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EFFICACY OF AN EXPERIMENTAL MODEL OF NON-ALCOHOLIC FATTY LIVER DISEASE BASED ON A HIGH-FAT DIET WITH CHOLESTEROL

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The purpose of the study was to establish the effectiveness and informativeness of the experimental model of non-alcoholic fatty liver disease based on high-fat diet and cholesterol in the experiment. The studies were performed on 60 white nonlinear adult male rats, weighing 180–200 g at the beginning of the experiment. During the experiment, the use of high-fat diet and cholesterol for 60 days resulted in an increase in body weight, rat body mass index, liver mass and liver mass index, increase in activity of alanine aminotransferase and aspartate aminotransferase, hyperglycemia. Morphological changes of liver tissues were characterized by fatty degeneration of hepatocytes, which had the form of microvesicular and macrovesicular vacuolation with deformation of the nuclei and their displacement to the periphery of the cell, a change in the size of the sinusoids. It is important to bring the experimental model as close as possible to the mechanisms of non-alcoholic fatty liver disease in humans.

Key words: liver, obesity, animal model, rats.

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ДІЄВІСТЬ ЕКСПЕРИМЕНТАЛЬНОЇ МОДЕЛІ НЕАЛКОГОЛЬНОЇ ЖИРОВОЇ ХВОРОБИ ПЕЧІНКИ НА ОСНОВІ ВИСОКОЖИРОВОЇ ДІЄТИ ТА ХОЛЕСТЕРИНУ

Метою роботи було встановити дієвість та інформативність експериментальної моделі неалкогольної жирової хвороби печінки на основі високожирової дієти та холестерину в експерименті. Дослідження проведені на 60 білих нелінійних статевозрілих щурах-самцях, з масою на початок експерименту 180–200 г. У процесі проведення експерименту використання високожирової дієти та холестерину протягом 60 днів призвело до збільшення маси тіла, індексу маси тіла щурів, маси печінки та індексу маси печінки, підвищенням активності аланінамінотрансферази й аспартатамінотрансферази, гіперглікемії. Морфологічні зміни тканин печінки характеризувалися жировою дистрофією гепатоцитів, яка мала вигляд мікровезикулярної та макровезикулярної вакуолізації з деформацією ядер і зміщенням їх до периферії клітини, зміною розмірів синусоїдів. Важливим є максимальне наближення експериментальної моделі до механізмів виникнення неалкогольної жирової хвороби печінки у людини.

Ключові слова: печінка, ожиріння, модель на тваринах, щури.

This study is a fragment of the research project "Features of compensatory and adaptive processes in various diseases and injuries of humans and animals, clinical and experimental justification of new surgical treatment methods" state registration No.: 0118U007342.

The study of the mechanisms of steatohepatitis and liver cirrhosis, assessment of hepatotoxicity of various substances, study of hepatoprotectors and the use of modern cellular technologies are impossible without an adequate model of non-alcoholic fatty liver disease in laboratory animals.

Non-alcoholic fatty liver disease (NAFLD) is considered a hepatic manifestation of metabolic disease and covers a range of liver pathologies [10, 14]. With the progression of NAFLD there is inflammation of the liver and early fibrosis, which mark the transition to non-alcoholic steatohepatitis (NASH) [10]. End-stages of NAFLD include inflammation, fibrosis, or cirrhosis of the liver. There is an increased risk of hepatocellular carcinoma (HCC) [10].

One of the main obstacles to the study of NAFLD is the lack of appropriate models on which to investigate the disease.

The study of pathogenetic and sanogenetic aspects of liver disease is usually carried out using experimental models, accompanied by profound structural changes in organ tissue, similar to such disorders in humans, but which are formed in a shorter period of time [1]. Because NAFLD is a systemic disease, its in vitro modeling using simple two-dimensional cell cultures is of limited interest. However, in each of the known models of hepatitis, liver cirrhosis, steatohepatosis and other liver diseases is often based on a toxic factor initiating liver damage. Highly toxic carbon tetrachloride (CCl4) is most used. It is administered subcutaneously [7], intragastrally or intraperitoneally [3] 1–3 times a week, with a dose ranging from 0.05 to 0.30 ml/kg. When CCl4 is administered, it is more often dissolved in olive oil [3]. The duration of administration can vary considerably (from 9 to 30 weeks), but usually the picture typical of cirrhosis (micro- or macronodular) is recorded after 8–12–15 weeks of induction. Simulations of toxic hepatopathy, fatty degeneration and liver cirrhosis contribute to rapid organic liver damage. However, the use of toxins has purely specific trigger mechanisms for the development of liver disease, which differ from the real causes that contribute to the pathogenesis of NAFLD in humans.

