

EXPERIMENTAL RESEARCHES**BIOCHEMICAL AND HISTOLOGICAL CHANGES IN TWO NON-ALCOHOLIC FATTY LIVER DISEASE MODELS OF DIFFERENT SEVERITY****ABSTRACT**

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Background. One of the priority areas of modern medicine, which unites the interests of various specialists (therapists, cardiologists, gastroenterologists, endocrinologists), is the study of the pathogenesis and clinical manifestations of non-alcoholic fatty liver disease (NAFLD), which is widespread and of unconditional social significance. The search for adequate experimental models of NAFLD that reflect the severity of liver damage is of paramount importance for studying its etiology and pathogenesis.

The aim of the study. To compare biochemical and histological changes in experimental models of NAFLD of varying severity.

Materials and methods. Two NAFLD model versions were used: a light one – non-alcoholic steatosis (NAS) and a severe variant – non-alcoholic steatohepatitis (NASH). The following biochemical parameters were measured: enzyme activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (AP), plasma glucose concentration, total protein (TP), total bilirubin (TBil) and its conjugate fraction (CB), plasma concentrations of homocysteine (HC), total cholesterol (TC), triacylglycerides (TG), catalase (Cat), superoxide dismutase (SOD) and malondialdehyde (MDA).

Results. When used in a model of steatohepatitis, liver function was impaired to a significantly greater extent than in the model of steatosis; this difference was manifested in a statistically significant increase in ALT, AST, AP, TC, TBil, MDA ($p < 0.001$) and a decrease in Cat, SOD ($p < 0.05$). This is confirmed by the development of more pronounced symptoms of disorders of pigment and lipid metabolism, cytolytic and cholestatic syndromes, significant activation of lipid peroxidation and depression of the antioxidant system when modeling non-alcoholic steatohepatitis. Various degrees of severity of morphological changes in the experimental groups were revealed.

Conclusion. The study showed the priority of determining biochemical markers, including the levels of ALT, AST, OBIL, TG, MDA and SOD to optimize laboratory methods for diagnosing the severity of liver dystrophy.

The practical originality of the results lies in the optimization of the methodology for laboratory diagnosis of the severity of the pathological process in NAFLD.

Key words: non-alcoholic fatty liver disease, hepatic steatosis, steatohepatitis, metabolism, rats, lipid peroxidation, malondialdehyde, superoxide dismutase

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БИОХИМИЧЕСКИЕ И ГИСТОЛОГИЧЕСКИЕ ИЗМЕНЕНИЯ НА ДВУХ МОДЕЛЯХ НЕАЛКОГОЛЬНОЙ ЖИРОВОЙ БОЛЕЗНИ ПЕЧЕНИ РАЗЛИЧНОЙ СТЕПЕНИ ТЯЖЕСТИ

РЕЗЮМЕ

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Введение. Одним из приоритетных исследований современной медицины, объединяющих интересы различных специалистов (терапевтов, кардиологов, гастроэнтерологов, эндокринологов), является изучение патогенеза из заболеваний неалкогольной жировой болезни печени (НАЖБП), для которой характерна распространённость и безусловная инновационность. Поиск адекватных экспериментальных моделей НАЖБП, отражающих степень тяжести повреждения печени, имеет первостепенное значение для изучения её этиологии и патогенеза.

Цель исследования. Определение биохимических маркеров для определения степени тяжести неалкогольной жировой болезни печени.

Материалы и методы. В эксперименте использовались два варианта модели НАЖБП: лёгкий – неалкогольный стеатоз (НАС), тяжёлый – неалкогольный стеатогепатит (НАСГ). Измеряли следующие биохимические показатели: активность ферментов аланинаминотрансферазы (АЛТ), аспартатаминотрансферазы (АСТ), лактатдегидрогеназы (ЛДГ), щелочной фосфатазы (ЩФ), глюкозы в плазме, общего белка (ОБ), общего билирубина (Обил) и его прямых соединений (ПБ), состояния в плазме гомоцистеина, холестерина (ОХ), триацилглициеридов (ТГ), каталазы (Кат), супероксиддисмутазы (СОД) и малонового диальдегида (МДА).

