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CHANGES IN THE MICROSCOPIC ORGANISATION OF THE SPLEEN OF ADULTS AND OLD RATS UNDER CONDITIONS OF CHRONIC HYPERHOMOCYSTEINEMIA

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Annotation. *Homocysteine (Hc), a product of methionine metabolism, can negatively affect internal organs' structural and functional parameters, including the spleen. The study aims to study the microscopic changes in the spleen of adults and old rats under conditions of chronic hyperhomocysteinemia (HHc). Experiments were performed on 44 white male rats (adult rats aged 6-8 months and old rats aged 24-26 months). The animals were divided into control and experimental groups (11 individuals in each group) during the experiment. Chronic HHc was achieved by administering D, L-thiolactone homocysteine hydrochloride to experimental group animals at a dose of 200 mg/kg of body weight intragastrically (intravenously) in a 1% starch gel solution once a day for eight weeks. After the end of the experimental simulation of chronic hyperhomocysteinemia, the animals were removed from the experiment by anaesthetising by decapitation and using thiopental anaesthesia. Histological preparations were studied using an SEO SCAN light microscope. It was found that under conditions of chronic HHc in adult rats, densification and disorganisation of the fibres of the dense connective tissue of the capsule and trabeculae of the spleen, vacuolisation of the cytoplasm of endotheliocytes of large-diameter vessels were noted. T-cells of the white pulp were subject to death by apoptosis, and B-cells of lymphoid nodules and marginal zones showed signs of marked proliferation. Modelling persistent GHz in old rats led to changes in the spleen's stromal and parenchymal structural elements. The capsule of the organ lost the clarity of its contours and was blurred and thickened. Lightening zones characterised periarterial sheaths due to the massive death of T-lymphocytes. The number of bright germinal centres and plasma cells increased. The number of macrophages containing lipofuscin inclusions increased in the red pulp. All these changes were caused by the irritating effect of excessive doses of homocysteine, particularly oxidative stress and nitrosylation, which suppresses the mechanisms of cell adaptation to this stress and hypomethylation of cell DNA.*
Keywords: *hyperhomocysteinemia, spleen, lymphocytes, white pulp, rats.*

Introduction

Organs of hematopoiesis and immune protection form one of the most reactive systems of the body, which can quickly respond to the action of damaging factors of exo- and endogenous origin, ensuring the maintenance of the stability of the internal environment of the body and the development of adaptation mechanisms [5, 15]. This system is of key importance in controlling the biological individuality of each person. The loss of these properties leads to multiple autoimmune, allergic and oncological diseases [4, 11].

Diseases of hematopoietic organs and immune protection are the most common among children. At this age, the immune system is vulnerable and exposed to the harmful effects of the environment due to its functional immaturity [20]. In mature people, long-term exposure to negative environmental factors leads to stimulation of the lymphatic system, and this especially applies to the peripheral organs of immune protection, which can lead not only to secondary immunodeficiency conditions but also to distant consequences, such as malignant neoplasms of lymphoid tissue [9, 24]. Therefore, it is essential to study the influence of factors of various genesis on the features of morpho-functional changes in immune defence organs, including the spleen.

Homocysteine (Hc), a product of methionine metabolism, can negatively affect internal organs' structural and functional parameters, which was proven in scientific experiments on laboratory animals [18, 19]. According to most authors, its normal level in the body is within 5-15 ?mol/l [16, 17]. It was established that the concentration of Hz varies with age and sex. Compared to children, its content is two times higher in adults. During life, the level of Hc in the blood increases, correlating with the concentration of vitamin B12 and folates [12]. Usually, it is important for the body, as it maintains the content of the essential amino acid methionine at a constant level. However, a significant increase in its concentration has several negative effects [13, 14, 21]. The problem of impaired metabolism of sulfur-containing amino acids has become the focus of many researchers. There was interest in the study of this issue earlier when scientific experiments demonstrated how methionine metabolism disorders lead to severe congenital defects of the central nervous system. Still, it has been established that this condition has a systemic effect on the body. In Ukraine, disorders of the metabolism of sulfur-containing amino acids are registered in 10% of adults and 2% of children and adolescents. In patients with cardiovascular diseases, this indicator varies between 40-43% [1].

Thus, it is urgent to study in depth the morphological changes of internal organs, including the spleen, at the microscopic level under hyperhomocysteinemia (HHc).

