Protective Effect of Transplantation of Neonatal Liver Cell Nuclei on the Model of Acute Toxic Hepatitis

A. A. Runovich, Yu. I. Pivovarov, T. E. Kuril'skaya, A. S. Sergeeva, I. V. Babushkina, and S. L. Bogorodskaya

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We present the results of comparative analysis of functional and morphological changes in the liver of animals with experimental CCl_4-induced hepatitis under conditions of transplantation of neonatal liver cells and nuclei. It was found that transplantation of neonatal liver cell nuclei in acute toxic hepatitis provides better functional and structural state of the target organ.

Key Words: *transplantation of neonatal liver cell and nuclei; toxic hepatitis*

Identification of structural components of tissue transplants determining functional and structural repair of the damaged tissue is a key to understanding of the mechanisms of pathological process regression after cell therapy.

Transplanted cells can activate intrinsic endogenous mechanisms controlling reparative processes in the damaged organ due to the presence of regulatory components in the transplant [3]. Under conditions of liver pathology, transplanted cells, in particular isolated hepatocytes, activate the function of remaining hepatocytes in the recipient via production of regulatory peptides (first of all, growth factors), rather than increase liver cell pool [7,19]. Experimental studies demonstrated the possibility of modulating the cytokine profile and state of the recipient with liver pathology by exogenous administration of growth factors [21]. High efficiency of hepatocyte microsomal enzyme systems and liver cytosol containing mitochondrial and microsomal fractions in the treatment of liver diseases was reported [1,2,6]. Positive results of transplantation of total cytosol and its termostable fraction from human embryonic tissues on pro/antioxidant status of the liver were observed in rats with acute CCl_4-induced hepatitis [10]. These data suggest that cell-free stage-specific factors are promising hepatoprotectors in experimental liver pathology.

However, the sanogenetic role of individual components of the transplant is poorly studied. In light of this, identification of the most effective cell components and evaluation of their effects on the damaged organs are important problems.

Here we studied the effect of transplantation of neonatal liver cell nuclei on the course of acute toxic hepatitis.

MATERIALS AND METHODS

Experiments were carried out on 150 outbred male rats weighing 180-250 g.

Toxic hepatitis was modeled by single subcutaneous injection (into the thigh) of CCl_4 in a dose of 0.4 ml 50% oil solution per 100 g body weight (group 1, control).

In group 2 (reference group), a suspension of liver cells from 2-day-old rabbits was used as the transplant. After mechanical homogenization of the liver from a neonatal rabbit, the cell suspension was obtained by enzymatic disintegration of the homogenate with collagenase followed by routine washout with Hanks saline [1]. Cell integrity was evaluated by trypan blue exclusion test in a Goryaev chamber.

Group 3 animals (the main experimental group) received liver cell nuclei isolated by differential cen-

Research Center of Reconstructive and Repair Surgery, Siberian Division of the Russian Academy of Medical Sciences, Irkutsk, Russia. ***Address for correspondence:*** sergeeva1111@yandex.ru. A. S. Sergeeva

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trifugation of liver cell suspension [5]. Liver cell suspension (10 ml, 10^6 cells/ml) was mixed with 20 ml isosmotic isolation medium, transferred to homogenizer, and homogenized using a large and then small pistils (3 times each). The tubes were equilibrated with isolation medium and centrifuged at 1500 rpm for 10 min; the supernatant was placed on ice. The pellet was resuspended with a brush in 20 ml isolation medium, homogenized, equilibrated with medium, and centrifuged at 1500 rpm for 10 min. The supernatant was added to the first portion (on ice). The pellet was again resuspended with a brush in 5 ml isolation medium and centrifuged at 3000 rpm for 10 min. Since the supernatant could contain cell nuclei, it was twice centrifuged at 3000 rpm for 10 min.

Thus, the animals received all components of neonatal liver cells (or their nuclei), except connective tissue stroma. In all cases, the cell and nuclear transplants was examined by histological methods.

The nuclei and cells of the neonatal liver were transplanted subcutaneously (into the hip) in a dose of 500,000 cells or nuclei per 100 g body weight in 0.5 ml 0.9% NaCl immediately after CCl_4 injection into the contralateral hip. The animals were decapitated on days 1, 3, 5, 7, and 10.