Результаты. На моделях стеатогепатита функция печени нарушается значительно большей степени, чем при стеатозе; этот фактор проявился в динамике повышения АЛТ, АСТ, ЩФ, ОХ, Обил, МДА ($p < 0,001$) и снижения Кат, СОД ($p < 0,05$), что способствует развитию более выраженных проявлений пигментного или липидного обмена, цитолитических и холестатических синдромов, активации ПОЛ и депрессии антиоксидантной системы при моделировании неалкогольного стеатогепатита. Также выявлена различная степень выраженности морфологических изменений в экспериментальных группах.

Выводы. Исследование показало приоритетность определения биохимических маркеров, в том числе уровней АЛТ, АСТ, Обил, ТГ, МДА и СОД, для оптимизации лабораторной методики диагностики степени тяжести дистрофии печени.

Практическая оригинальность результатов заключается в оптимизации методологии лабораторной диагностики степени тяжести патологического процесса при НАЖБП.

Ключевые слова: неалкогольная тяжёлая болезнь печени, стеатоз печени, стеатогепатит, обмен веществ, крысы, перекисное окисление липидов, малоновый диальдегид, супероксиддисмутаза

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a clinical and laboratory syndrome characterized by profound disorders of lipid metabolism, morphologically manifested by lipid deposition in hepatocytes [1]. The current classification of NAFLD includes three stages: non-alcoholic steatosis (NAS), non-alcoholic steatohepatitis (NASH) and liver cirrhosis.

Currently, NAFLD is not only the predominant liver pathology in the world, but also a component of the metabolic syndrome [2]. Recent screening studies in Russia found NAFLD in 27 % of people, with 80 % of these patients diagnosed with NAS, 17 % with NASH, and 3 % with cirrhosis [3]. Up to 80 % of all cases of liver cirrhosis in Russia are directly caused by NAFLD [4]. Manifestations of NAFLD and metabolic syndrome occur in 30 % of all therapeutic patients in Russia [5]. The increased incidence of NAFLD directly correlates with increased cardiovascular and endocrine pathology [6, 7]. This high prevalence of NAFLD is associated with modern trends in nutrition and the prevalence of a sedentary lifestyle among the population [8].

One theory for excess lipid accumulation in hepatocytes is a decrease in the oxidation of free fatty acids (FFA) in mitochondria, as well as an increase in the delivery of FFA to the liver. Progressive accumulation of FFAs causes direct damage to cell membranes, activation of lipid peroxidation (LPO), oxidative stress, chronic inflammation (NASH), collagenogenesis and progressive fibrosis.

The lack of effective methods for the treatment and prevention of NAFLD is due to insufficient understanding of its etiology and pathogenesis. Liver biopsy is still the gold standard for diagnosing NAFLD. But its use is not always appropriate, and it cannot be used in all patients [9]. Thus, the relevance of the study lies in the validation of existing models of liver damage, as well as elucidation of aspects of the development of the pathological process over time using a number of biochemical indicators. Considering the above, we determined the purpose of this study: to identify biochemical markers to determine the severity of non-alcoholic fatty liver disease.

EXPERIMENTAL SECTION

Materials and methods

Prior to the experiment, the study plan, standardized operating procedures and accompanying documentation were subjected for ethical review and subsequently approved by the Local Ethical Committee of the Ministry of Health of Russian Federation (protocol No. 1/1 dated January 16, 2017).

The study involved 120 male albino rats with body mass 220–240 g divided into three groups:

1. Controls ($n = 24$) – intact healthy animals tested for reference blood parameters. They were fed with standard food rations and had free access to water.

2. "Liver steatosis" ($n = 48$) – rats that were fed with standard rations identical to those of the controls but received 10 % fructose solution instead of water [10].

3. "Steatohepatitis" ($n = 48$) – rats that throughout the entire study were fed with food briquettes consisting of 21 % protein, 5 % animal fat, 60 % fructose, 8 % cellulose, 5 % minerals and 1 % vitamins. This routine was shown in our previous morphologic studies to cause in 3–4 weeks severe hepatic fibrosis [11].

Restrictions on access to food, diets and drinking conditions were not introduced. Throughout the study, the control group (healthy, intact animals) was fed with a complete extruded and granulated food specially designed for feeding laboratory rodents (Laborator-korm LLC, Russia). Before feeding the animals, the food was sterilized. Water for the animals was filtered and, after filling the drinking bottle, irradiated with ultraviolet light for 5 minutes.

