Association between hyperhomocysteinemia, proliferation and polyploidization of hepatocytes

Natalya N. Chuchkova*, Ksenia A. Pazinenko, Marina V. Smetanina

Address: Department of Medical Biology, Izhevsk State Medical Academy, Izhevsk, Russia

Email: Natalya N. Chuchkova – mig05inbx.ru

Ksenia A. Pazinenko, k.pazinenko@yandex.ru

Marina V. Smetanina, lisenok0910@rambler.ru

Abstract. Hyperhomocysteinemia is a factor in the development of non–alcoholic fatty liver dystrophy. Aim of research is to find out the effect of hyperhomocysteinemia on the regenerative process of liver cells.

Materials and methods. The histological structure of the liver was studied in Rattus norvegicus Berk white rats (n=25) (control group – 10 animals, comparison group with moderate hyperhomocysteinemia – 15 animals). On histological sections stained with hematoxylin and eosin, the following were calculated: the number of nuclei (per 100 μm^2), the proportion of binuclear cells (%), the area (in μm^2) and the diameter (in μm) of the nucleus; the nuclear cytoplasmic index was calculated. We using Immunohistochemical stain by antibodies to detect the expression of the Ki-67 marker (rabbit IgG, 1:200; Cell Marque Corporation, USA), the number and intensity of expression of Ki-67-positive cells in the field of view of the microscope were determined. The data of the group indicators were analyzed using analysis of variance (ANOVA) by SPSS software. The differences between the groups were considered statistically significant at $p<0{,}05$. Results. In the liver of animals with moderate hyperhomocysteinemia, the presence of two processes was revealed at the same time. It is reactive-dystrophic (the presence of periportal leukocyte-lymphocyte infiltrates, the appearance of cells in a state of dystrophy and necrosis) and regenerative (an increase in the core area from 52,51±4,5 to 56,68±5,58 μm^2, nuclear-cytoplasmic ratio, an increase in the number and intensity of Ki expression-67+ cells). The presence of hepatocytes with very large nuclei (polyploid), which make up 12,5% of the entire population is characteristic of

homocysteine-induced liver pathology. Conclusion. Hyperhomocysteinemia, along with a decrease in the number and dystrophy of individual hepatocytes, leads to an increase in the diameter and area of the cell nucleus, an increase in the intensity of proliferation, the appearance of polyploid nuclei, which increases the regenerative potential of the liver and provides a crucial role in the homeostasis of the gland. Finding data obtained require further research to determine the "critical point" of the transition, which will allow modulating the functions of liver tissue.

Keywords: hyperhomocysteinemia, hepatocytes, proliferation, polyploidy

Introduction**.** In a healthy liver, hepatocytes are mostly at rest [1]. It was found that 84,1% of hepatocytes do not multiply in the liver over a span of 9,5-9,7 months [2]. It is also known that the liver has a high capacity for reparative regeneration and the proliferative activity of hepatocytes increases with resection and damage to the gland, but the mechanisms and biomarkers of drug damage to the liver are less well known [3, 4]. Numerous studies have shown that in the adult liver there is a connection between polyploidy and various cellular stresses, such as metabolic overload, DNA damage, chemical or drug damage to the liver [5, 6, 7]. Hyperhomocysteinemia is a factor of non–alcoholic fatty liver dystrophy [8]. In studies, there are indications of the relationship between non-alcoholic liver damage and the formation of polyploid cells [9, 10].

Aim of research is to find out the effect of hyperhomocysteinemia on the regenerative process of liver cells.

Materials and methods. The research was carried out on white rats Rattus norvegicus Berk of both sexes (25 animals) with a body weight of 200-220 gram in the autumn period (October-November), in the afternoon. The study was approved of the Committee on Biomedical Ethics of the Izhevsk State Medical Academy of the Ministry of Health of the Russian Federation (№ 656 of 23.04.2019). The animals were divided into 2 experimental groups: № 1 – intact control (n=10) – kept in a vivarium on a standard diet (extruded feed with free access to water); № 2 – comparison group (n=15) – animals with experimental moderate form of hyperhomocysteinemia (HHC) [11].