The *study* aims to find the microscopic changes in the

spleen of adults and old 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 performance of medical and biological research using animals by the "General Principles of Work on Animals" approved by the 1st National Congress on Bioethics (Kyiv, Ukraine, 2001) and agreed with the provisions "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, France, 1986) [3].

The Bioethics Committee of the National Pirogov Memorial Medical University, Vinnytsya, Ukraine, confirmed that the work complied with ethical principles (protocol № 3 dated 17.10.2019).

Experiments were performed on 44 white male rats (adult rats aged 6-8 months and old rats aged 24-26 months) obtained from the vivarium of National Pirogov Memorial Medical University, Vinnytsya. Laboratory rats were kept under normal vivarium conditions with a 12-hour day/night regime; water and balanced pelleted feed were received ad libitum by established norms.

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

For light-optical examination, pieces of spleen were taken from prematurely weighed animals of all groups. The selected samples were fixed in a 10% formalin solution. The next stage was the dehydration of samples in alcohols of increasing concentration and pouring them into paraffin blocks. Sections with a thickness of 3 ?m were made from the finished blocks and stained with hematoxylin and eosin [2, 8]. Histological preparations were studied with the help of an SEO SCAN light microscope and photo-documented with the help of a Vision CCD Camera with a system of the image output from histological preparations.

The study is a fragment of research works of the Departments of Biological and General Chemistry, as well as the Department of Histology of VNMU named after E. Pirogov: "The influence of exogenous and endogenous factors on the exchange of hydrogen sulfide and associated metabolic processes in normal and pathological conditions" (state registration number 0113U006461), "The role of exogenous and endogenous sulfur-containing compounds in the mechanisms of damage to internal organs and cytoprotection in various pathological conditions" (state registration number 0119U001142).

Results. Discussion

The assessment of the state of the spleen of mature animals from the group with the simulation of chronic hyperhomocysteinemia showed more pronounced signs of abnormalities in the morphology and, accordingly, the functioning of this organ than in young rats.

When examining the stroma, we noted a slightly changed density and disturbed organisation of the fibres of the dense connective tissue of the capsule and trabeculae (Fig. 1 A). Due to the delamination of the fibres and the appearance of small vacuoles between them, the capsule generally looks blurred; its boundaries are unclear. If you pay attention to the cells, it is noticeable that the nuclei of fibroblasts within the capsule are not as organised and parallel to each other as in control; some nuclei have a slight swelling, which is reflected in their more uneven and pale colour and change in shape - it becomes irregular, due to that the nuclei look heterogeneous. It is also necessary to note vacuolisation as a sign of oedema in the subcapsular zone of the splenic pulp (purple arrows).

It is also worth paying attention to the state of the endothelium in the large vessels of the spleen - signs of vacuolisation are observed in the epithelial lining cells, which is a typical manifestation of the pathological state of the cells (Fig. 1. B). As the literature knows, nitrosylation is one mechanism that influences excessive homocysteine concentration. Endothelial cells can detoxify homocysteine by releasing nitric oxide, accompanied by an increase in the level of Nos3 mRNA [10]. This is a protective mechanism against the adverse effects of homocysteine. At the same time, it is noted that chronic exposure to high levels of homocysteine leads to a decrease in the production or availability of NO, which entails unimpeded oxidative damage mediated by homocysteine and the formation of peroxynitrite.

Homocysteine can affect the activity of glutathione peroxidase, thus changing the microenvironment during the spread of reactive oxygen species. Endothelial glutathione peroxidase catalyses the reduction of hydrogen peroxides and lipids to the corresponding alcohol, preventing oxidative inactivation of NO. In turn, homocysteine reduces glutathione peroxidase's mRNA level, inhibiting similar protective mechanisms [6].

That is why the presence of vacuolated cytoplasm and deformation of the endothelium of the large vessels of the spleen can be explained by nitrosylation caused by the long-term effect of excessive amounts of homocysteine.