Blood samples (6 ml) were collected into vacutainers through a transcutaneous heart puncture into Monovette vacuum systems, after which the animals were euthanized. Samples from control animals were taken on day 1 of the experiment and from the rats of "Liver steatosis" and "Steatohepatitis" groups – on days 21, 28 and 37 of the experiment. Previously, the animals of these groups were combined into three subgroups of 16 rats each.

Biochemical blood tests were carried out using generally accepted methods using a StatFax 3300 analyzer and a set of reagents from Parma LLC (Russia). Studies included: glucose concentration (Glu), total plasma proteins (TP), total bilirubin (TBil) and conjugate bilirubin (CB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDG), alkaline phosphatase (AP), homocysteine (HC), total cholesterol (TC), triacylglycerides (TAG) levels. The intensity of LPO was judged by changes in the concentration of malondialdehyde (MDA), which was determined colorimetrically with thiobarbituric acid [12]. The state of the antioxidant system was assessed by catalase concentration, determined by the method of M.A. Korolyuk et al., 1988 [13] and superoxide dismutase (SOD) concentration, which was determined by the adrenaline autoxidation method [14].

Histological examination was carried out by light microscopy, hematoxylin-eosin staining, magnification 20x. A different degree of severity of morphological changes in the experimental groups was revealed. All experimental groups share signs of fatty degeneration of hepatocytes.

All the results were statistically processed with the help of SPSS for Windows 13.0 package. All the resulting data are presented as mean \pm standard error ($M \pm SE$). Kolmogorov – Smirnov criterion was used to determine the character of data distribution. To describe quantitative characteristics that do not correspond to the law of normal distribution, the nonparametric Mann – Whitney test was used. The obtained data are presented as median, lower and upper quartiles (Me, quartiles [25 %–75 %]). Using Friedman's χ^2 test (with a distribution other than normal), $p < 0.05$ (probability of at least 95 %) was accepted as a significant level of difference, which is standard for biomedical experiments.

RESULTS AND DISCUSSION

Starting from day 21, the animals from "Steatohepatitis" group displayed total bilirubin blood plasma concentration increase due to direct bilirubin fraction that demonstrated a valid constant increase during the entire experiment ($p = 0.037$). This increase reflects a progressive hepatic dysfunction alongside steatohepatitis development. The absence of statistically valid parallel increase of total bilirubin blood concentration during the entire experiment in comparison to control group ($p = 0.363$) confirms this thesis.

Liver steatosis unlike steatohepatitis had caused moderate impairment of pigment metabolism with slow but reliable total bilirubin blood concentration increase ($p = 0.040$) without substantial fluctuations of conjugate bilirubin concentration testifying to mild hepatocytes dysfunction (Table 1).

The analysis of data on the activity of cellular enzymes characterizing cytolytic liver impairment in blood of animals with steatohepatitis (ALT and AST) had revealed a synchronous reliable increase reaching a statistically valid level of difference in comparison to the control group from the very beginning of the experiment (ALT: $p < 0.001$, AST: $p < 0.001$), with a continuous increment during the entire experiment (Fig. 1).

Hepatic transaminases activity in "Liver steatosis" group demonstrated a slow increase. It was only on day 37 that they have reached statistically valid difference from the control group (ALT: $p = 0.001$; AST: $p = 0.002$). Cytolytic syndrome intensity in case of steatosis was much lower which was confirmed by a lower ALT and AST level in the animals of this group in comparison with "Steatohepatitis" group (AST level lower by 8.5 IU/l ($p = 0.011$), ALT level – by 13.4 IU/l ($p = 0.004$)) (Fig. 1). This fact confirms validity of two chosen NAFLD models of varying severity.

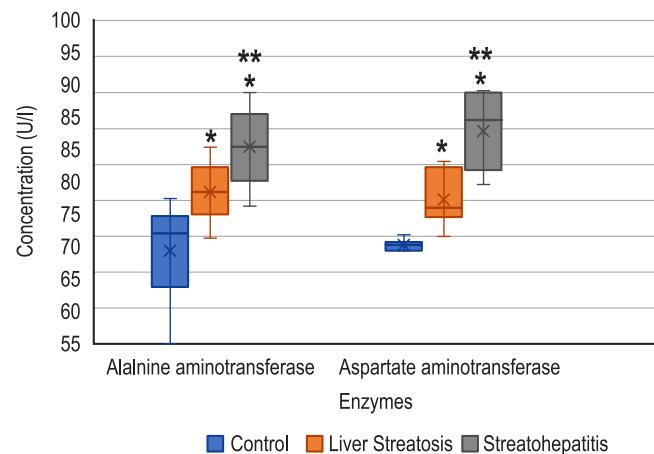


FIG. 1.