The animals were kept in compliance with the "Rules for carrying out work using experimental animals" (Order of the Ministry of Higher and Secondary Special Education of the USSR № 742 11.13.1984) and the Interstate Standard "Guidelines for the maintenance and Care of Laboratory Animals (2016). The liver was extracted after euthanasia of animals, fixed in 10% formalin, poured into Histomix paraffin medium, serial organ sections were prepared. Some histological preparations were stained with hematoxylin and eosin to assess the histo- and cell-structure of the tissue, the other part was stained immunohistochemically using a set of antibodies to detect the expression of the Ki-67 marker (rabbit IgG, 1:200; Cell Marque Corporation, USA). We used a mixture of second antibodies associated with Alexa Fluor 488 (anti-rabbit IgG 1:300; Webcam, USA) and Alexa Fluor 647 (anti-mouse IgG 1:300; Abcam, USA) for double immunofluorescence staining. On histological sections stained with hematoxylin and eosin, the following were calculated: the number of nuclei (per 100 μm^2), the proportion of binuclear cells (%), the area (in μm^2) and the diameter (in μm) of the nucleus; the nuclear cytoplasmic index was calculated. The number of Ki-67-positive cells was estimated in the field of view of a microscope at 400x magnification in 10 random fields of view on each 5 slice. We measured luminous intensity of the immunoresponsive product (in units) on frontal slices using morphometric programs Image ProInsite 8.0, Image ProPlus 6.0 (MediaCybernetics). The slices were studied using a Nikon ECLIPSE E200 fluorescent microscope.

The statistical method was used to determine the arithmetic mean (M), its error of mean (m). The results of the study were checked for the normality of the distribution using the criterion Shapiro-Wilk's. The data of the group indicators were analyzed using analysis of variance (ANOVA) by SPSS software. The differences between the groups were considered statistically significant at $p<0,05$.

Results and their discussion

The level of homocysteine in the blood of experimental rats with hyperhomocysteinemia was 28,9±2,65 umol/l, in control animals was 8,5±0,6 umol/l. Pathohistological analyze of the liver of animals with HHC revealed the presence of two simultaneous processes in the tissue – reactive-dystrophic and

regenerative in nature. Reactive-dystrophic processes included the presence of periportal leukocyte-lymphocyte infiltrates, the appearance of cells in a state of dystrophy and necrosis. Significantly (by 1,75 times, $p<0,05$) the density of hepatocyte cells per unit area decreased (from 448,1±23,3 in control animals to 256,2±15,5 μm^2 in rats with HHC), the area of hepatocyte cytoplasm decreased by 1,55 times (from 326,03±19,69 to 210,19±27,2 μm^2). At the same time, the average area of a single nucleus increases from 52,51±4,5 to 56,68±5,58 μm^2, the nuclear-cytoplasmic ratio increases by 1,65 times (from 0,17±0,04 to 0,28±0,06, $p <0,05$). To assess the regenerative possibility of liver cells, we performed an immunohistochemical analysis of the expression of the Ki-67 protein in animals with hyperhomocysteinemia. In the control group of animals, the Ki-67 protein is present in a small part of the cells (11,5±1,11 pcs/μm^2), the intensity of the histochemical product is insignificant (39,85±1,86 relative units in the field of view). While hyperhomocysteinemia leads to an increase in cells expressing the proliferation marker by 2,63 times (30,3±4,09 pcs/ μm^2), and to an increase in the intensity of expression by 1,68 times (67,01 ± 1,62 relative units in the field of view).

The proliferation index in hyperhomocysteinemia increases by 4 times: from 0,03±0,01 in the population of control hepatocytes to 0,12± 0,07 relative units at HHC ($p<0,05$). It is known that after toxic liver damage caused by paracetamol, the cells surrounding the necrosis zone undergo proliferation, but are accompanied by desynchronization of the cell cycle during regeneration [4]. In addition, recent studies reveal the role of Ki-67 in the regulation of the cell cycle as a whole, the maintenance of heterochromatin and the assembly of the perichromosomal layer on mitotic chromosomes [12, 13]. Characteristic of homocysteine-induced liver pathology is the presence of hepatocytes with very large nuclei and a high nuclear-cytoplasmic ratio (0,296±0,17 vs 0,197±0,12 – control), which make up 12,5% of the entire population. No such cells were observed in the control. Presumably, such hepatocytes are polyploid. Polyploid hepatocytes provide regenerative and adaptive possibilities of the liver, provide protection of the gland from oxidative stress and genotoxic damage [5,14]. The fact of the appearance of large-nuclear (presumably polyploid) hepatocytes can

be associated with an increase in the proliferation index. It is known that hepatocyte polyploidy is the result of both nuclear polyploidy (an increase for DNA per nucleus) and cellular polyploidy (an increase in the number of nuclei per cell), while cells can decrease in size [5].

Conclusion. The multidirectional changes in the hepatocyte population obtained by us: on the one hand, a decrease in the number of cells, dystrophic changes in them, on the other hand, an increase in the diameter and area of cell nuclei in part of cells, an increase in the intensity of proliferation, the appearance of polyploid nuclei, indicate various mechanisms of the effect of increased homocysteine content on liver cell populations, on the one hand, its total cytotoxic effect and, at the same time, increase the regenerative potential of the liver, providing a crucial role in the homeostasis of the gland. The data obtained require further research to determine the "critical point" of the transition to an in-depth pathological process, which will allow modulating certain functions of liver tissue.