In this regard, to study the mechanisms of liver disease, to develop new approaches to their prevention and treatment using modern advances in science and technology, researchers need experimental models that are closest to real clinical conditions. Obesity caused by diet is the most common risk factor for developing NAFLD in humans. Excessive fat intake is a pathogenetic mechanism for the development of hepatic steatosis, with the introduction of cholesterol in the diet putting extra strain, which contributes to the development of inflammation. In addition, increasing the term of alimentary exposure allows form a severe degree of steatohepatitis and starts the mechanisms of fibrogenesis [4, 8].

The purpose of the study was to develop a model of fatty degeneration and liver fibrosis caused by natural factors.

Material and methods. Experimental studies were performed on 60 white nonlinear sexually mature male rats, weighing 180–200 g at the beginning of the experiment. Animal keeping and experiments were carried out in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 2005), "General ethical principles of animal experiments", adopted by the Fifth National Congress on Bioethics (Kyiv, 2013). The research protocols were approved by the Bioethics Committee of National M.I. Pirogov Memorial Medical University (protocol No. 6 of June 20, 2019) and do not contradict the bioethical standards of animal experiments.

Prior to the experiments, the animals were quarantined for 10 days. During this period, the animals received a full standard semi-synthetic starch-casein diet (SSSCD). Subsequently, the animals were divided into 2 groups: control – 30 intact animals, which continued to be fed the same diet, and experimental – 30 rats, which created a model of NAFLD, for which kept for 90 days on a high-calorie diet high in fat and high cholesterol (HFHC). The HFHC diet contained about 30 % fat (mostly saturated lipids) from the addition of cholesterol (obtained by mixing 2 g of cholesterol and 10 g of lard with 88 g of granules of a normal balanced diet) [12]. Cholesterol was included in the diet as one of the factors that activate lipid disorders, fatty infiltration of the liver. Dietary cholesterol has been shown to play an important role in the progression of NAFLD in NASH, causing inflammation and fibrosis [9], and has a potent effect even in small doses. An atherogenic diet is more effective in rats than in mice, as it can cause NASH, fibrosis, and cirrhosis in rats at 9 weeks [11].

The animals were systematically supervised and cared for. Feeding was carried out twice a day. Water was not restricted. In the room where the laboratory animals were kept, the temperature was constantly maintained at 24–25° C.

During the experiment, regular weekly weighing of animals was performed to monitor the dynamics of changes in body weight of rats in grams (g). Body length (from nose to anus) was determined in centimeters (cm) in all rats. Body weight and body length were used to determine the body mass index (BMI). BMI was calculated by the formula:

$$\text{BMI (g/cm}^2\text{)} = \text{Body weight}/(\text{Body length})^2. \quad (1)$$

All animals under thiopental anesthesia (40 mg/kg) were bled for biochemical examination. The blood was centrifuged for 15 min at 1500 g. Aliquots of serum were collected in Erpendorf microtubes and stored at -20 ° C until the study. In blood serum was determined by standardized methods: the activity of enzymatic markers of cytolysis - alanine aminotransferase (ALT), aspartate aminotransferase (AST).

All animals under thiopental anesthesia (40 mg/kg) underwent liver tissue samples for morphological examination. The liver was weighed on analytical balances. The relative weight of the liver was calculated by the formula:

(Liver weight/Body weight)*100 %. (2)

Macroscopic evaluation and description of animal liver tissues was performed after their removal.

Assessment of the morphological state of the liver in the experiment was performed based on histological examination by staining drugs with hematoxylin-eosin, Sudan III.

The micropreparations were studied using an SEO CCAN light microscope and photo-documented using a Vision CCD Camera with a histological preparation image output system.

Statistical analysis of the obtained results was performed using the program "STATISTICA 5.5" using non-parametric methods of evaluation of the obtained results. Differences between the compared samples were determined using the Mann-Whitney U test. The level of reliability of statistical indicators was taken as $p<0.05$.