When examining the white pulp, even at small magnifications of the microscope, a characteristic picture of periarterial sheaths formed by T-cells catches the eye - they appear to be "eaten by moths". This appearance is characteristic of these elements of the white pulp due to the partial death of T-lymphocytes by apoptosis between

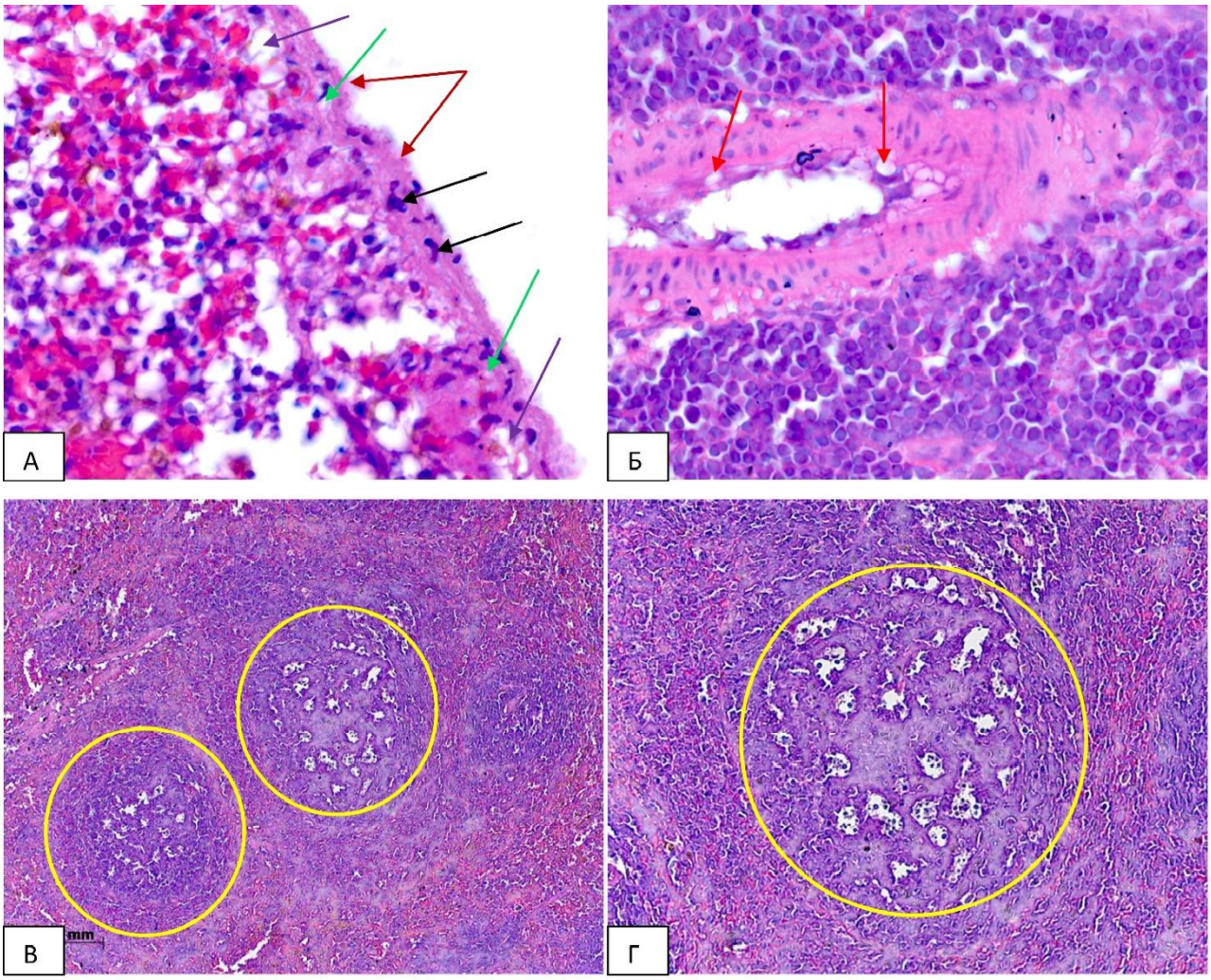

Fig. 1. Stroma and white pulp of mature rats from the group with chronic hyperhomocysteinemia. A: blurred collagen fibres of the capsule (brown arrows); vacuoles in the capsule (green arrows); subcapsular vacuolisation (purple arrows); nuclei of fibroblasts (black arrows); B: vacuolisation of the endothelium (red arrows); B, D: moth-eaten periarterial sheaths (yellow ovals). A, B x1000, B x200, D x400.

unaffected cells, which creates a dark background with numerous white holes from dead neighbours (Fig. 1 B, D).