In the liver, the following parameters were evaluated: relative content of hepatocytes with fatty degeneration; relative area of necrotic zones (in %); cell index (S cell number/S section – 10,000 μ^2); absolute number of apoptotic bodies (a.b.) in 30 sections; content of apoptosis and proliferation markers Bcl-2, Bcl-Xs, PCNA, Ki-67, and p53 (measured using the corresponding monoclonal antibodies Bcl-2 Oncoprotein clon 124, Dako Cytomatio, Bcl-X_S clon NCI, NCI-PCNA clon PC10, Novocastra; Ki-67 Antigen clon MIB-1, p53 Protein clon DO-7, Dako Cytomatio). The degree of apoptosis inhibition and DNA reparation were evaluated using apoptosis inhibition coefficient (Bcl-2)/(Bcl-Xs) and proliferation coefficient (PCNA)/(Ki-67).

Visualization, treatment, and morphometry of liver samples were performed using a Quantimet 550IW computer-assisted video system (Leica), Leica Q-Win16 morphometry software, and Video-Test-Marter 4.0 image-analysis software.

The state of an intracellular protective mechanism was evaluated by the content of heat-shock proteins in the liver tissues. The total pool of Hsp70 and inducible Hsp72 and Hsp60 isoforms were assayed by immunoblotting with the corresponding primary antibodies (Sigma, StressGen). For detection of a specific amino acid sequence in the structure of primary antibodies raised against a certain family of heat-shock proteins, second alkaline phosphatase-conjugated antibodies were used. Scanning and analysis of membranes was performed using Sigma Scan Pro software. Relative protein content was expressed in relative units (arb. units).

Activity of liver enzymes was assessed by the dynamics of ALT, AST, and lactate dehydrogenase (LDH) activities (mmol/mg tissue/min) on an Ultrospec-4050 spectrophotometer. Moreover, MDA content in the liver tissue was measured using thiobarbituric acid [4].

Significance of differences was analyzed using nonparametric Mann–Whitney *U* test and Wilcoxon *W* test for linked variables. The differences were significant at $p<0.05$.

RESULTS

Acute toxic hepatitis in control animals was accompanied by structural disturbances in the liver tissue typical of this experimental model (necrosis and fatty degeneration of hepatocytes) and maximally pronounced on day 3 of the experiment (Table 1). Cell infiltration of the liver tissue and transaminase AST and ALT activities also peaked at this term. Moreover, an increase in the content of Hsp70 and Hsp60 was observed on day 1; an elevation of LDH activity and maximum MDA concentration were recorded on day 5. However, on day 10 of the experiment all studied parameters did not exceed the corresponding values in intact animals. Functional recovery of the liver was accompanied by alleviation of destructive changes.

In experimental animals receiving transplantation of neonatal liver nuclei, ALT and AST activities were higher and lipid peroxidation (MDA) was less pronounced than in controls and rats receiving liver cells over 7 days of the experiment (Table 2). Moreover, the degree of fatty degeneration of the liver was minimum in this group throughout the observation period (Table 3).

Bcl-2 belongs to a family of cell proteins and is a structural and functional analogue of human CED-9 [13,20], a membrane-associated protein located on mitochondrial and perinuclear membranes and modulating their permeability for cytochrome C, thus inhibiting apoptosis [14]. Its role consists in the maintenance of cell survival and proliferation.

During the first day of toxic hepatitis, intensive apoptosis of liver cells was observed in all groups, but in rats receiving transplantation of liver cell nuclei this process was less pronounced (15.6 a.b., 5-34; $p<0.05$) than in controls (43.5 a.b., 7-47) and animals receiving cell transplantation (28.5 a.b., 3-40). The coefficient of apoptosis inhibition (Bcl-2)/(Bcl-Xs) on day 3 of observation was considerably higher in rats receiving nuclei than in other groups (Fig. 1). The latter can be explained by more intensive synthesis of Bcl-2 protein: the level of Bcl-2 protein in rats receiving liver

cell nuclei was 1.05 arb. units *vs.* 0.81 (0.77-1.0) and 0.65 (0.42-0.93) arb. units in the control group and in rats receiving cells, respectively.

