ORIGINAL RESEARCH

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MODELING OF LIVER HYPOXIA IN AN *IN VIVO* EXPERIMENT

We studied an effect of the portal triad blood flow reduction on liver enzymes, the organ status and survival of laboratory mice. The study included Balb/c mice divided into groups (n=6 for each group): group 1 – controls, blood and liver were collected; group 2 – liver blood flow reduction for 20 minutes, blood and liver were collected 2 hours after the procedure; group 3 – liver blood flow reduction for 20 minutes, blood and liver were collected 24 hours after the procedure; group 4 – liver blood flow reduction for 20 minutes, and the follow-up during the next 14 days to assess the survival. The blood flow was reduced by occlusion of the portal triad: the animals underwent laparotomy, then the portal triad was isolated and a needle with suture material was brought under it; weights were attached to the ends of the suture material, and the vessels were occluded due to the tension of the ligature. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in both groups with reduced blood flow were statistically significantly higher than in the control group ($p<0.05$), and also statistically significantly higher in the group with blood sampling 24 hours after reperfusion, compared to the group with blood sampling 2 hours after reperfusion ($p<0.05$). Histological examination showed signs of ischemic damage in tissues, and an increase in the number of vessels in liver samples of animals with blood flow reduction, compared to the control. The animal survival after the procedure was over 80%, which was satisfactory, but nevertheless indicated the need for such a number of animals that will allow statistical processing of the results even if some animals die. The results of the study demonstrated the model as an important tool for the study of ischemic and hypoxic-associated pathological liver states.

Keywords: hypoxia, oxygenation, liver, liver diseases, *in vivo* models.

Introduction. Insufficient supply of tissues with oxygen and the resulting hypoxia promote many diseases, and in some cases they are the main factors in the development of some pathological conditions [3, 7, 10]. Inadequate tissue oxygenation leads to oxidative stress, inflammatory processes, and dysfunction of key units in lipid and protein metabolism [6, 9]. Recent studies have demonstrated the importance of hypoxia in the development of liver diseases, such as steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma [9]. In addition, cancer studies revealed the key role of the hypoxic microenvironment in the formation of a metastatic niche, epithelial-mesenchymal transition, resistance to therapy, and a more malignant phenotype of tumor cells [8].

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To understand the course of various biological processes and the influence of the environment, the use of the "corresponding microenvironment – cell culture" system is the first step towards obtaining fundamental ideas about the complex interaction between the microenvironment and cells [2, 5]. 2D cell culture has been an important tool for understanding the mechanisms of cell behavior *in vivo* for more than a century; however, 2D culture fails to replicate the physiology of real tissues. Culturing of cell monolayers leads to a change in the tissue-specific architecture (forced polarized adhesion, a flattened shape), transformation of mechanical and biochemical signals, and a two-dimensional contact with neighboring cells [4]. In this regard, an *in vivo* study is required to confirm the phenomenon or mechanism observed *in vitro* [1, 4]. There are various experimental approaches to control oxygenation in *in vivo* studies of the hypoxia effects, including the use of special chambers and exposure of animals to a gas mixture with a reduced concentration of O2, temperature modification, pharmacological drugs or substances, and reduction of blood flow to study area [5]. One of the most effective methods of liver hypoxia modeling involves clamping blood vessels, namely, occlusion of the portal triad, including the hepatic artery, hepatic vein, and bile duct [11]. However, the method is technically complex; in addition, we have not found literature data on the safe duration of reduced blood flow in the liver, since a long-term study implies high survival of laboratory animals after the procedure, and the preservation of the main functions of the liver during several days to several weeks.

In this regard, the purpose of this study was to reveal the effect of portal triad occlusion on the liver state and survival of laboratory mice.

Material and methods. *Animals and their maintenance.* The study included female Balb/c mice aged 9-12 weeks with an average weight of 25 g. The animals were bred in the vivarium of the Experimental Laboratory Center, National Medical Research Centre for Oncology. Mice were kept in conventional open cages with free access to food and water. All manipulations in the study were performed in accordance with the ethical principles established by the European Convention for the Protection of Vertebrate Animals used for Experimental or other Scientific Purposes (ETSN 123, Strasbourg, March 18, 1986). The study protocol was approved by the local bioethical committee of the National Medical Research Centre for Oncology.

Technique for reducing blood supply to the liver by the portal triad occlusion. Liver blood flow was reduced using the technique proposed by Zimmerman et al. (2012) [11]. Laparotomy was performed to provide access to the liver and its blood vessels. The stomach and intestines were displaced caudally with a wet cotton swab, and the right lobe of the liver was displaced closer to the diaphragm for a clear view of the portal triad. After its visual identification, a needle with suture material (polypropylene 4/0) was brought under the portal triad, including the he-

patic artery, hepatic vein and bile duct. At each end of the suture material, a weight of 3 grams was fixed on a special holder providing reduced blood flow in the vessels of the portal triad due to the tension of the suture material. Eppendorf tubes filled with water were used as the weight. After 20 minutes, the weights were removed, and the surgical wound was sutured in layers with a continuous suture.