Literary sources point to one of the factors of the negative impact of chronic hyperhomocysteinemia, precisely in lymphocytes. An increase in plasma homocysteine level is associated with parallel hypomethylation of lymphocyte DNA. Violating the non-random pattern of DNA methylation can lead to inappropriate gene expression and promote the development of pathology in lymphocytes and T-cells in particular [7, 22, 23].

B cells of lymph nodes and marginal zones, in turn, show signs of proliferation - there are germinal centres and mature plasma cells within the white and red pulp (Fig. 2 A). At the same time, the marginal zone of nodules is not expanded but has slightly blurred borders (Fig. 2 B, C). A significant number of lymphoblasts and lymphocytes indicates a reactive response of these cells to the chronic effect of elevated homocysteine levels [26].

In the red pulp itself, processes are noted, which, as in the experimental group of young animals, testify to the benefit of increasing the level of oxidative stress - among other formed elements of the blood, there are macrophages with golden-brown inclusions of lipofuscin pigment (Fig. 2 D). As mentioned earlier, the appearance of this pigment is evidence of high levels of lipid oxidation, which leads to the presence of reactive oxygen species in the tissue [25].

When examining the state of the spleen of old rats that have experienced chronic hyperhomocysteinemia, pathological manifestations are observed in both stromal and parenchymal elements. When reviewing the stroma, we noted specific changes even in its coarse component, which was not observed in any experimental group except for mature animals from the experimental group. The boundaries of the capsule look blurred due to the indistinct contours of collagen fibres (Fig. 3 A). In general, the capsule appears somewhat thickened due to the loose arrangement

