METHODS

Simulation of Nonalcoholic Steatohepatitis in Rats

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The new model of nonalcoholic steatohepatitis in rats is based on alimentary effects of hypercaloric diet including much cholesterol and beef fat. This model reproduces structural and functional disorders in the liver, including hepatocyte fatty degeneration, parenchymatous necrosis and fibrosis paralleled by dyslipidemia and is intended for studies of the mechanisms of formation, progress, and therapy of liver diseases of noninfectious origin.

Key Words: *liver; steatohepatitis; experimental model*

Experimental models of fatty disease of the liver are widely used for studies of the mechanisms of disease formation, clearing out the contribution of environmental and visceral factors to development of pathological processes. They are convenient for detailed studies of the pathogenesis and finding the means for rational therapeutic interventions and prevention.

The pathogenetic and sanogenetic aspects of liver diseases are usually studied on experimental models, reproducing deep structural changes in the organ tissues, similar to those in humans but forming within a shorter period. Each of the known models of hepatitis, cirrhosis of the liver, fatty hepatosis, and other liver diseases is based on the toxic factor initiating hepatic injuries [2,3] and differing from the real causes of liver pathogenesis in humans. This impedes extrapolation of experimental data on the mechanisms of hepatic disease development on the patients. Hence, experimental models close to the actual clinical conditions as much as possible are to be created for studies of the mechanisms of hepatic disease formation, development of new approaches to their treatment and prevention.

It is known that irrational nutrition and alcohol abuse are the main causes initiating noninfectious diseases of the liver [1]. The possibility of development of fatty hepatic disease under conditions of unbalanced hypercaloric nutrition suggests the development of a model of nonalcoholic steatohepatitis (NASH) by feeding animals a hypercaloric hepatogenic ration. The aim of our study was to create an experimental model of diet-induced NASH in rats.

MATERIALS AND METHODS

The study was carried out on adult male Wistar rats initially weighing 173.0±5.6 g. Several similar experimental conditions have been reproduced; all variants of modifications of the reproduced models were analyzed on the basis of histological studies of liver tissue. The models providing stable structural disorders in the liver associated with fatty infiltration and development of hepatic necrosis without mortality of experimental animals were selected.

Nonalcoholic steatohepatitis was induced by using a fat-rich ration promoting the development of hepatic disease (Table 1). The diet included cholesterol, a fac-

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tor stimulating lipid disorders and fatty infiltration of the liver.

Fat load (beef fat) increasing the caloricity of hepatitis-inducing diet, served as the pathogenetic factor. The rats received hypercaloric diet for 180 days.

Four groups of animals, 10 per group, were formed. Control group consisted of intact animals on standard ration. Experimental animals received experimental ration: group 1 for 30 days, group 2 for 90 days, and group 3 for 180 days. The animals were kept in a vivarium in accordance with the common regulations on the design, equipment, and regimens of experimental biological clinics [6]. The rats were decapitated under ether narcosis in 30, 90, and 180 days.

Biometric parameters were evaluated: body weight before and after experiment, liver weight, and visceral fat weight (Table 2). Lipid metabolism was studied by total cholesterol (TCH), triglycerides, HDL cholesterol (HDL CH). The concentrations of LDL CH and VLDL CH and atherogenicity index were calculated. Serum glucose level and activities of ALT, AST, lactate dehydrogenase (LDH) were measured on an FP-901 biochemical analyzer using Allwex kits. Blood for analyses was collected after overnight fasting from the cervical vein after decapitation. Histological studies were carried out on preparations from small fragments of the central part of the right lobe of the liver, fixed in 10% neutral formalin prepared on 0.07 M phosphate buffer (pH 6.98). The tissues were dehydrated in ascending ethanols and embedded in paraplast. The sections (7 μ) were sliced on an MZP-01 microtome (TECHNOM), stained with hematoxylin and eosin after Romanowskii and with picrofuchsin after van Gieson for visualization of collagen fibrils [4]. The