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) level changes (IU/l) in rats with liver steatosis and steatohepatitis. Boxplots showing hormonal and metabolic differences between "Liver Steatosis" groups and "Control" group: * – differences from the "Control" group are statistically significant (Mann – Whitney test); ** – differences from the "Liver Steatosis" group are significant (Mann – Whitney test)

A significant discrepancy was revealed in the dynamics of biochemical blood parameters of experimental animals, characterizing the condition of the liver between groups with different severity of the process. Liver functions in the "Steatohepatitis" group were significantly more disturbed than in the "Liver steatosis" group: disorders of pigment and lipid metabolism, as well as cytolytic and cholestatic syndromes and hyperhomocysteinemia in the former group were much more pronounced than in the latter one. The evaluation supports the validity of fructose-induced NAFLD models.

The used high-carbohydrate (60 % fructose of the total feed mass – "Steatohepatitis" group) and lipid-rich

TABLE 1

INDICATORS OF PIGMENT METABOLISM IN RATS WITH NAFLD OF VARYING SEVERITY (ME [25 %; 75 %]) IN EXPERIMENTAL GROUPS

Groups	Observation period (days)	n	Indicators studied	
			Total bilirubin, $\mu\text{mol/l}$	Conjugate bilirubin, $\mu\text{mol/l}$
Control	0 ⁽¹⁾	24	10.0 [4.8; 15.1]	1.1 [0.69; 1.7]
	21 ⁽²⁾	16	12.2 [4.4; 22.1]	1.1 [0.62; 1.6]
Liver steatosis	28 ⁽³⁾	16	12.3 [8.9; 14.9]	1.6 [1.1; 2.0]
	37 ⁽⁴⁾	16	15.4 [13.7; 16.5]	1.1 [0.62; 1.6]
Steatohepatitis	21 ⁽⁵⁾	16	14.5 [12.8; 16.2]	1.0 [0.68; 1.2]
	28 ⁽⁶⁾	16	19.4 [10.1; 29.2]	1.3 [1.01; 2.0]
	37 ⁽⁷⁾	16	28.1 [24.2; 33.1]	1.6 [1.1; 2.0]

Note. ²⁻⁷ – measurements within groups on days 21, 28, 37 were made using the Friedman's test ($p < 0.005$); ^{1-2, 1-3, 1-4, 1-5, 1-6, 1-7} – measurements within groups on days 21, 28, 37 were made using the Mann – Whitney test; statistically significant differences ($p < 0.005$) were observed in the groups 1–4, 1–5, 1–6, 1–7 by the level of total bilirubin.

diet led to the rapid formation of pathological processes (5 weeks) compared to other used models [15–17]. However, such a diet also led to the formation of serious pathological conditions in the cardiovascular system and liver of rats, which is confirmed by the 30 % mortality of animals at the end of the study (day 37). In studies by other authors [18, 19], no mortality was reported.

Lethality rate, in our opinion, can be used as an integral parameter for assessing the severity of the pathological process and the intensity of the studied models. The high-carbohydrate (60 % fructose by weight), lipid-rich diet caused rapid development of pathological processes, leading to a 32 % mortality rate by the end of the experiment (day 37).

In studies by other authors, high levels of ALT and AST in the experimental groups were similar to our data [20–23]. However, the degree of disturbances in lipid and pigment metabolism is significantly higher according to our results.

LDG blood levels in "Steatohepatitis" group demonstrated a reliable moderate increase ($p < 0.001$). LDG blood levels in "Liver steatosis" group did not substantially differ from those of the control animals. Comparison of mean LDG blood levels in "Steatohepatitis" and "Liver steatosis" groups revealed that LDG values in rats with liver steatosis were reliably lower by 13.4 IU/l than in the animals with steatohepatitis ($p = 0.026$).

AP blood levels in "Steatohepatitis" group demonstrated a reliable moderate increase in comparison with the control group ($p < 0.001$) which is a cholestatic syndrome biochemical marker. Glucose levels in "Steatohepatitis" group grew slowly during the experiment and demonstrated a valid difference with control ($p = 0.015$), while Glu levels in "Liver Steatosis" group did not differ statistically from those in the control group (Fig. 2). Homocysteine blood concentration increase, an important hepatic and endothelial dysfunction marker, was statistically valid in "Steatohepatitis" group ($p = 0.001$), but not in "Liver steatosis" one.