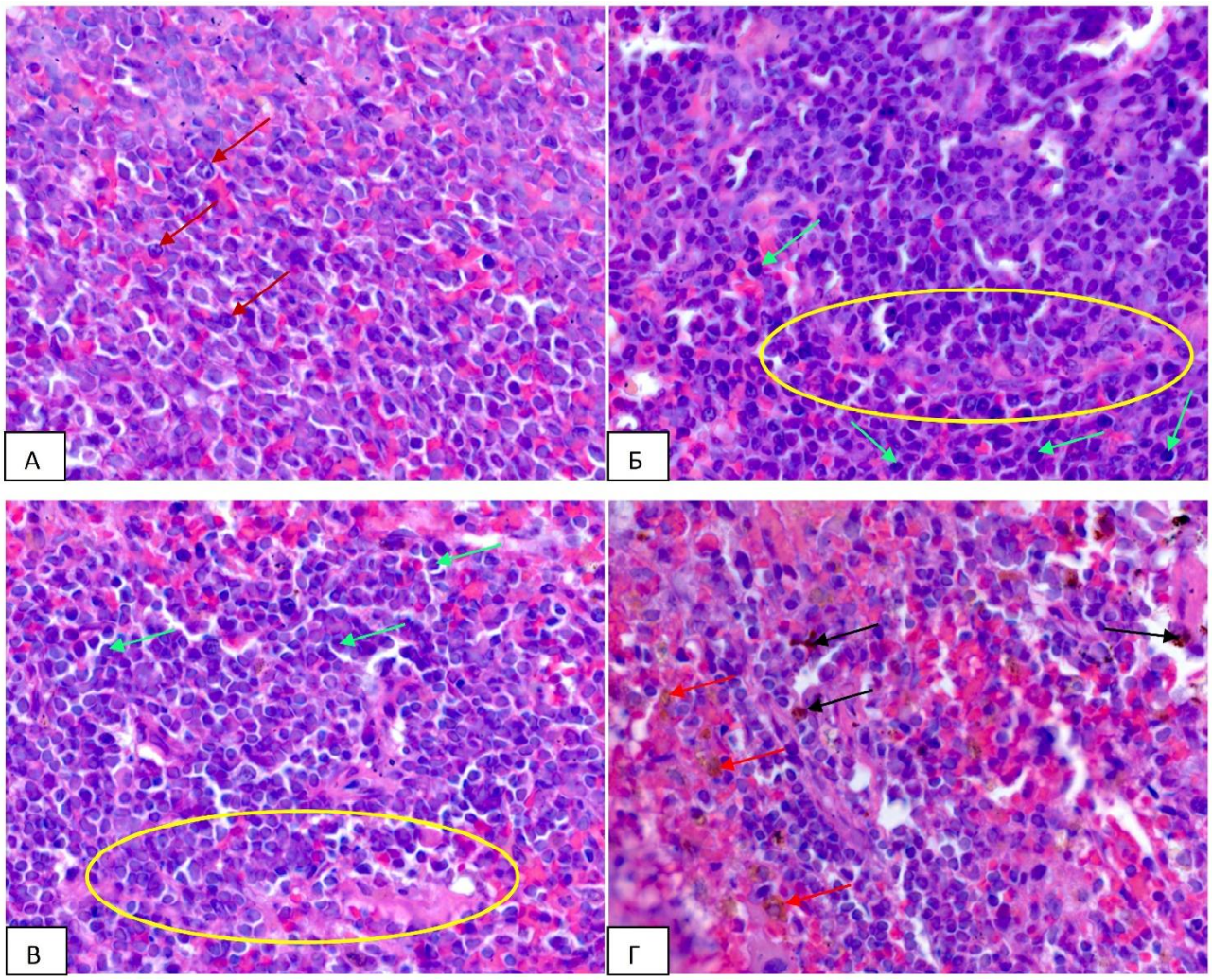

Fig. 2. Splenic parenchyma of mature rats from the group with chronic hyperhomocysteinemia. A: plasma cells (brown arrows); B, C: marginal zone of lymph nodes (yellow ovals); lymphocytes (green arrows); D: macrophages with hemosiderin (black arrows); macrophages with lipofuscin (red arrows). x1000.

of these fibres. Not all fibroblasts of dense connective tissue have a normal morphology because not all nuclei are flattened; some are rounded with perinuclear swellings in the cells. A somewhat loose structure characterises the delicate stroma penetrating the white and red pulp - reticulocytes are also characterised by indistinct borders, perinuclear swellings and unevenly coloured, somewhat granular cytoplasm. These characteristic signs of cell impression are likely due to the increased generation of reactive oxygen species caused by chronic exposure to elevated homocysteine concentrations. Such radicals have a toxic effect on cells and cause oxidation of phospholipids, accompanied by a violation of membrane structures within the cell and in the plasmalemma. The expansion of the cisterns of the granular endoplasmic reticulum, which fights intoxication, is manifested in the appearance of light vacuoles in the cytoplasm of cells (Fig. 3 B).

The parenchymatous elements of the spleen in old animals also do not remain unchanged. Periarterial sheaths formed by T-lymphocytes have numerous bright holes, which make these areas look like moth-eaten areas (Fig. 3B). Light vacuolated zones are present even directly around the wall of the central arteries (Fig. 3D). This is likely due to the partial death of the T cells due to the parallel hypomethylation of their DNA associated with chronically elevated plasma homocysteine levels that affected these and other splenic cells for months. Also, we cannot rule out the already mentioned negative impact of oxidative stress, which also caused intoxication of both the stroma and the parenchyma of this immune organ for months. One of the responses to the totality of these pathological influences among T cells is apoptosis. Further absorption of apoptotic bodies by macrophages (Fig. 3 B) increases the number of these cells in the periarterial sheaths while adding light spots to the general dark background of these zones.

B-cells often respond to external stimuli by proliferation, increasing the number and area of light germinal centres and the appearance of new ready-to-work plasma cells