Euthanasia. Animals were euthanized by decapitation with the following collection of biological material (blood and liver tissue).

Distribution by groups. All animals were divided into the following groups:

group 1 (n=6) – intact controls; animals were euthanized on day 1 of the experiment, and blood and liver were collected;

group 2 (n=6) – liver blood flow reduction for 20 minutes; animals were euthanized 2 hours after the procedure, and blood and liver were collected;

group 3 (n=6) – liver blood flow reduction for 20 minutes; animals were euthanized 24 hours after the procedure, and blood and liver were collected;

group 4 (n=6) – liver blood flow reduction for 20 minutes; animals were observed during the next 14 days to assess the procedure tolerability, and then were euthanized without collecting the biological material.

Anesthesia. All surgical procedures were performed using anesthesia: veterinary preparations Xila 20 mg/kg, and Zoletil-100 50 mg/kg.

Biochemical tests. Liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined in blood plasma. ALT was determined using the automatic biochemical analyzer VETSCANVS2, and AST was determined using the automatic biochemical analyzer VITROS 5600.

Histological examination. The liver was fixed in 10% formalin for 24 h and embedded in paraffin; histological microsections were prepared and stained with hematoxylin and eosin by the standard method.

Statistical analysis. The data were analyzed using the STATISTICA 10.0 program. Data were presented as mean ± standard error of the mean; comparison was performed using Student's t-test; differences were considered statistically significant at $p<0.05$.

Results and discussion. During the experiment on vascular occlusion with suture material and weights, we achieved a reduction in liver blood flow, visually confirmed by a change in the color of the liver (Figure 1).

According to the literature data, a system of suspended weights is less traumatic than vascular clamps, especially when reapplying the instrument for multiple cycles of ischemia-reperfusion to achieve the effects of hypoxia-reoxygenation, since even the thinnest clamps can cause significant damage [11]. However, we identified some special aspects that must be taken into account during the procedure, namely, avoiding too deep immersion of the needle when bringing it under the portal triad due to the risk of damage to the underlying vena cava. There is also a risk of damage to the triad by the cutting edges of the needle; to prevent this, the portal triad should be preliminarily isolated and held with tweezers, while bringing the needle with the suture material under it. Ischemia and hypoxia are not synonymous conditions, since well perfused tissues sometimes are not normoxic, and poorly perfused tissues sometimes are not hypoxic [3]. In this regard, we performed histological examination of the liver tissue to confirm the development of hypoxia in response to the blood flow reduction.

Histological examination of the liver of intact animals revealed normal tissue architecture without signs of inflammation

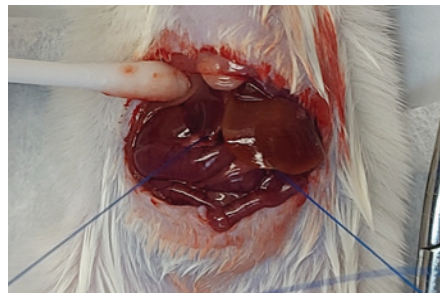

Fig. 1. Murine blood flow reduction with the portal triad occlusion

and necrosis, and without hepatocyte edema (Figure 2A). Samples obtained from animals with reduced blood flow demonstrated signs of ischemic liver damage: the tissue was characterized by an increase in the intercellular space, a noticeable edema of hepatocytes, decreased density of the cytoplasm, a large number of cells with karyolysis, and focal areas of necrosis (Figure 2B).

Also, the histological preparation obtained from an animal with induced liver hypoxia showed a much larger number of blood vessels (Figure 3B) than a corresponding preparation from an intact animal (Figure 3A), which can probably be interpreted as a phenomenon aimed at

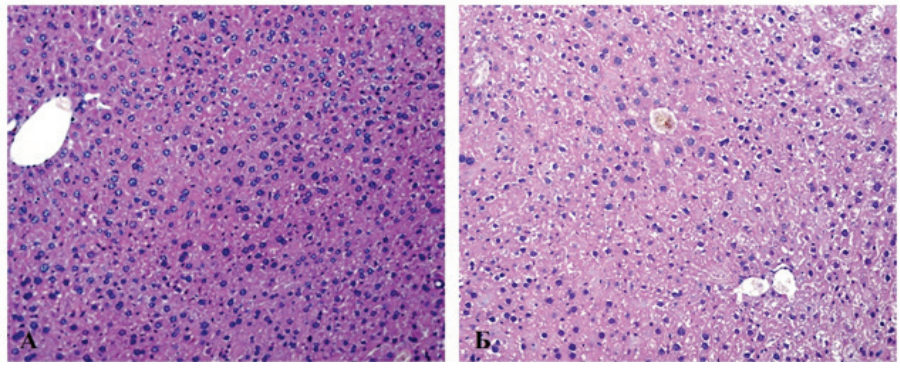

Fig. 2. Histological preparations of the Balb/c mouse liver. А – liver of an intact animal; Б – the animal's liver after a 20-minute blood flow reduction. Hematoxylin and eosin staining. Magnification: ×200