Results of the study and their discussion. It was found that animals after the creation of the NASH model develop a syndrome of cytolysis and impaired protein synthesis. There was a significant increase in ALT activity, which was 111.55±4.02 U/l, which is 35.38 % more than in animals of the control group ($p<0.001$). ACT activity was 29.02 % higher than in control animals ($p<0.05$). Feeding after the creation of the full-fledged SSSCD model of NASH showed a lower activity of ALT by 14.38 % and ACT by 13.12 % compared to animals on HFHC diet.

The initial weight of rats (control 1) taken into the experiment after quarantine for 10 days, when the animals received a full SSSCD, was 205.0±6.1 g.

The weight of rats (control 2), which received a full SSSCD for another 60 days increased 1.41 times and amounted to 288.7±3.1 g

The weight of rats (control 3) that received a full SSSCD for another 90 days increased one and a half times and amounted to 309.0±8.2 g

During the experiment, the use of HFHC diet for 60 days led to an increase in body weight by 1.66 times, and after 90 days – by 1.87 times compared to baseline body weight (control 1), with a BMI greater than by 1.32 and 1.39 times respectively. Compared to the weight of rats that received a full SSSCD for 90 days (control 3), the body weight of rats with the model of NAFLD, which were kept for 90 days on HFHC diet was by 1.24 times greater, and BMI was by 1.25 times greater (table. 1).

During the experiment, the weight of the liver increased significantly. After using HFHC diet for 60 days, the relative weight of the liver was by 1.14 times greater than control 1, and after using HFHC diet for 90 days was by 1.18 times greater than control 3.

Table 1

Changes in body weight, body mass index and relative liver mass after the development of the NAFLD model in rats

	Body weight (g)	BMI (g/cm^2)	Relative liver weight (%)
Control-1 (1 day) (n=10)	205.0± 6.1#	0.62± 0.03	2.82± 0.09
Control-2 (60 day) (n=10)	288.7± 6.7*	0.69± 0.03	2.88± 0.06
Control-3 (90 day) (n=10)	309.0± 8.2*	0.69± 0.03	2.90± 0.07
NAFLD model (60 day) (n=15)	340.3± 5.6*Δ#	0.82± 0.05*Δ#	3.21± 0.05*Δ#
NAFLD model (90 day) (n=15)	382.4± 3.3*Δ#	0.86± 0.04*Δ#	3.33± 0.10*Δ#

Note: * – statistically significant difference with indicators in animals control-1 ($p<0.05$); Δ – statistically significant difference with indicators in animals control-2 ($p<0.05$); # – statistically significant difference with indicators in animal control-3 ($p<0.05$);

It was found that animals develop a syndrome of cytolysis and protein synthesis after the creation of the NAFLD model. There was a significant increase in ALT activity on the 60th day of feeding HFHC diet, which was 111.55±4.02 U/l, which is 35.38 % more than in animals of the control group ($p<0.001$). ACT activity was 29.02 % higher than in control animals ($p<0.05$).

Increased biochemical markers of liver tissue damage indicate the presence of structural and functional changes in hepatocytes with the development of cytolysis and cholestasis in the occurrence of NASH. An increase in glucose levels of 29.07 % was observed in rats of the experimental group after 60 days of HFHC diet feeding. To confirm the effectiveness of the model, a morphological study of rat liver after 60 days of HFHC diet feeding, significant accumulation of lipids in hepatocytes in the form of large droplets mainly in the central vein, which caused cell hypertrophy, narrowing of sinusoidal lumens. Central venous plethora was observed.

Fatty hepatocyte dystrophy took the form of microvesicular and mainly macrovesicular vacuolation, which was manifested in the presence of large lipid droplets in the cytoplasm of hepatocytes. There was a visible increase in the size of hepatocytes compared with the control group of animals. The filling of the cytoplasm with fat droplets caused deformation of the nuclei and their displacement to the periphery of the cell (fig. 1).

A significant proportion of hepatocytes showed signs of dystrophy. Cells became hypertrophic, changing their shape to more rounded in parallel with the decrease in basophilia and vacuolation of the cytoplasm by lipid inclusions. The greatest accumulation of lipids was observed in hepatocytes near the portal zones, and lipid vacuoles were found in both large and small sizes. With predominantly macrovesicular vacuolation, the cytoplasm had an optically empty appearance, the nucleus shifted to the periphery of the cell (fig. 2). There are hepatocytes, the cytoplasm of which is filled with lipids. The nature of the histological picture of liver tissue at 60 days of feeding HFHC diet can be identified as steatohepatitis.