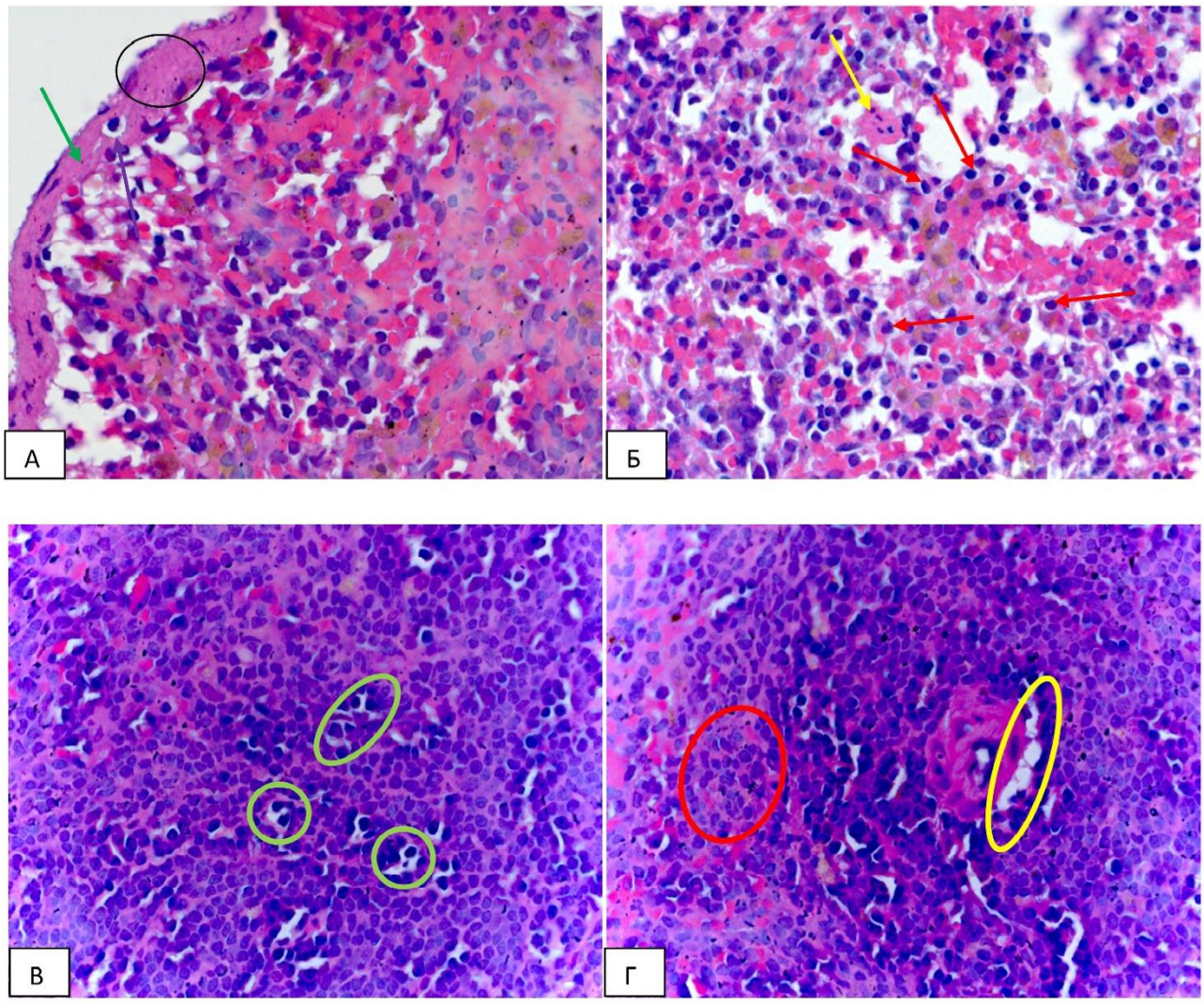

Fig. 3. Stroma and parenchyma of the spleen of old rats from the group with chronic hyperhomocysteinemia. A: collagen fibres (green arrow); capsule thickening (black oval); oedema in the perinuclear zone of the fibroblast (purple arrow); B: perinuclear swelling in reticular cells (red arrows); a macrophage with absorbed apoptotic bodies (yellow arrow); B: lumens of the periarterial sheath with apoptotic bodies (green ovals); D: perivascular vacuolisation near the central arteries (yellow oval); germinal centre (red oval). x1000.

inside the nodules and their marginal zones. However, in old animals, one should not forget such a factor as the general suppression of the immune system with age; B-cells are no longer so reactive in their response.

A detailed examination of the state of the B-cell component of lymph nodes allows us to note the presence of isolated germinal centres, which indicates the reaction of B-cells to chronic hyperhomocysteinemia (Figs. 3 D, 4 B). The direct consequence of the appearance of germinal centres is the formation of plasma cells in lymph nodes (Fig. 4 A). These cells are characterised by a nucleus slightly shifted to the periphery. Nevertheless, the white pulp is diluted with many reticular cells and fibroblasts, noticeable by the increased eosinophilic background around lymphoblasts, lymphocytes, and plasma cells. Marginal zones lose clarity, so the transition between white and red pulp is unclear (Fig. 4 B, C). The visual blurring of this transition is also facilitated by the infiltration of B cells into the red pulp, which is accompanied by their appearance between red blood cells and groups of platelets (Fig. 4. B).