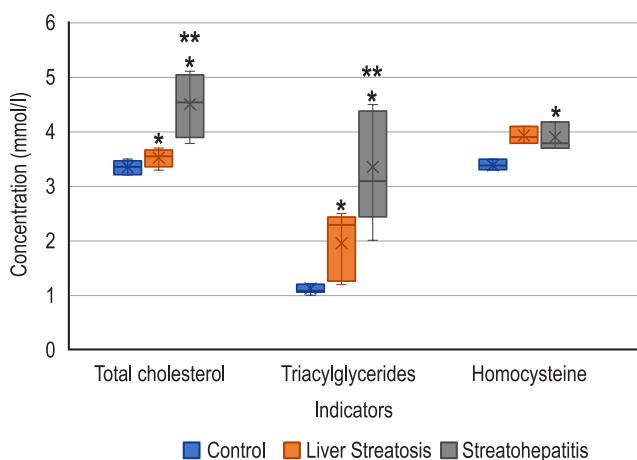


FIG. 2.

Total cholesterol, triacylglycerides and homocysteine level changes (mmol/l) in rats with liver steatosis and steatohepatitis. Box-plots showing hormonal and metabolic differences between "Hepatic Steatosis" groups and "Control" group: * – differences from the "Control" group are statistically significant (Mann – Whitney test); ** – differences from the "Liver Steatosis" group are statistically significant (Mann – Whitney test)

Fatty liver dystrophy in both experimental models was assessed in the present study is based on profound metabolic disorder with hypercholesterolemia and hypertriglyceridemia (Fig. 2). Total cholesterol blood concentration in "Steatohepatitis" group increased considerably in comparison with control from the very beginning of the experiment ($p < 0.001$) with a parallel even more substantial rise of TAG blood levels ($p < 0.001$); TAG/TC ratio increasing from 0.53 on day 21 up to 0.79 on day 37 (TAG/TC ratio in control group was 0.52).

The rats in "Liver steatosis" group also demonstrated an increase of TC and TAG blood levels (Fig. 2). However, the increase was slower and not as high as in "Steatohepatitis" group (TC: $p = 0.003$; TAG: $p = 0.002$). TAG/TC ratio in this group changed from 0.46 on day 21 to 0.67 on day 37 of the experiment.

It is assumed that the pathogenesis of NAFLD is based on a pronounced imbalance of lipid metabolism with the formation of hypercholesterolemia and hypertriglyceridemia [24, 25]. In our studies, on day 37 of observation, the TG level in the "Steatohepatitis" group became 300 % higher than in the "Control" group. This is slightly higher than in the experiment of Z. Ackerman [11]: on day 35 of observations, the indicator increased by 223 %. The level of TC by the end of the experiment increased by 167 %, while in studies by the same author this figure increased by 89 % [11].

Serious metabolic disorders accompanying the development of NAFLD in experimental animals were reflected by biochemical blood plasma changes causing lipid peroxidation and considerable antioxidant system depression. These disorders were represented by a progressive increase of MDA blood concentration in both NAS and NASH models with a parallel decrease of basic antioxidant system enzymes activity (catalase, SOD) (Table 2).

MDA blood concentration in "Steatohepatitis" group grew quickly and reliably ($p < 0.001$) from the very beginning of the experiment reflecting increased lipid peroxidation (Table 1). The intensity of LP in the rats from "Liver steatosis" group was way lower than in the animals with NASH: MDA blood concentrations in rats with NAS grew slowly ($p = 0.010$) but by the end of the study (day 37) MDA mean value was statistically higher by 9.8 mmol/l than in the control group ($p = 0.001$) although lower by 11 mmol/l than in rats with NASH.

Parallel to lipid peroxidation activation in both experimental groups basic antioxidant system enzymes (SOD and catalase) considerably decreased their concentration. SOD blood concentration in "Steatohepatitis" group demonstrated a precipitous drop ($p < 0.001$) with a synchronous decrease of blood catalase concentration from the very beginning of the experiment ($p = 0.001$).

The same enzymes' blood concentration in "Liver steatosis" group decreased slower (SOD: at day 28 ($p = 0.002$); catalase: at day 37 ($p = 0.009$) and not as substantial.