Further dynamic observation revealed more intensive accumulation of proliferation inductors (PCNA) against the background of reduced content of proliferation repressors (Ki-67) in rats receiving nuclei in comparison with the control and rats receiving cell transplantation, which led to higher proliferation coefficient (PCNA/Ki-67) in this group (Fig. 2). These findings suggest earlier and more pronounced reparative processes in the damaged liver in animals receiving transplantation of liver cell nuclei.

Processes of apoptosis and proliferation in the studied groups had different correlations with other studied parameters. The apoptotic processes in the control group and in animals receiving transplantation of liver cells had one strong correlation with the degree of necrosis (R=-0.97; p<0.001) and PCNA (R= -0.91; p<0.05), while in rats receiving nuclei three

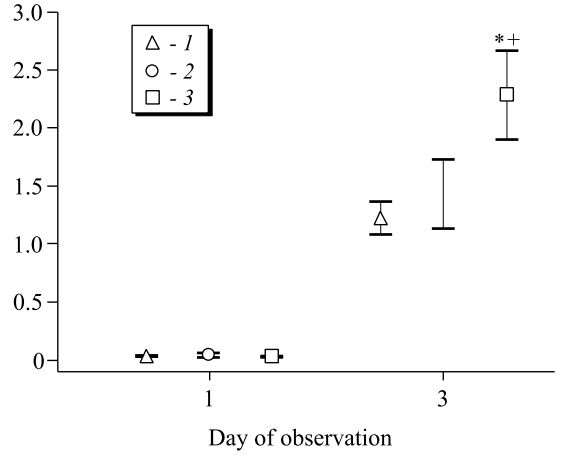

Fig. 1. Dynamics of apoptosis inhibition coefficient (Bcl-2/Bcl-Xs) in the liver of animals with toxic hepatitis (group 1; *1*) receiving transplantation of neonatal liver cells (group 2; *2*) and nuclei (group 3; *3*). Here and in Figs. 2, 3: p<0.05 in comparison with: *group 1, +group 2 (Mann–Whitney test).

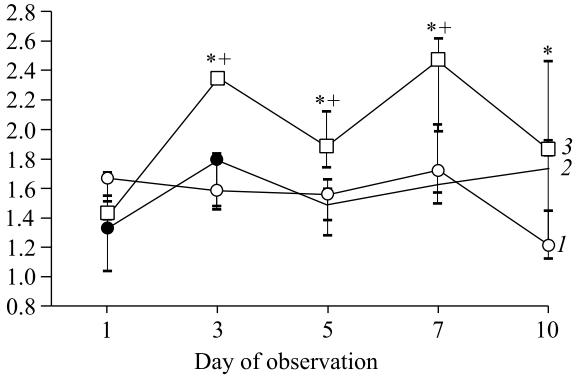

Fig. 2. Dynamics of proliferation coefficient (PCNA/Ki-67) in the liver of animals with toxic hepatitis (group 1; *1*) receiving transplantation of neonatal liver cells (group 2; *2*) and nuclei (group 3; *3*).

TABLE 1. Dynamics of Biochemical and Morphometric Changes and Content of Heat-Shock Proteins in the Liver of Rats with Experimental Toxic Hepatitis

Day of observation	LDH	AST	ALT	MDA	Necrosis area, %	Fatty infiltration of hepatocytes, %	Cell index	Hsp70	Hsp72	Hsp60
Intact animals	600.8 (552-693)	208.8 (187-249)	45.6 (28-47)	103.5 (93-151)	-	-	-	0.35 (0.1-0.8)	0.1 (0.01-0.10)	1.1 (1.1-1.4)
Day 1	630 (596-834)	216 (201-247)	46.5 (40-53)	110.5 (97-122)	2.2 (1.75-3.00)	38.4 (36.1-41.2)	19 (13.3-20.0)	1.1* (0.6-1.5)	0.25 (0.10-0.65)	2.7* (2.0-3.3)
Day 3	754 (724-787)	276* (256-296)	60.2* (39.5-56.0)	88 (73-105)	29.2+ (27.3-31.4)	77.3+ (69.3-86.2)	35.2+ (28.7-38.0)	0.53 (0.01-1.40)	0.0 (0.0-0.2)	1.05 (0.75-1.20)
Day 5	820* (762-1035)	231 (210-276)	46.4 (33.0-54.4)	182* (69-501)	20.8+ (19.0-2.8)	55+ (46-69)	20.8 (17.9-27.3)	0.2 (0.1-0.8)	0.0 (0.0-0.1)	1.7 (1.0-2.1)
Day 7	837 (643-903)	222 (182-277)	37 (36.0-62.6)	122 (86-162)	15.5+ (12.7-17.7)	28.6+ (26.9-34.0)	15.0 (11.6-18.0)	0.4 (0.1-0.8)	0.0 (0.0-0.0)	3.0* (1.8-5.05)
Day 10	668 (592-749)	254 (181-294)	45 (34.5-53.5)	85.5 (57.5-336.0)	2.7 (1.40-4.49)	11.4+ (9.0-15.4)	7.7+ (4.6-9.0)	0.07* (0.03-0.55)	0.0 (0.0-0.0)	1.5 (0.5-2.2)