The assessment of the state of the red pulp also confirms the age-complicated pathological effect of chronic hyperhomocysteinemia on the spleen. Among the red-shaped blood elements located in the venous sinuses and Billroth cords, a significant number of macrophages are noted, which dispose of old and affected elements, accumulating hemosiderin when digesting haemoglobin (Fig. 4 B). The increase in the number of macrophages containing lipofuscin inclusions in their cytoplasm is also worth noting. This increase indicates the natural processes of organ ageing and the increase in oxidative stress caused by the accumulation of reactive oxygen species under chronic hyperhomocysteinemia (Fig. 4D).

Conclusions and prospects for further

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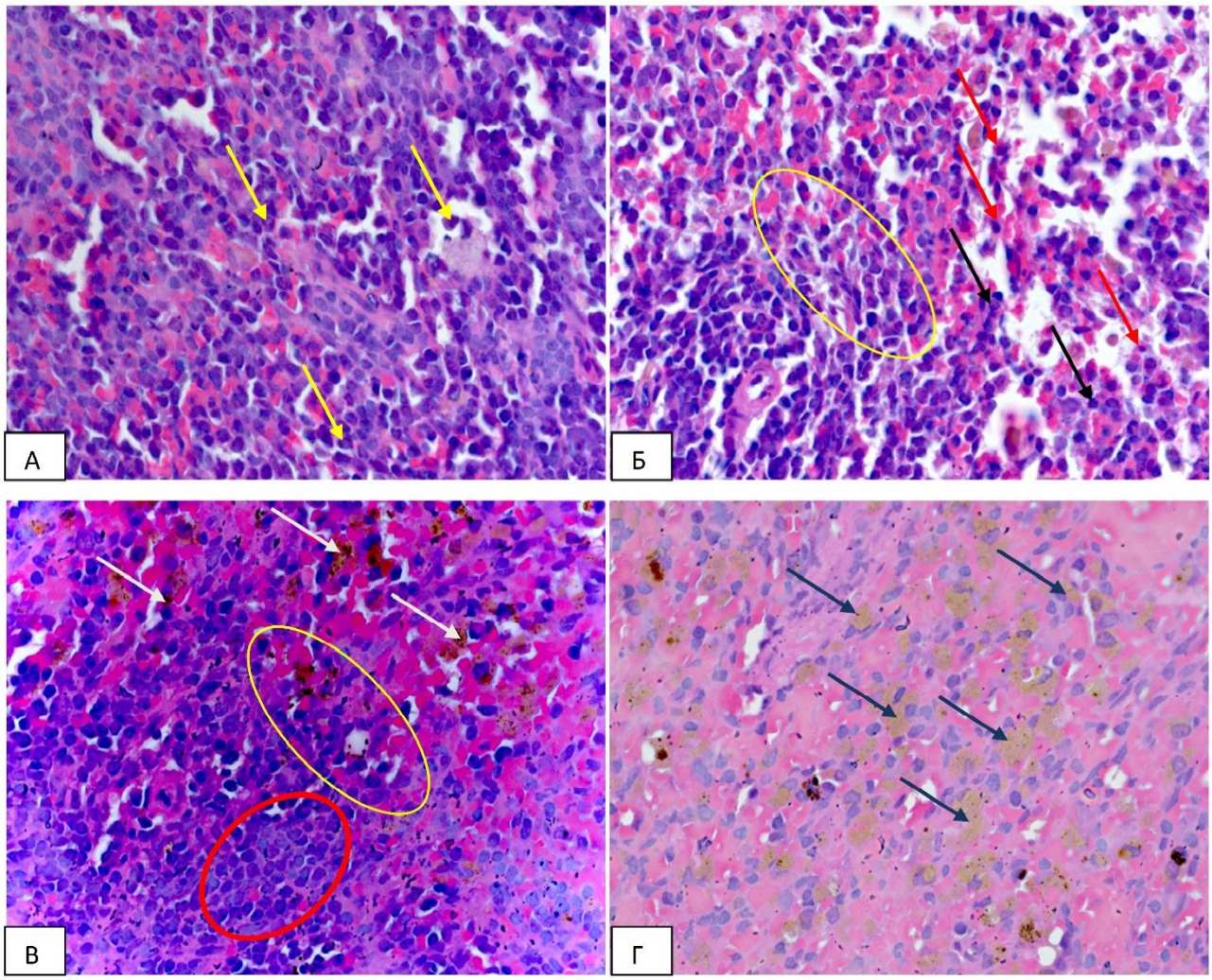

