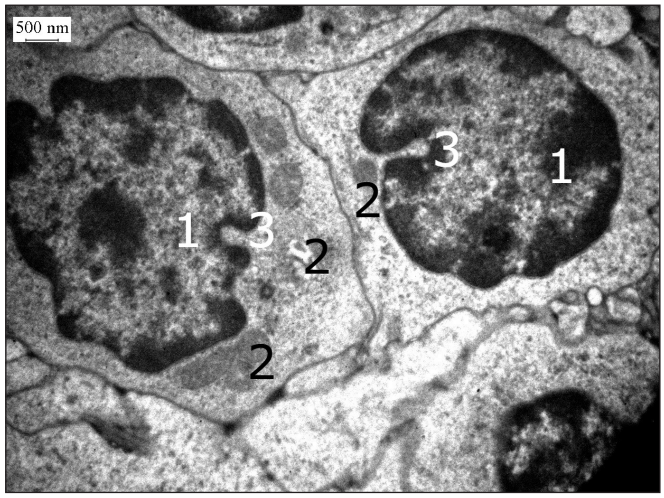

Fig. 4. Ultrastructural organization of rat's cortical substances. Thymocytes. General dehydration, 3rd day. Electronic photography. Magnification: ×8000. Designation: 1- nucleus 2-mitochondria 3-karyolem with intussusceptions.

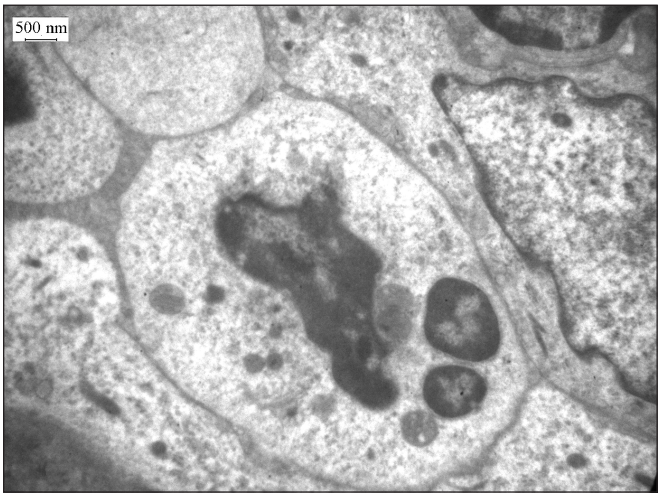

Fig. 5. Ultrastructural organization of rat cortical substances. Karyorrhexis. General dehydration, 3rd day. Electronic photography. Magnification: ×8000.

medulla ratio of the thymus. A decrease in the value of this ratio is a recognized, quite sensitive histological marker of negative effects, in particular toxic, infectious, etc. of a nature that causes the involution of the organ [16-18]. The normal cortex: medulla ratio noted by us in the thymus of animals that were in a state of a mild degree of general dehydration indicates that the functional microenvironment of thymocytes is preserved in general and there is a sufficient possibility of releasing their mature forms from the thymus.

At the same time, we found signs of initial cortical atrophy: focal delymphatization, decrease in the density of thymocytes in the cortical zone, cells with signs of karyopyknosis, karyolysis and cytoplasmic compaction, violation of the nuclear-cytoplasmic ratio. Morphological signs of increasing thymocyte apoptosis processes are also quite indicative, although it should be noted that many cortical thymocytes are immature, short-lived, and subject to apoptosis and phagocytosis by macrophages in normal conditions [19]. One way or another, the loss of cellularity by the thymus, with a disturbed or preserved microarchitecture of the organ, is a sign of atrophy. The latter leads to a decrease in the elimination of naïve T-lymphocytes and a decrease in the expression of T-cell receptors, which are an integral part of the processes of positive selection (recognition of "own" ligands, development of T cells) and negative selection (induction of cell death), and therefore

changes the further fate of T-lymphocytes, associating it with autoimmunity [20, 21]. In general, autoimmunity develops as a consequence of cortical atrophy, when positive selection, which is autoimmune in nature, is supplemented by violations of negative selection and selection of regulatory T cells [22]. In our opinion, atrophic changes in the thymus in conditions of a mild degree of general dehydration of the body can be associated, as a cause, with the development of accelerated immunological aging, the consequences of which will be a decrease in anti-infective and post-vaccination immunity, suppression of immune surveillance of tumors, development of autoimmune processes, chronic systemic inflammation, which in turn will contribute to many degenerative diseases, metabolic disorders and cardiovascular pathology [23,24].

An important structural component of the thymus is the subcapsular zone, quite noticeable at later stages of embryonic development, but weakly expressed in the postnatal thymus of rats. This zone ensures the creation of a kind of gradient that determines the direction of migration of T-lymphocytes in the process of their maturation. After all, it is known that precursor cells of T-lymphocytes migrate to the thymus in a wave-like manner, so that the successful maturation and exit of one group of cells provides space for the next [25, 26].

Subcapsular epithelial cells produce thymic hormones, cytokines, chemokines, neuropeptides and extracellular matrix components. Also, in the thymus of rats, there are areas free of subcapsular epithelium, which contain immature T-lymphocytes and are considered an alternative route for such cells, moving along which immature T-lymphocytes do not come into contact with stromal elements, and therefore to some extent avoid the influence of positive and negative selection [27,28]. The expansion of the subcapsular zone of the thymus of rats in the experimental group due to areas free of stromal elements that we discovered may be accompanied by an increase in the number of potentially autoreactive T- lymphocytes that do not die in the thymus and enter the pool of peripheral lymphocytes, increasing the risk of developing autoimmune processes in the body. An increase in the number of small lymphocytes in the expanded subcapsular zone probably illustrates the acceleration of the processes of proliferation and maturation of thymocytes with an earlier entry into the peripheral blood flow of immunocompetent cells. This occurs together with a decrease in transcriptional activity, as evidenced by a lower amount of euchromatin in lymphocytes of the thymus of animals under conditions of a mild degree of general dehydration.

Ultrastructural changes in the thymus under the influence of dehydration, in particular, such as intussusception and partial lysis of the karyolem of lymphoblasts, swelling of mitochondria, disorientation, and reduction of their cristae, are signs of the cellular aging process, which is essentially associated with the involution of the thymus [29].

CONCLUSIONS

General dehydration of a mild degree in the experiment is accompanied by a cell- mediated response of the central link of immunogenesis and results in morphological changes in the thymus, which are atrophic in nature with a typical pattern of remodeling of the organ's microstructure, which corresponds to cellular aging and the associated sign of accelerated involution.

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Conflict of interest:
The Authors declare no conflict of interest.

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