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SUBMICROSCOPIC CHANGES IN THE SPLEEN OF MATURE RATS UNDER CONDITIONS OF CHRONIC HYPERHOMOCYSTEINEMIA

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In mature rats with hyperhomocysteinemia, pronounced destructive processes in the spleen were observed. A high degree of apoptosis of lymphocytes of the white pulp of the spleen and proliferation of B-lymphocytes into mature plasma cells were noted. The number of lysosomes and phagosomes increased in macrophages, and their cytoplasm contained fragments of destroyed formed blood elements. A significant number of lymphoblasts characterised the active immune response of the organ to hyperhomocysteinemia. The red pulp of the spleen was characterised by increased disintegration of erythrocytes and platelets and accumulation of lipofuscin granules in the cytoplasm of macrophages. Endothelial cells of sinusoidal capillaries were also changed.

Key words: hyperhomocysteinemia, spleen, white pulp, lymphocytes, rats.

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СУБМІКРОСКОПІЧНІ ЗМІНИ СЕЛЕЗІНКИ ЗРІЛИХ ЩУРІВ ЗА УМОВ ХРОНІЧНОЇ ГІПЕРГОМОЦИСТЕЇНЕМІЇ

У зрілих щурів при гіпергомоцистеїнемії спостерігали наявність виражених деструктивних процесів в селезінці. Відмічали високий ступінь процесів апоптозу лімфоцитів білої пульпи селезінки, проліферацію В-лімфоцитів в зрілі плазматичні клітини. В макрофагах зростала чисельність лізосом, фагосом, цитоплазма їх містила фрагменти зруйнованих формених елементів крові. Активна імунна відповідь органу на гіпергомоцистеїнемію характеризувалась наявністю значної кількості лімфобластів. Червона пульпа селезінки відрізнялась посиленим розпадом еритроцитів та тромбоцитів, накопиченням гранул ліпофусцину в цитоплазмі макрофагів. Змін зазнавали також ендотеліальні клітини синусоїдних капілярів.

Ключові слова: гіпергомоцистеїнемія, селезінка, біла пульпа, лімфоцити, щури.

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Hyperhomocysteinemia is a metabolic disorder characterized by an increase in the level of homocysteine in the blood plasma. It is a potential risk factor for the onset and progression of pathological conditions. Homocysteine is a product of methionine metabolism. The latter enters the body with dietary proteins and undergoes some biochemical transformations. First, it is alkylated by ATP to form S-adenosylmethionine. With the help of the enzyme cytosol-5-methyltransferase, the latter transfers its methyl group to cytosine in DNA, thereby forming S-adenosylhomocysteine. The enzyme adenosylhomocysteine hydrolase (SAHase) breaks it down to adenosine and homocysteine. S-adenosylmethionine is an essential intermediate product of these reactions. It participates in DNA, RNA, proteins, lipids, creatine, phosphatidylcholine, and adrenaline methylation. Its role in modifying xenobiotics has been noted [7, 8].

Typically, homocysteine is utilized in the body in the reactions of remethylation, transsulfuration and desulfuration. The transsulfuration pathway begins with the condensing of two molecules of homocysteine and serine, forming cystathionine. This reaction is catalyzed by the pyridoxal phosphate-dependent enzyme cystathionine-β-synthase [8, 13]. Subsequently, cystathionine is converted to cysteine, ammonia and α-ketobutyrate under cystathionine-γ-lyase. During the remethylation processes, methionine is resynthesised from homocysteine using cobalamin-dependent methionine synthase. There is also another remethylation pathway involving the enzyme betaine-homocysteine-S-methyltransferase. Both enzymes are cytosolic and are characterized as zinc-dependent methyltransferases. Methionine synthase is present in all body tissues and uses N-5-methyltetrahydrofolate as a methylating group. Betaine-homocysteine-S-methyltransferase uses an endogenous choline derivative – betaine and is present to a greater extent in the liver, adrenal cortex, brain tissue, skeletal muscles, and placenta. When utilizing homocysteine by desulfuration, it is converted into hydrogen sulfide [5, 13].

The leading causes of hyperhomocysteinemia include genetic mutations of enzymes that catalyze homocysteine metabolism reactions; bad habits – smoking, alcoholism, excessive coffee consumption; impaired kidney function; eating protein-rich foods; insufficient intake of vitamins; diseases of the gastrointestinal tract – gastritis, gastric ulcer, ulcerative colitis, Crohn's disease, celiac disease, enteritis; oncopathology – breast cancer, ovarian cancer, pancreatic cancer, lymphoblastic leukemia [4, 5, 9].