Fig. 4. Stroma and parenchyma of the spleen of old rats from the group with chronic hyperhomocysteinemia. A: plasma cells (yellow arrows); B: reticular cells (red arrows); B cells in the red pulp (black arrows); B: germinal centre (red oval); macrophages with hemosiderin inclusions (white arrows); B, C: marginal zones (yellow ovals); D: macrophages with lipofuscin inclusions (blue arrows). x1000.

development

1. Under conditions of chronic HHc in adult rats, densification and disorganisation of dense connective tissue fibres of the capsule and trabeculae of the spleen and vacuolisation of the cytoplasm of endotheliocytes of large-diameter vessels were noted. T-cells of the white pulp were subject to death by apoptosis, and B-cells of lymphoid nodules and marginal zones showed signs of marked proliferation.

2. Simulation of persistent HHc in old rats led to changes in the spleen's stromal and parenchymal structural elements. The organ's capsule lost the clarity of its contours and became blurred and thickened. Lightening zones characterised periarterial sheaths due to the massive death of T-lymphocytes. The number of bright germinal centres and plasma cells increased. The number of macrophages containing lipofuscin inclusions increased in the red pulp.

A promising direction is the significant expansion and deepening of research on the understanding of the development of pathological processes under conditions of hyperhomocysteinemia in the spleen of rats of various ages.

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ЗМІНИ МІКРОСКОПІЧНОЇ ОРГАНІЗАЦІЇ СЕЛЕЗІНКИ ДОРОСЛИХ І СТАРИХ ЩУРІВ ЗА УМОВ ХРОНІЧНОЇ ГІПЕРГОМОЦИСТЕЇНЕМІЇ

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Анотація. *Гомоцистеїн (Гц), що є продуктом метаболізму метіоніну, здатен чинити негативний вплив на структурно-функціональні параметри внутрішніх органів, у тому числі селезінки. Метою дослідження є вивчення мікроскопічних змін селезінки дорослих і старих щурів за умов хронічної гіпергомоцистеїнемії. Досліди виконано на 44 білих щурах-самцях (дорослі щури віком 6-8 місяців і старі щури віком 24-26 місяців). Під час експерименту тварин розподілили на контрольну та дослідну групи (по 11 особин у кожній групі). Хронічну ГГц досягали введенням D,L-тіолактон гомоцистеїну гідрохлориду тваринам дослідної групи в дозі 200 мг/кг маси тіла внутрішньошлунково (в/шл) на 1% розчині крохмального гелю 1 раз на добу протягом 8 тижнів. Після закінчення термінів експериментального моделювання хронічної гіпергомоцистеїнемії, тварин виводили з експерименту, знеживлюючи методом декапітації та використанням тіопенталового наркозу. Гістологічні препарати вивчали за допомогою світлового мікроскопа SEO SCAN. Виявлено, що за умов хронічної ГГц у дорослих щурів відмічали ущільнення та дезорганізацію волокон щільної сполучної тканини капсули і трабекул селезінки, вакуолізацію цитоплазми ендотеліоцитів судин великого діаметру. Т-клітини білої пульпи підлягали загибелі шляхом апоптозу, а В-*

клітини лімфоїдних вузликів і маргінальних зон проявляли ознаки вираженої проліферації. Моделювання стійкої ГГц у старих щурів призводило до змін стромальних і паренхіматозних структурних елементів селезінки. Капсула органу втрачала чіткість контурів, була розмитою, потовщеною. Периартеріальні піхви характеризувались зонами просвітлення внаслідок масивної загибелі Т-лімфоцитів. Зростала кількість світлих гермінативних центрів та плазмоцитів. В червоній пульпі збільшувалась чисельність макрофагів, що містили включення ліпофусцину. Всі ці зміни були викликані подразнюючою дією надмірних доз гомоцистеїну, зокрема оксидативним стресом, нітрозилюванням, що пригнічує механізми адаптації клітин до цього стресу та гіпометилюванням ДНК клітин.

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