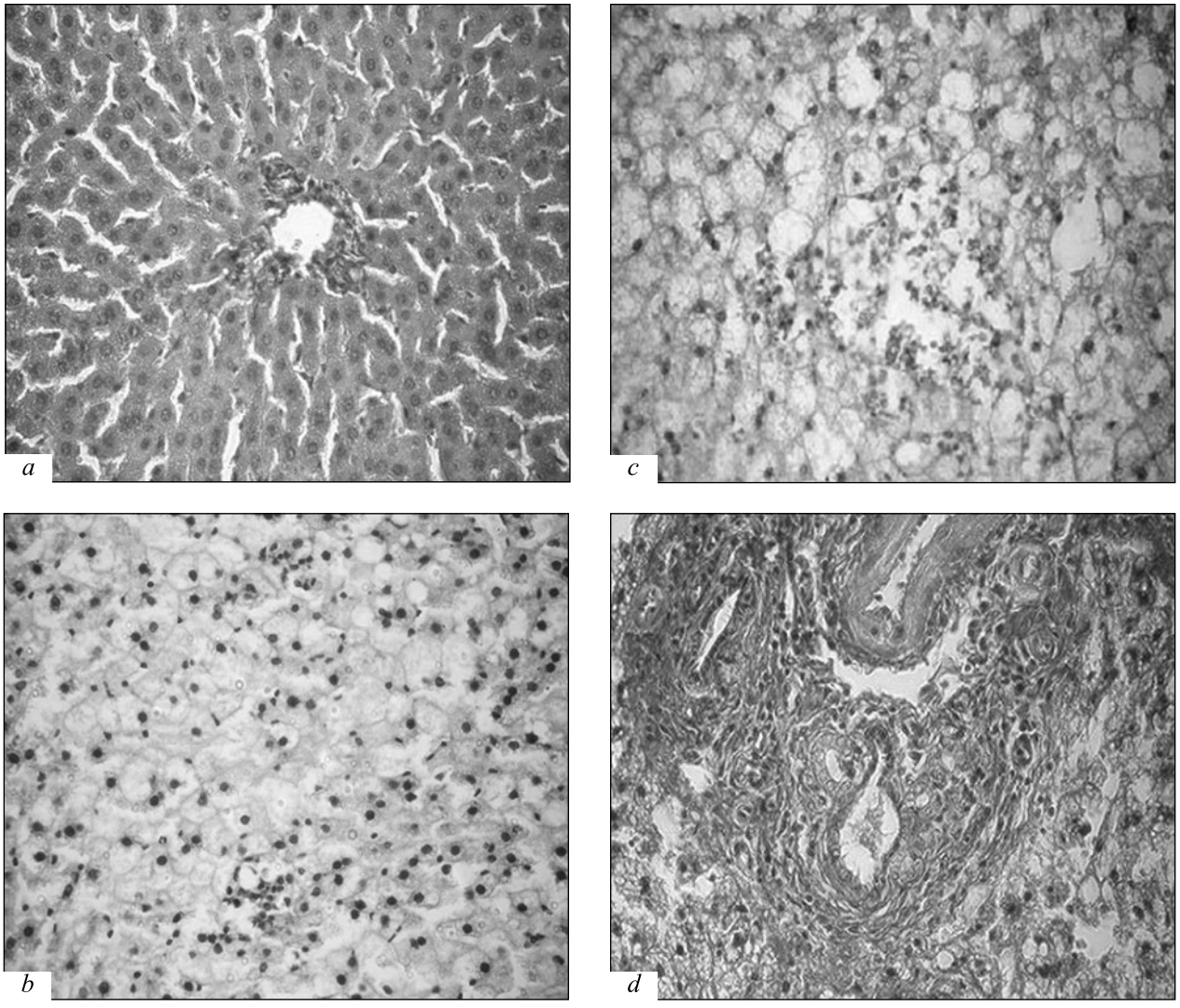

Fig. 1. Histological structure of rat liver, ×40. *a*) control; *b*) group 1 (NASH simulation, 30 days), fatty degeneration of hepatocytes; *c*) group 2 (NASH simulation, 90 days), focus of hepatic tissue necrosis; *d*) group 3 (NASH simulation, 180 days), hepatic tissue fibrosis. Hematoxylin and eosin staining (*a*-*c*), van Gieson staining (*d*).

preparations were examined under a Carl Zeiss microscope (×10, ×40, and ×90).