Metabolic disorders in animals that accompany the development of NAFLD in our studies lead to a decrease in the activity of the body's antioxidant system and activation of LPO [26]. This is reflected in a progressive increase

TABLE 2

ANTIOXIDANT SYSTEM ENZYMES ACTIVITY AND PEROXIDATION INTENSITY IN RATS WITH TWO NAFLD MODELS, ME [25 %; 75 %]

Groups	Day of the experiment	n	Biochemical parameters		
			SOD, IU/ml	Catalase, mmol/l	MDA, mmol/l
Control	0 ⁽¹⁾	24	6.4 [6.4; 6.6]	0.15 [0.13; 0.17]	9.5 [9.1; 9.7]
	21 ⁽²⁾	16	5.9 [5.0; 7.5]	0.15 [0.14; 0.16]	13.9 [13.1; 14.7]
Liver steatosis	28 ⁽³⁾	16	5.8 [3.9; 7.3]	0.14 [0.13; 0.15]	16.8 [15.6; 17.0]
	37 ⁽⁴⁾	16	4.6 [4.4; 4.8]	0.13 [0.12; 0.14]	18.9 [18.4; 19.4]
Steatohepatitis	21 ⁽⁵⁾	16	5.8 [3.9; 7.3]	0.14 [0.13; 0.15]	15.5 [13.9; 17.1]
	28 ⁽⁶⁾	16	4.6 [3.7; 5.0]	0.1 [0.9; 0.11]	19.8 [16.7; 22.0]
	37 ⁽⁷⁾	16	4.0 [2.9; 4.9]	0.07 [0.06; 0.08]	29.9 [26.3; 33.5]

Note. ²⁻⁷ – measurements within groups on days 21, 28, 37 were made using the Friedman's test ($p < 0.05$); ^{1-2, 1-3, 1-4, 1-5, 1-6, 1-7} – measurements within groups on days 21, 28, 37 were made using the Mann – Whitney test. Statistically significant differences ($p < 0.05$) were observed in the groups 1–3, 1–4, 1–5, 1–6, 1–7 by the level of superoxide dismutase; in the groups 1–4, 1–5, 1–6, 1–7 – by the level of catalase; in the groups 1–3, 1–4, 1–5, 1–6, 1–7 – by the level of malondialdehyde.

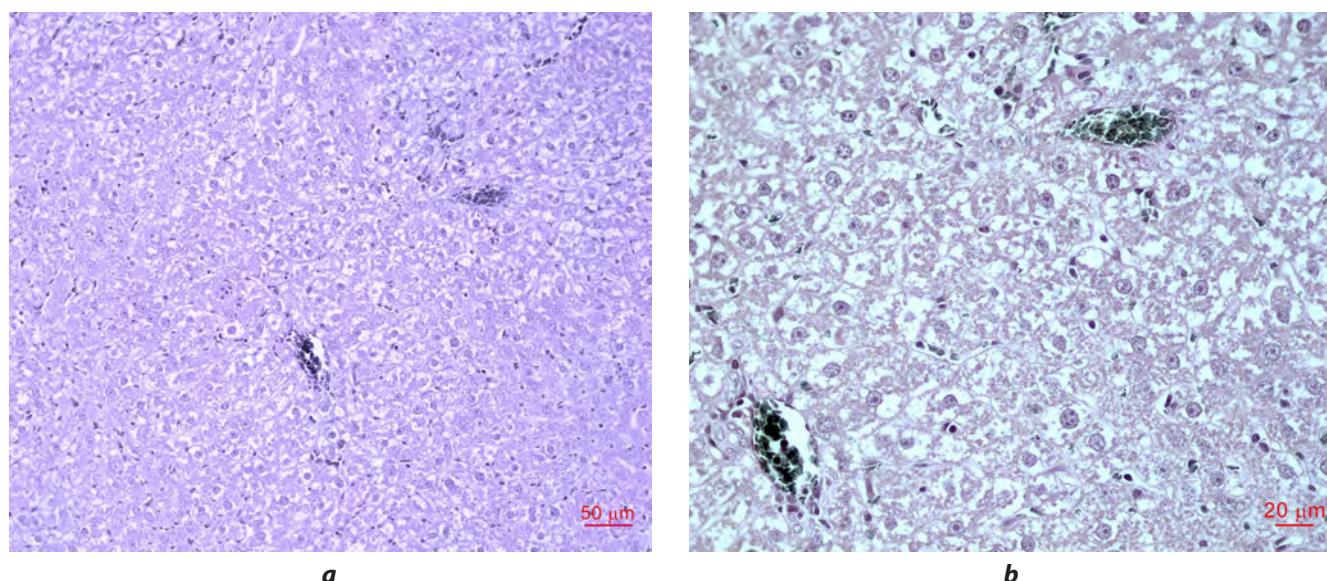


FIG. 3.