Genetic causes of hyperhomocysteinemia are relatively rare, but they cause the development of severe pathologies and even death in childhood. Congenital defects of the genes methylenetetrahydrofolate reductase, cystathionine-β-synthase and methionine synthase have been studied [10].

The role of hyperhomocysteinemia in the pathogenesis of many diseases is currently being actively studied. Its negative effect on the endothelium of the vascular wall, participation in the development of complications of cardiovascular and cerebrovascular diseases, diseases of the respiratory, excretory, endocrine, and immune systems, etc., have been established [11].

The purpose of the study was to establish submicroscopic changes in the spleen of mature rats under conditions of chronic hyperhomocysteinemia.

Materials and methods. Modelling of chronic hyperhomocysteinemia was carried out through experimental studies on laboratory rats in compliance with international recommendations on the conduct of biomedical research using animals by the "General Principles of Animal Research", approved by the I National Congress on Bioethics (Kyiv, Ukraine, 2001) and agreed with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, France, 1986) [7]. The Bioethics Committee of National Pirogov Memorial Medical University, Vinnytsya, approved that the work complied with ethical principles (protocol No. 3 dated 17.10.2019).

The experiments were performed on 22 white male rats aged 6–8 months, obtained from the National Pirogov Memorial Medical University, Vinnytsya. Laboratory rats were kept in normal vivarium conditions with a 12-hour day/night regime, and water and balanced granulated feed were received ad libitum following the established norms.

Animals were divided into control and experimental groups (11 individuals in each group) during the experiment. Chronic hyperhomocysteinemia was achieved by administering D, L-thiolactone homocysteine hydrochloride (Acros Organics, Italy) to animals of the experimental group at a dose of 200 mg/kg body weight intragastrically (i.v.) in a 1 % starch gel solution (1 ml/100g of rat weight) once a day for 8 weeks. After the end of the experimental modelling of chronic hyperhomocysteinemia, the animals were removed from the experiment, euthanized by decapitation and using thiopental anesthesia (sodium thiopental 100 mg/kg i.p.).

Pieces of spleen 0.5–1 mm in size were fixed in 2.5 % glutaraldehyde solution in phosphate buffer pH 7.2–7.4. Subsequently, they were introduced into the Epon-Araldite mixture according to the generally accepted method [2, 6]. Sections were made from the obtained blocks and stained with toluidine blue and Hayat. After field microscopy of thin sections, ultrathin sections were made using LKB III (Sweden) and Reihart (Austria) ultramicrotomes, which were contrasted with a 2 % solution of uranyl acetate and lead citrate. The sections were examined and photographed under a PEM125K electron microscope with a magnification of 6–20 thousand times.

Results of the study and their discussion. Modelling of persistent hyperhomocysteinemia in adult rats led to more pronounced shifts in the ultrastructure of the spleen of animals. The lumens of sinusoidal capillaries were dilated. They showed accumulations of formed blood elements, namely erythrocytes, platelets, and granulocytes. Endothelial cells of the vessel walls had hyperchromic nuclei, heterochromatin in the form of a dense rim was located under the karyolemma, and light areas of euchromatin were in the center. In some places, the contours of the nuclear envelope of endothelial cells were indistinct and slightly blurred. The nuclei were located mainly eccentrically and had a rounded shape. Mitochondria, ribosomes and numerous vacuoles were found in the cytoplasm of endothelial cells. Erythrocytes and platelets in the lumens of blood vessels of the spleen were often in the stage of degradation.

Electron microscopic studies of the spleen of mature animals revealed that the white pulp, as in young rats, was more affected by hyperhomocysteinemia than the red pulp. A distinctive feature was the significant death of T-lymphocytes by apoptosis. Most lymphocytes had a rounded shape. Their nucleus was located in the center; in some cells, the nuclei were wrinkled. The karyolemma was characterised by a violation of its integrity; its numerous invaginations were noticeable, and the chromatin was pronouncedly condensed. The cytoplasm surrounded the nucleus of lymphocytes in the form of a rim and contained giant swollen mitochondria, in which the destruction of cristae was observed. The tubules of the granular endoplasmic reticulum were also swollen and dilated (Fig. 1).