Note. *p<0.05 in comparison with: *intact animals (Mann–Whitney test), +experimental day 1 (Wilcoxon test). Here and in Tables 2-4 the data are shown as medians and percentiles 25-75 (in parentheses).

correlations were found (ALT R=-0.88; Ki-67 R=0.87; PCNA R=-0.90; p<0.005).

Heat-shock proteins play a leading role in reparation and degradation of cell proteins, while disturbances in Hsp system can be a cause of dysfunction and damage to organs and tissues [11,15]. In light of this, some authors consider heat-shock proteins as a part of intracellular stress-limiting system protecting the cells from damage [8,9,18].

We detected more pronounced increase in the content of heat-shock proteins (total Hsp70 and inducible Hsp72 and Hsp60 forms) in animals receiving transplantation of nuclei, especially by the 3rd day of the experiment (Table 4), which attests to more pronounced induction of intracellular defense mechanisms.

p53 protein is known as a tumor growth suppressor or cell genome keeper. Lengthening of the intracellular half-life of p53 under the effect of DNA damage, hypoxia, or some other pathogenic factors led to its accumulation and potentiation of its transcription effects and to deceleration of cell mitosis [7,17]. Our experiments showed that the level of this protein in the liver of rats receiving nuclei was higher than in controls and in animals with cell transplantation, especially on day after CCl_4 injection (Fig. 3). This association of considerable p53 increase with the period of maximum morphofunctional changes in this organ was probably related to protection and maintenance of normal function of preserved liver cells, rather than with activation of apoptosis.

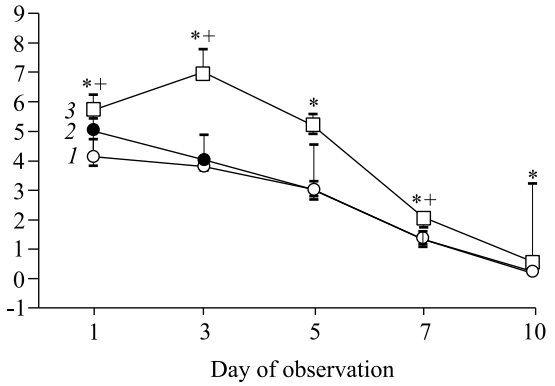

Fig. 3. Dynamics of p53 in the liver of animals with toxic hepatitis (group 1; *1*) receiving transplantation of neonatal liver cell (group 2; *2*) and nuclei (group 3; *3*).

Thus, transplantation of neonatal liver cell nuclei under conditions of toxic hepatitis led to better preservation of the enzyme systems realizing transami-

TABLE 2. Comparative Data on Enzyme Activities and Content of Peroxidation Intermediates in the Liver of Animals with Toxic Hepatitis under Conditions of Transplantation of Neonatal Liver Cells and Nuclei