Histological changes in "Liver steatosis" group: **a** – hematoxylin and eosin staining, magnification 10x; **b** – hematoxylin and eosin staining, magnification 40x

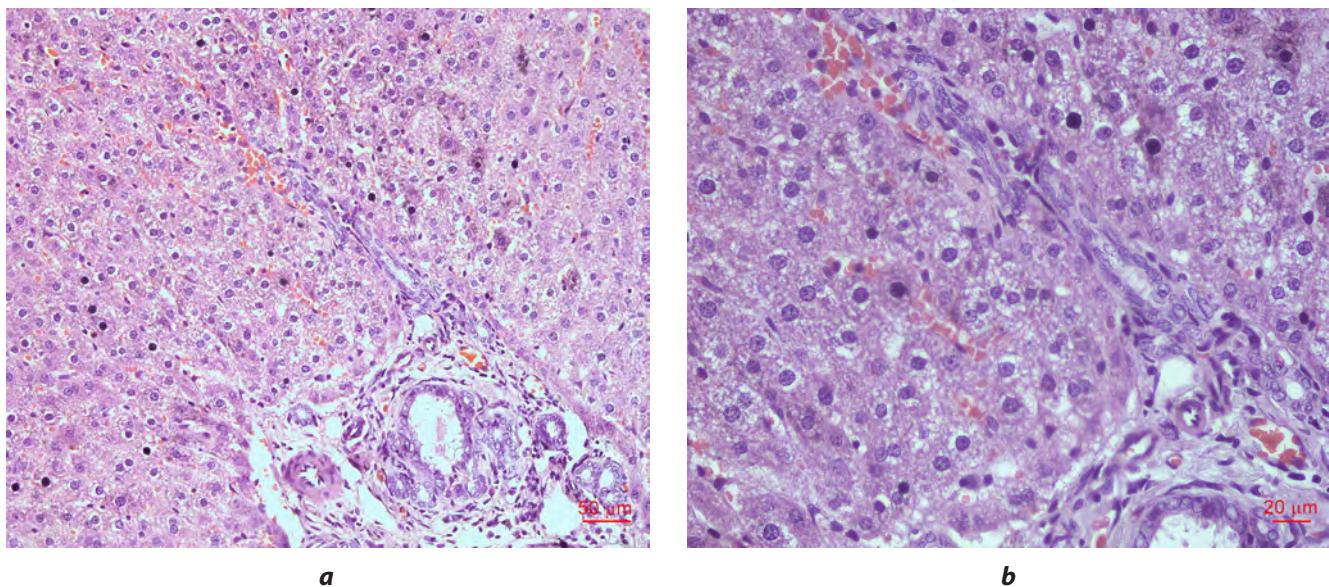
in the level of MDA in the blood of rats in both models of NAFLD and a decrease in the content of the main antioxidant enzymes (catalase, SOD), which is comparable with the results of studies by other authors. [27]. Lujan P.V. et al. also observed a significant decrease in the level of antioxidant enzymes SOD and catalase against the background of NAFLD [28].

In the experimental "Liver steatosis" group, large droplet fatty degeneration is observed, which is characterized by the presence of large lipid droplets in the cytoplasm of hepatocytes with a displacement of the nucleus to the cell periphery (Fig. 3).