In the germinal center of lymphoid nodules, the proliferation of B-lymphocytes was noted, and the predominance of mature plasma cells was observed. The latter were subject to ultrastructural rearrangements. In most plasma cells, the nucleus was bean-shaped, and the nuclear pores were expanded.

Heterochromatin formed lumps and clusters, which were located under the nucleolemma. In the light courtyards of plasma cells, swollen mitochondria with destroyed cristae and expanded tubules of the granular endoplasmic reticulum were observed. It should be noted that in this experimental group of animals, the number of macrophages increased significantly, and their functional activity increased. Despite their significant number, macrophages often underwent dystrophic changes. Their nuclei were pyknotic and had a deepening of the karyolemma, and the latter locally lost signs of integrity. Heterochromatin was located marginally in the form of lumps. The cytoplasm of macrophages was filled with numerous lysosomes, phagosomes, and inclusions. Fragments of erythrocytes, platelets, lipofuscin granules, and ferritin accumulations were noted. Mitochondria increased in size and were fluoresced, and cristae were barely discernible. In the white pulp of the spleen of adult rats, an increase in the number of lymphoblasts and light and dark lymphocytes was detected, which indicated an active immune response of the spleen to the increase in the concentration of homocysteine in the blood plasma (Fig. 2).

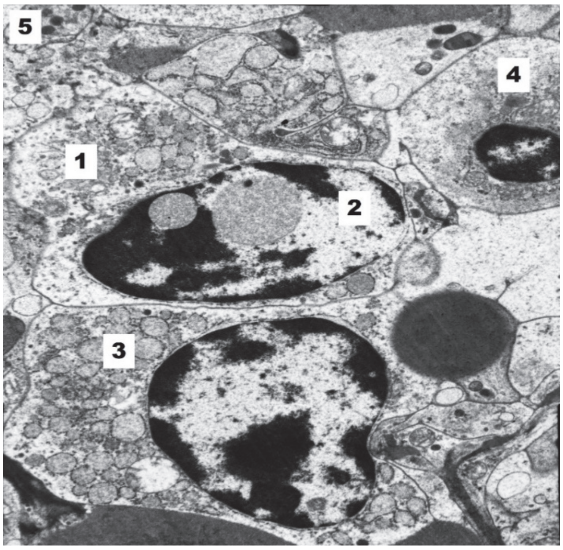

Fig. 1. Electron microscopic changes in the spleen of an adult rat with hyperhomocysteinemia. Macrophage cytoplasm (1), macrophage nucleus (2), plasma cell (3), lymphocyte (4), platelet (5). Magnification. ×9000.

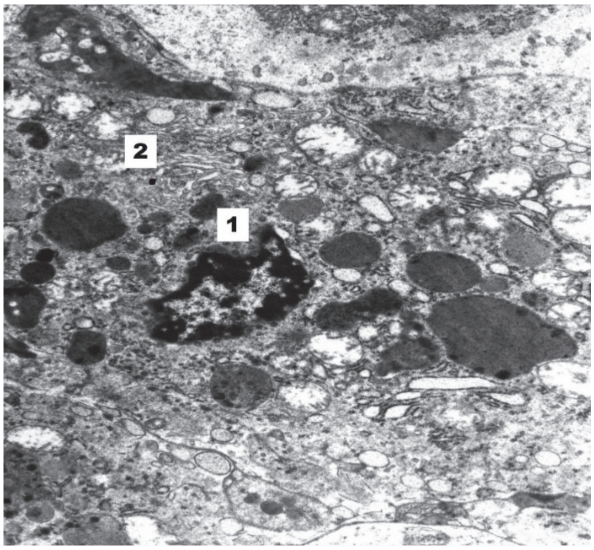

Fig. 2. Submicroscopic changes in an adult rat's spleen under hyperhomocysteinemia conditions. Macrophage nucleus (1), macrophage cytoplasm (2). Magnification. ×9000.

In the red pulp of the spleen, erythrocytes, platelets, and granulocytes (mainly neutrophils) were noted. Eosinophils with large granules were also present. Macrophages, which occupied one of the main places in the red pulp of the organ under these conditions, were characterised by hyperchromic, eccentrically located nuclei. In the cytoplasm of these cells, both secondary lysosomes and residual bodies with a large amount of detritus were noted. Fragments of phagocytised material, including red blood cells and platelets, were detected. Lipofuscin inclusions were observed (Figs. 3, 4).