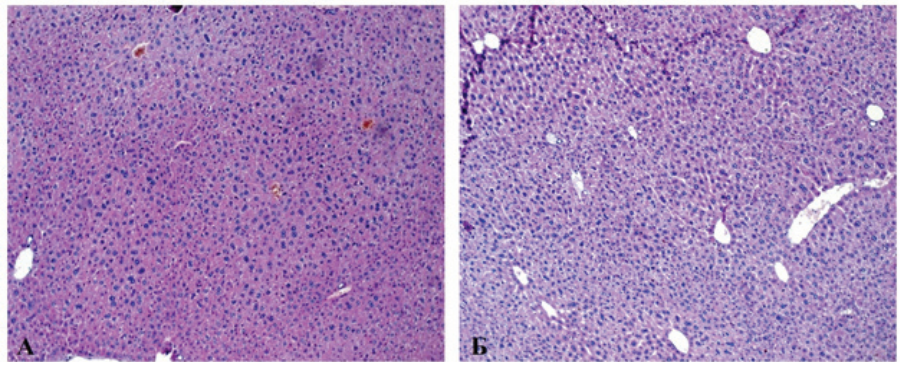

Fig. 3. Histological preparations of the Balb/c mouse liver. А – liver of an intact animal; Б – the animal's liver after a 20-minute blood flow reduction 24 hours after reperfusion. Hematoxylin and eosin staining. Magnification: ×100

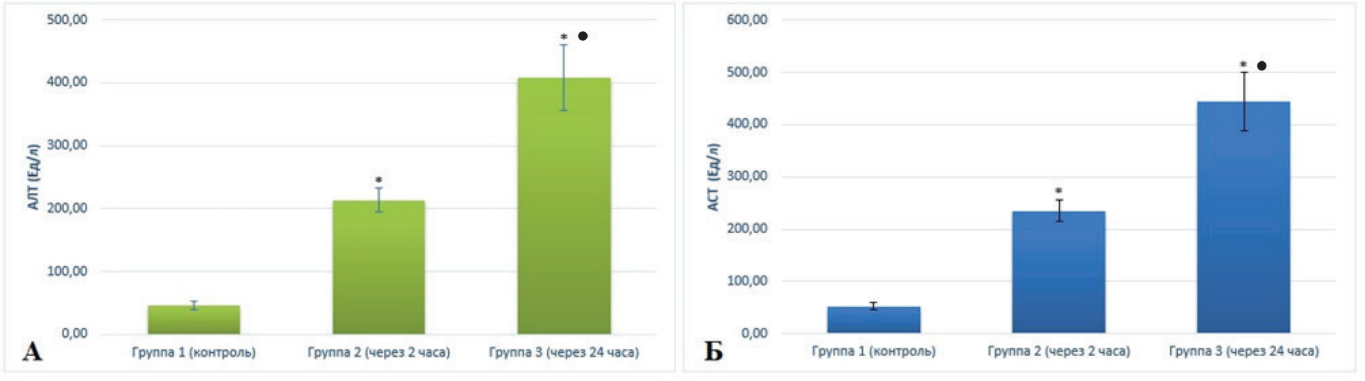

Fig. 4. Levels of liver enzymes in blood plasma in mice from groups 1-3. А – ALT levels; Б – AST levels. Note: * – statistically significant differences in enzyme levels between groups 2 and 3 and group 1 ($p<0.05$). ● – statistically significant differences in enzyme levels between group 2 and group 3 ($p<0.05$)

compensating for the resulting low tissue oxygenation.

The results of the study showed that the levels of liver enzymes in both groups with reduced blood flow were statistically significantly higher than in the control group ($p<0.05$). They were also statistically significantly higher in group 3 (24 hours after reperfusion) compared to group 2 (2 hours after reperfusion) ($p<0.05$). (Figure 4).

Determination of liver enzyme levels showed that ALT in intact mice was 40.8±6.3 U/L, in group 2 - 213.3±18.7 U/L, and in group 3 - 408.3± 52.9 U/L, which, respectively, was 5.2 and 10.0 times higher than in control animals. The level of AST in intact mice was 52.4±6.6 U/L, in group 2 - 235.3±20.7 U/L, and in group 3 - 443.8±56.4 U/L, which, respectively, was 4.5 and 8.4 times higher than in the control group. Elevated levels of liver enzymes in blood plasma were associated with ischemic cell damage; in addition, the amount of ALT and AST in the blood plasma of experimental animals increased during the day.

We also evaluated the survival rate of animals after the portal triad occlusion. One animal out of six (group 4) died the next day after the procedure, while the rest did not show signs of deterioration in health throughout the entire observation period. On the one hand, the demonstrated survival rate of more than 80% is a satisfactory result, but on the other hand, it indicated that careful planning of the number of animals is required in further experiments with technique in order to avoid losses that may preclude statistical processing of the results.

Conclusion. The results demonstrated that a 20-minute reduction of the portal triad blood flow contributes to severe hypoxia of the liver tissues, but at the same time, it is characterized by high survival rates of animals, which allows considering this method as an indispensable tool for liver pathology research.

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