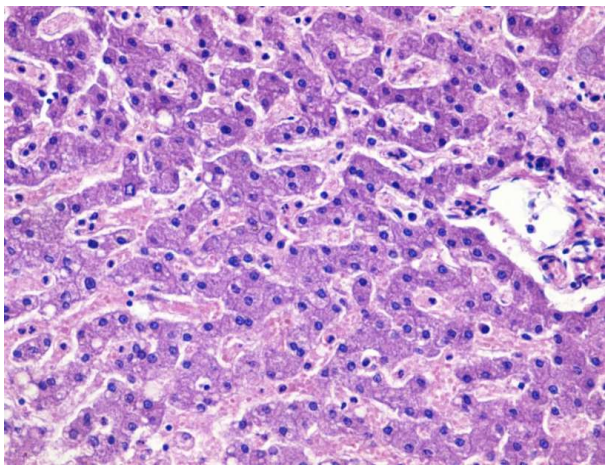

Fig. 1. Rat liver for 60 days of feeding HFHC diet. Diffuse predominantly microvesicular fatty degeneration of hepatocytes. Hematoxylin-eosin, x 400.

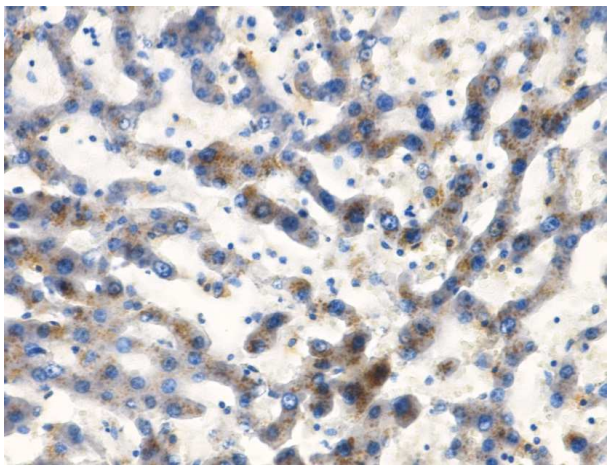

Fig 2. Liver of a rat on the 60th day of feeding HFHC diet. Preferably macrovesicular fatty degeneration of hepatocytes. Sudan III, x400. Sudan III, x 400.

Thus, the use of HFHC diet for self-feeding rats led to the development in experimental animals of steatohepatitis, characterized by the phenomena of membrane destruction, signs of fatty, hydropic dystrophy, violation of the histological architecture of hepatocytes. Such histological changes are also characteristic of people with NAFLD. There are changes in the biochemical parameters of the blood, which increase depending on the duration of the experimental diet: hyperenzymemia, hyperglycemia. It is important to bring the experimental model as close as possible to the mechanisms of NAFLD in humans. The obtained data are consistent with the results of other researchers [1, 5].

Submicroscopic studies have shown that experimental NAFLD (model using HFHC diet) in the liver on the background of microcirculation disorders develop significant changes in plasma, nuclear and intracellular membranes of endothelial cells and hepatocytes [6]. According to the literature, the inclusion in the high-fat diet of cholesterol promotes the progression of processes from steatosis to NASH and may act synergistically with lipids, causing NASH, possibly by directly damaging organelle membranes and increasing the production of reactive oxygen species. In turn, this leads to hepatocellular damage, which contributes to inflammation, hepatocellular decay and, ultimately, activates the mechanisms of recovery, including fibrogenesis [13].

The use of the model of NAFLD based on HFHC diet allows to conduct experiments that in the clinic are almost impossible to perform [2]. The sequence of identified processes allows to establish one or another link of pathological mechanisms of NAFLD development and to determine the strategy of hepatotropic treatment.

Conclusions

1. The use of HFHC diet contributed to the development of obesity and NAFLD in rats, which was characterized by an increase in body weight, body mass index of rats, liver weight and liver mass index, increased ALT and ACT activity, hyperglycemia.

2. Morphological changes of liver tissues were characterized by fatty degeneration of hepatocytes, which had the form of microvesicular and macrovesicular vacuolation with deformation of the nuclei and their displacement to the periphery of the cell, changing the size of the sinusoids.

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