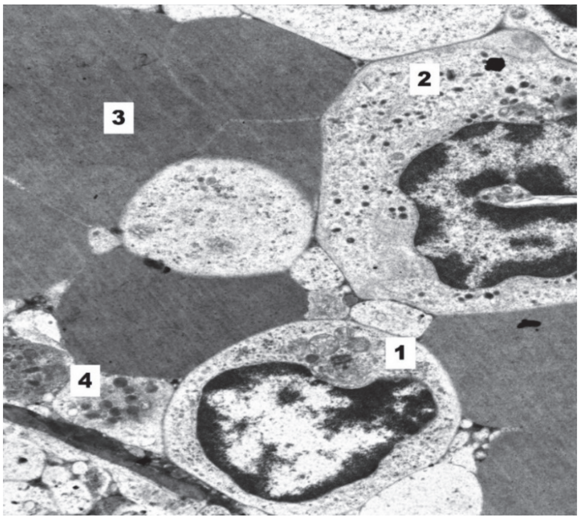

Fig. 3. Ultrastructural changes in the red pulp of the spleen of an adult rat with hyperhomocysteinemia. Lymphocyte (1), macrophage (2), erythrocyte (3), platelet (4). Magnification. ×9000.

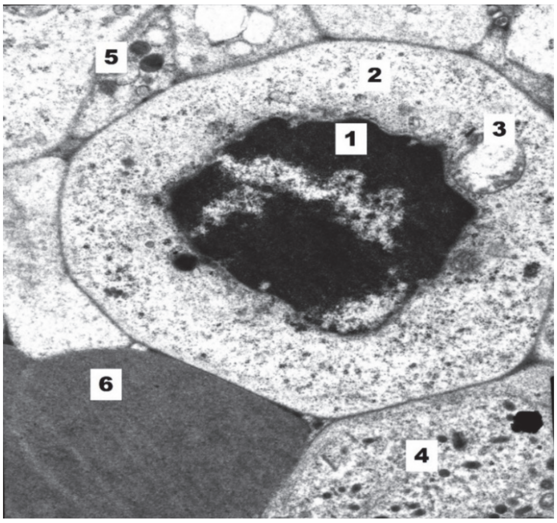

Fig. 4. Electronogram of an adult rat's spleen under hyperhomocysteinemia conditions. Lymphocyte nucleus (1), lymphocyte cytoplasm (2), mitochondria (3), neutrophil (4), platelet (5), erythrocyte (6). Magnification. ×9000.

Experimental studies on animals have made it possible to establish the main causes of homocysteine toxicity on the body. To date, scientists have identified the main ones, namely: activation of oxidative stress and endoplasmic reticulum stress, disruption of gene expression, homocysteinelation of proteins, stimulation of the inflammatory mediators' production and fibrosis [9]. The role of homocysteine in the pathogenesis of many diseases is being actively studied.

It has been established that hyperhomocysteinemia affects the components of the innate and acquired link of the immune defence system. In particular, it was found that lymphocytes express NMDA receptors on their surface, which can interact with homocysteine. Under its influence, the number of ROS and the content of Ca2+ and ATP in cells increases. In addition, hyperhomocysteinemia stimulates the production of IFN-γ and TNF-α by lymphocytes, involving protein kinase, NADPH oxidase and NO synthase. The consequence of such an effect is the death of lymphocytes by apoptosis. The authors note that constant stimulation of immune system cells in the bloodstream under these conditions leads to its depletion. According to other authors, homocysteine interacts with inotropic glutamate receptors activated by NMDA. Activation of these receptors leads to increased entry of Ca2+ ions into the cell and an increase in the level of ROS, activation of lipid peroxidation processes and apoptosis of lymphocytes. It should be noted that the blockade of NMDA receptors of T cells by homocysteine inhibits mRNA synthesis and increases the secretion of INF-γ, TNF-α, and IL-10. Its single administration is accompanied by a pronounced decrease in T lymphocytes and their subpopulations. According to the authors, this is due to an increase in the expression of adhesion molecules to both endothelial cells and lymphocytes, which ensures the adhesion of lymphocytes to the vascular wall and thereby reduces the pool of circulating lymphocytes. Some may be eliminated due to the activation of NMDA receptors with the subsequent development of apoptosis. However, under conditions of chronic hyperhomocysteinemia in animals, an increase in the number of T lymphocytes at the expense of T cytotoxic ones was recorded. When hyperhomocysteinemia is administered once a day for a week in animals, adaptation mechanisms are activated, allowing the inactivation of aminothiol as a xenobiotic. High doses of homocysteine damage the tissue structures of the spleen, which is accompanied by the development of autoimmune reactions, as evidenced by a sharp increase in the concentration of all cytokines, especially IL-17A [3].