Conflict of interest information: the authors declare no conflict of interest.

References:

1. *Michalopoulos, G.K. & Bhushan, B. Liver regeneration: biological and pathological mechanisms and implications. Nat Rev Gastroenterol Hepatol, 18(1):40-55, 2021.*

2. *Chen, F., Jimenez, R.J., Sharma, K., Luu, H.Y., Hsu, B.Y., Ravindranathan, A., Stohr, B.A. & Willenbring, H. Broad distribution of hepatocyte proliferation in liver homeostasis and regeneration. Cell Stem Cell, 26(1):27-33.e4, 2020.*

3. *Clemens, M.M., McGill, M.R. & Apte, U. Mechanisms and biomarkers of liver regeneration after drug-induced liver injury. Adv Pharmacol., 85:241-262, 2019.*

4. *Bhushan, B., Gunewardena, S., Edwards, G. & Apte, U. Comparison of liver regeneration after partial hepatectomy and acetaminophen-induced acute liver failure: A global picture based on transcriptome analysis. Food Chem Toxicol, 139:111186, 2020.*

5. Donné, R., Saroul, M., Maillet, V., Celton-Morizur, S. & Desdouets, C. La polyploïdie hépatique - Dr Jekyll ou Mr Hyde [Hepatic polyploidy: Dr Jekyll or Mr Hyde]. Med Sci (Paris), 35(6-7):519-526, 2019.

6. Lin, S., Nascimento, E.M., Gajera, C.R., Chen, L., Neuhöfer, P., Garbuzov, A., Wang, S. & Artandi, S.E. Distributed hepatocytes expressing telomerase repopulate the liver in homeostasis and injury. Nature, 556(7700):244-248, 2018.

7. Campana, L., Esser, H., Huch, M. & Forbes, S. Liver regeneration and inflammation: from fundamental science to clinical applications. Nat Rev Mol Cell Biol., 22(9):608-624, 2021.

8. Vatsalya, V., Gala, K.S., Hassan, A.Z., Frimodig, J., Kong, M., Sinha, N. & Schwandt, M.L. Characterization of Early-Stage Alcoholic Liver Disease with Hyperhomocysteinemia and Gut Dysfunction and Associated Immune Response in Alcohol Use Disorder Patients. Biomedicines, 9(1):7, 2020.

9. Н.Н. Чучкова, Н.В. Кормилина, П.В. Смирнов Морфофункциональная оценка нуклеолярного аппарата гепатоцитов и лимфоцитов брыжеечных лимфоузлов крыс при измененном характере питания // Вестник УдГУ. Серия Биология. - №3. – 2016. –. N.N. Chuchkova, N.V. Kormilina, P.V. Smirnov. Morfofunkcional'naya ocenka nukleolyarnogo apparata gepatocitov i limfocitov bryzheechnyh limfouzlov krys pri izmenennom haraktere pitaniya //Vestnik UdGU. Seriya Biologiya. - №3. – 2016. – 98-104.

10. Gentric, G., Maillet, V., Paradis, V., Couton, D., L'Hermitte, A., Panasyuk, G., Fromenty,. B, Celton-Morizur, S. & Desdouets, C. Oxidative stress promotes pathologic polyploidization in nonalcoholic fatty liver disease. J Clin Invest., 125(3):981-92, 2015.

11. Pazinenko, K.A., Chuchkova, N.N., Smetanina, M.V., Pazinenko, O.A., Panteleev, K.E., Ivanov, G.V. & Kormilina N.V. Dynamics of Biochemical and Cytological Parameters of Rat Blood in Simulated Chronic Alimentary Methionine-Induced Homocysteinemia. Journal Biomed, 17(2):46-57, 2021.

12. Cuylen, S., Blaukopf, C., Politi, A.Z., Müller-Reichert, T., Neumann, B., Poser, I., Ellenberg, J., Hyman, A.A. & Gerlich, D.W. Ki-67 acts as a biological surfactant to disperse mitotic chromosomes. Nature, 535(7611):308-312, 2016.

13. Sun, X. & Kaufman P.D. Ki-67: more than a proliferation marker Chromosoma, 127(2):175-186, 2018.

14. Matsumoto, T., Wakefield, L., Tarlow, B.D. & Grompe, M. In vivo lineage tracing of polyploid hepatocytes reveals extensive proliferation during liver regeneration. Cell Stem, 26(1):34-47, 2020.