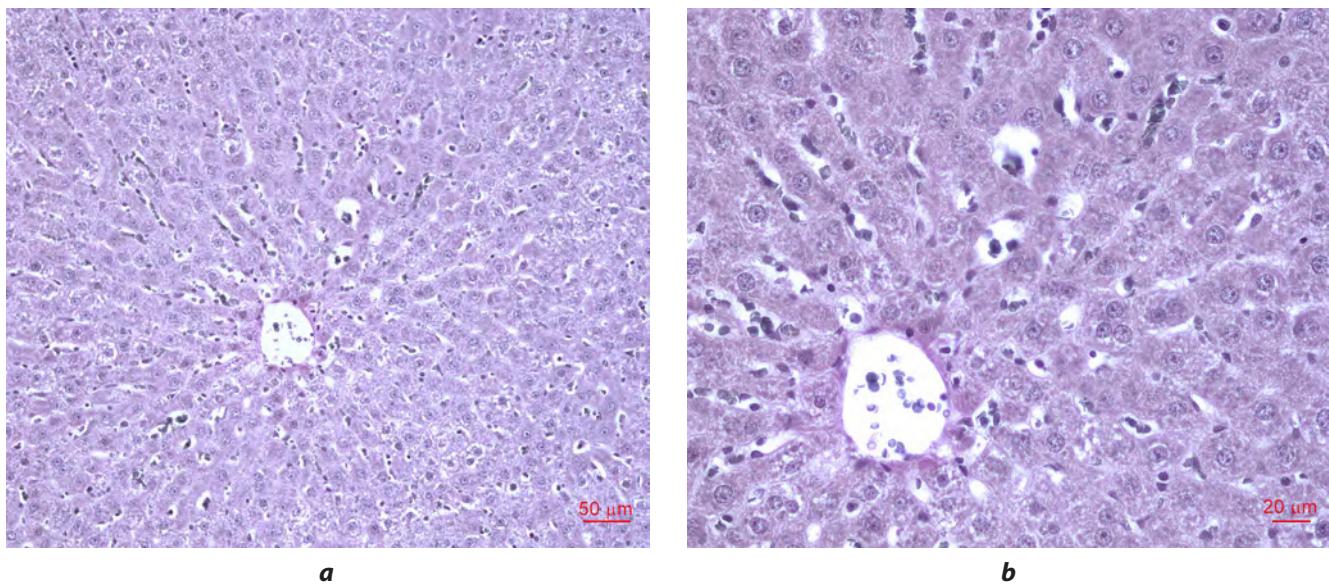
Signs of liver tissue degeneration are most pronounced in the "Steatohepatitis" group (Fig. 4). Signs of balloon dys-

trophy, apoptosis of hepatocytes are noticeable in comparison with the control (Fig. 5) and the "Liver steatosis" group. Small droplet fatty degeneration was revealed: there are a lot of small lipid droplets in hepatocytes, the nucleus is located in the center of the cell. Hepatocytes are also found in a state of balloon dystrophy. Focal centrilobular necrosis often develops with small droplet steatosis. Hyaline bodies of Mallory are detected with different frequency. The inflammatory infiltrate inside the lobules contains neutrophils, lymphocytes, and histiocytes.

The used models of steatosis and steatohepatitis were characterized by the development of fatty liver in experimental animals, bilirubinemia, cholesterolemia, activation lipid peroxidation and suppression of antioxidant mech-

**FIG. 4.**

Histological changes in "Steatohepatitis" group: a – hematoxylin and eosin staining, magnification 10x; b – hematoxylin and eosin staining, magnification 40x

**FIG. 5.**

Histological changes in the Control group: a – hematoxylin and eosin staining, magnification 10x; b – hematoxylin and eosin staining, magnification 40x

anisms, cytolytic and cholestatic syndromes. The severity of metabolic disorders depended on the severity of the disease being modeled.

The results of the study prove the possibility of using biochemical markers of NAFLD (ALT, AST, TC, TAG, MDA, SOD) for more accurate diagnosis of the severity and stage of development of liver pathology, as well as for monitoring the effectiveness of therapy.

CONCLUSIONS

- Both NAFLD models studied caused disorders of the hepatobiliary, endocrine and cardiovascular systems.

The intensity of these disorders depended on the severity of the model used and was maximum when modeling steatohepatitis (60 % fructose in the diet) and less pronounced when modeling steatosis (10 % fructose solution instead of drinking water).

- High mortality rates in both models of NAFLD confirm the adequate severity of both models of NAFLD, as well as a direct correlation of dysmetabolic changes and disorders of compensatory mechanisms.

- In the model of steatohepatitis, liver functions were impaired to a much greater extent than in steatosis; this difference was manifested in more pronounced symptoms of disorders of pigment and lipid metabolism, the severity of cytolytic and cholestatic syndromes, significant acti-

vation of lipid peroxidation and depression of the antioxidant system.

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Conflicts of interest

The authors declare no conflict of interest.

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