When homocysteine was exposed to lymphocytes in patients with coronary artery disease, it was found that Fas receptors of T-helper cells and their ligands CD178 were activated during their growth in the culture of peripheral blood cells. An early marker of apoptosis was the expression of APO 2.7. Subsequently, negatively charged phospholipids, mainly phosphatidylserine, appeared on the cell membrane. At the same time, the concentration of Bcl-2, which usually protects cells from apoptosis, decreased, leading to their death [1, 4, 5].

In the folate-induced model of hyperhomocysteinemia, instability of genomic DNA in the spleen of rats was revealed, which is expressed in a decrease in the indices of elastoplasticity and molecular weight, as well as an increase in its availability for hydrolysis by DNase I and EcoRI restriction enzyme. It is known that hyperhomocysteinemia can be involved in the metabolism of cell mitochondria, increasing the production of ROS and the level of Ca2+ ions, which leads to disorders of tissue respiration and an increase in the potential of organelle membranes. By being involved in the mitochondrial metabolism of T lymphocytes, it causes their reprogramming. These changes in T cells trigger the mechanisms of endoplasmic reticulum stress, manifested in the spleen by an increase in their markers – p-elf2α, p-PERK, IRE-1α, and XBP-1 [6].

Currently, a certain number of works are devoted to the study of homocysteine damaging effect on the endothelium of the vascular wall and its participation in the development of cardiovascular and cerebrovascular complications [14]. It has been established that in men with a homocysteine level exceeding the upper limit of normal by 12 %, the risk of myocardial infarction increases 3 times. An increase in its content in the blood by every 5 μmol/l above the norm leads to an increase in the probability of coronary pathology in men by 60 %, in women by 80 %, and cerebrovascular pathologies by 50 % in both men and women. Therefore, hyperhomocysteinemia is considered an independent risk factor for coronary and cerebral circulation disorders [8, 13]. There is information on endothelial destruction, hyperplasia of smooth myocytes in the aortic media. Vacuolar sarcoplasmic dystrophy, loss of myofibril striation, extensive areas of necrosis, and leukocyte infiltration of the myocardium have been observed. A thorough review of the scientific literature has shown that homocysteine causes endothelial cell damage and the development of thrombosis. The thrombogenic effect may be associated with inhibition of prostacyclin synthesis, activation of coagulation factor V, inhibition of protein C activation, and blockade of tissue plasminogen activator binding by endothelial cells. In addition, hyperhomocysteinemia is associated with platelet hyperaggregation [15]. The role of homocysteine in reducing the synthesis of the endothelium relaxing factor – NO, stimulation of peroxynitrite production, which causes the death of epithelial cells of the vascular wall has been established. Recent studies also demonstrate that during the oxidation of homocysteine, ROS are generated, which cause the development of oxidative stress [12]. It should be noted that in the vascular wall it stimulates lipid oxidation, as a result of which favorable conditions for atherogenesis arise [6].

Homocysteine thiolactone activates platelet aggregation induced by ADP, collagen and adrenaline. Hyperhomocysteinemia negatively affects nucleotide metabolism, leads to changes in the activity of platelet, hepatic and circulating enzymes of adenyl nucleotide metabolism due to impaired expression of the corresponding genes and their post-translational modification. By generating ROS, it inactivates nucleotide metabolism enzymes. Ultimately, impaired adenyl nucleotide metabolism creates conditions for platelet hyperreactivity [5].

Conclusion

In mature rats with hyperhomocysteinemia, pronounced destructive processes were observed in the spleen. A high degree of apoptosis of lymphocytes of the white pulp of the spleen and proliferation of B-lymphocytes into mature plasma cells were noted. The number of lysosomes and phagosomes in macrophages increased, and their cytoplasm contained fragments of destroyed formed blood elements. A significant number of lymphoblasts characterised the active immune response of the organ to hyperhomocysteinemia. The red pulp of the spleen was distinguished by increased disintegration of erythrocytes and platelets and accumulation of lipofuscin granules in the cytoplasm of macrophages. It should be noted that in the group of mature animals, endothelial cells of sinusoidal capillaries also changed.

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