The data were analyzed using Statistica 6.1 software (1203C series for Windows). The significance of differences between the means was evaluated by Wilcoxon's, White's, Kolmogorov–Smirnov's tests, for normal distribution by Student's *t* test.

RESULTS

In rats of experimental groups, body weight gain and increase of visceral fatty tissue and liver weights indicated the formation of obesity and liver hypertrophy.

Studies of histological sections of liver tissue confirmed the formation of NASH (Fig. 1). The girder structure of hepatocyte cords was impaired on day 30 of NASH simulation (Fig. 1, *b*). Signs of degeneration were found in an appreciable part of hepatocytes: hypertrophy and abnormal shape of the cells together with reduced basophilia and cytoplasm vacuolation. Liver cells tightly adhered to each other, which led to narrowing of the lumens of sinusoidal capillaries. The hepatocyte cytoplasm was filled with lipid vacuoles, large and small. In small-droplet vacuolation, the hepatocytes had a foamy cytoplasm, with vacuoles distributed evenly in the cytoplasm, without shifting the nuclei. In large-droplet vacuolation, the cytoplasm looked empty, with the nucleus shifted to the cell periphery. The histological picture of the liver on day 30 of NASH development could be identified as steatosis.

Foci of hepatocytes in a state of necrobiosis with lymphocytic macrophage infiltration were found in animals receiving high-fat diet for 90 days (Fig. 1, *c*). Sinusoidal capillaries in the periportal and central zones of the lobes were dilated, the central veins were plethoric. Slight mononuclear infiltration was seen near the bile ducts. Fatty degeneration of hepatocytes persisted. Moderate intralobular infiltration of the liver parenchyma was detected. Hence, high-fat experimental diet for 90 days led to the formation of steatohepatitis.

On day 180 of the experiment, the structure of the liver changed significantly. The area of necrotic foci increased (10-15% of total area of the studied liver sites), they were surrounded by lymphocytic macrophage infiltration; the girder structure of hepatic liver lobes was disordered (Fig. 1, *d*). Hepatocytes formed no trabeculae, were located chaotically; no sinusoids could be found. The large portal tracts were dilated at the expense of stroma growth, which was filled with inflammatory infiltration. Necrotic changes in the vessels were found in some portal tracts. The walls of these vessels were poorly discernible, in some cases destroyed. The bile ducts were destroyed, there were large foci of inflammatory infiltration. Collagen fibers in the portal zone were clearly seen in the preparations stained by van Gieson's method, which was a direct evidence of hepatic fibrosis.

Hypercaloric diet for 30 days led to the formation of alimentary dyslipidemia, manifesting by elevation of TCH, triglycerides, VLDL CH levels, atherogenicity index, and reduction of serum HDL CH level (Table 3). After 90 days of alimentary load triglycerides and VLDL CH reduced significantly (more than 2-fold). Experimental ration for 180 days led to progress of hyperlipidemia. A significant elevation of blood TSC and atherogenicity index in comparison with the controls were seen. These results characterized the time course of lipid metabolism disorders at all stages of experiment. Low VLDL CH levels in groups 2 and 3 served as criteria of metabolic disorders in the liver and confirmed the development of steatohepatitis [1]. Nonalcoholic steatohepatitis formed in the hyperglycemia, which was proven by high glucose level in the blood in all experimental groups. Activities of hepatic enzymes (ALT, AST, LDH) increased in animals receiving experimental ration, indicating impairment of hepatocytes and development of the cytolytical syndrome.

Hence, exposure to an alimentary hepatitis-inducing factor for 180 days led to the formation of NASH, associated with dyslipidemia, hyperfermentemia, and hyperglycemia. Lipid metabolism disorders, deposition of excessive amounts of easily oxidized fat in the liver stimulated LPO processes [5,9] and augmented structural and functional disorganization of the liver. These disorders are independent pathogenetic events in the common chain of mechanisms of formation of hepatobiliary diseases [9]. Lipid peroxides damage

TABLE 1. Daily Ration of Rats (g/100 g body weight)

Ingredients	Experimental ration	Common vivarium ration
Beef fat	4.25	0.5
Cholesterol	0.43	-
Sunflower oil	0.5	0.5
Grain mixture	5	5
Dry wheat bread	2	20
Oatmeals	1.3	1.3
Beef, category II	2	2
Degreased custard	0.8	0.8
Carrots	3.3	3.3
Greens	3.3	3.3
Brewer's yeast	0.05	0.05
Common salt	0.1	0.1

TABLE 2. Body Weights and Lengths of Rats with Experimental NASH (*M*±*m*)

Parameters		Control group	Experimental groups		
			1 (30 days of ER)	2 (90 days of ER)	3 (180 days of ER)
Body weight, g	before experiment	168.0±4.1	174/0±5.6	178.5±8.5	180±28
	after experiment	250.0±8.9	324.5±8.7***	361.4±12.2*	471.2±67.9**
Liver weight, g		8.16±0.23	16.75±0.64***	16.2±1.0***	27.9±4.1***
Visceral fat weight, g		5.91±0.68	15.71±0.96***	13.2±0.2***	12.5±3.4***

Note. Here and in Table 3: *p<0.05, **p<0.01, ***p<0.001 in comparison with the control. ER: experimental ration.

TABLE 3. Blood Biochemistrty in Rats with Experimental NASH (*M*±*m*)

Parameter, μmol/liter	Control group (n=10)	Experimental groups		
		1 (30 days of ER)	2 (90 days of ER)	3 (180 days of ER)
TCH	1.57±0.04	3.34±0.04***	1.68±0.08	2.04±0.17*
Triglycerides	1.12±0.04	1.95±0.06***	0.51±0.05***	1.17±0.08
HDL CH	0.67±0.04	0.26±0.02***	0.50±0.08	0.50±0.15
LDL CH	0.70±0.16	0.84±0.196	0.96±0.12	0.90±0.06
VLDL CH	0.65±0.19	1.14±0.29*	0.23±0.02**	0.20±0.03***
Atherogenicity index, arb. units	1.43±0.15	11.87±1.55***	2.46±0.35	7.4±0.8***
Glucose	5.4±0.1	8.7±0.2***	7.2±0.1***	12.0±0.3***
ALT	52.2±3.5	108.9±3.5***	42.4±1.1*	172±12***
AST	118.6±6.1	243.1±9.1***	130.8±14.4	165.6±2.5***
LDH	936±133	1799.1±176.8**	730±92	1285.0±56.1***

hepatocyte membranes and stimulate hepatic astrocytes with the development of fibrosis [1,5]. Hyperactivation of lipid peroxidation processes is paralleled by significant changes in the composition and degree of membrane phospholipid oxidation, this eventually leading to destruction of cell membrane, dysfunction of the receptor system, reduction of glucose tolerance, development of hyperglycemia and hyperfermentemia [7,8,10]. Hence, the suggested method for NASH simulation initiates the basic pathogenetic mechanisms triggering the fibrogenesis processes. The development of the suggested NASH model includes the majority of clinically significant factors of liver injury, most incident in the hepatitis pathogenesis: dyslipidemia, hyperglycemia, hyperfermentemia. This model can be used for studies of stages in the development of fatty hepatic disease characterized by the formation of steatosis, steatohepatitis, and fibrosis. This model is easy to perform, well reproducible, involves no lethal outcomes, and is intended for studies of the mechanisms of formation, progress, and therapy of hepatic diseases of noninfectious origin. Creation of this model opens the possibility of experiments which cannot be realized in a clinical setting.

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