Day of observation	Experimental series	LDH	AST	ALT	MDA
Day 1	CCl_4 and cells	762 (746-799)	216 (204-239)	41.2 (32.9-46.0)	56* (40.2-64.0)
	CCl_4 and nuclei	779 (743-837)	295*+ (249-391)	72.3*+ (39.5-79.0)	50.2* (45.6-95.0)
Day 3	CCl_4 and cells	831 (721-1028)	256 (179-313)	43.6 (31.2-56.8)	57.5* (52.5-69.0)
	CCl_4 and nuclei	955*+ (944-1370)	329*+ (314-410)	74.2*+ (69.2-79.0)	47.7* (45.6-53.0)
Day 5	CCl_4 and cells	832 (650-991)	236 (212-264)	39.5 (35.4-60.0)	65.8* (42.9-67.0)
	CCl_4 and nuclei	914 (890-941)	380*+ (266-387)	86.4*+ (56-120)	49.3* (29.2-95.0)
Day 7	CCl_4 and cells	812 (661-1022)	249 (207-331)	54.3 (36.2-69.2)	77.2* (65.7-92.0)
	CCl_4 and nuclei	839 (686-941)	259*+ (276-337)	92.2*+ (88-102)	16.4*+ (14.6-29.2)
Day 10	CCl_4 and cells	755 (592-749)	200 (163-247)	51 (34.6-77.4)	50* (45-124)
	CCl_4 and nuclei	664 (633-868)	247+ (212-299)	53.6* (52.7-54.8)	38.3*+ (34.7-73)

Note. Here and in Tables 3, 4: p<0.05 in comparison with: *toxic hepatitis, +transplantation of neonatal liver cells (Mann–Whitney test).

TABLE 3. Comparative Data on Morphometric Changes in the Liver of Animals with Toxic Hepatitis under Conditions of Transplantation of Neonatal Liver Cells and Nuclei at Different Terms of Observation

Day of observation	Experimental series	Necrosis area, %	Fatty infiltration of hepatocytes, %	Cell index
Day 1	CCl_4 and cells	1.9 (1.62-2.71)	37.5 (28.0-41.3)	18.3 (17.3-20.3)
	CCl_4 and nuclei	1.97 (1.87-2.10)	31.5* (31.0-32.5)	15.2+ (13.6-15.2)
Day 3	CCl_4 and cells	25.7 (22.6-29.6)	64.9 (59.5-71.5)	28.2 (25.0-32.5)
	CCl_4 and nuclei	26* (24.7-26.3)	60.2* (57.2-65.0)	24* (22.4-28.2)
Day 5	CCl_4 and cells	20.1 (13.3-21.7)	45.9 (37.3-55.6)	19.3 (18.0-21.3)
	CCl_4 and nuclei	19.2 (18.0-20.3)	42.4 (37.0-48.2)	17.0*+ (15.7-18.2)
Day 7	CCl_4 and cells	16.8 (14.3-17.2)	27.6 (26.3-29.4)	16.4 (13.7-17.5)
	CCl_4 and nuclei	15.3 (14.6-15.9)	22*+ (21.1-24.6)	12.7 (11.8-13.8)
Day 10	CCl_4 and cells	3.5 (1.2-5.2)	16.8 (13.5-26.8)	8.5 (7.6-10.2)
	CCl_4 and nuclei	2.1 (1.8-2.1)	8.9+ (8.00-9.25)	7.0 (4.5-8.3)

TABLE 4. Comparative Data on the Content of Stress-Proteins in the Liver of Animals with Toxic Hepatitis under Conditions of Transplantation of Neonatal Liver Cells and Nuclei at Different Terms of Observation

Day of observation	Experimental series	Hsp70	Hsp72	Hsp60
Day 1	CCl_4 and cells	2.9* (2.7-3.1)	2.1* (1.30-2.25)	2.4 (2.2-2.5)
	CCl_4 and nuclei	3.15*+ (3.10-3.25)	2.1* (2.05-2.20)	2.9+ (2.7-3.0)
Day 3	CCl_4 and cells	2.35* (1.25-3.05)	0.22* (0.20-0.35)	1.025 (0.95-1.10)
	CCl_4 and nuclei	3.02*+ (2.90-3.15)	0.4*+ (0.3-0.5)	3.17*+ (3.10-3.25)
Day 5	CCl_4 and cells	3.5* (3.40-3.85)	1.0* (0.9-1.1)	1.55 (1.40-1.65)
	CCl_4 and nuclei	3.05*+ (2.9-3.1)	0.65*+ (0.55-0.85)	1.55 (1.50-1.65)
Day 7	CCl_4 and cells	1.72* (0.60-1.85)	0.42* (0.35-0.50)	3.22 (3.1-3.3)
	CCl_4 and nuclei	2.17*+ (2.05-2.20)	0.42* (0.30-0.45)	2.85+ (2.7-3.0)
Day 10	CCl_4 and cells	1.95* (1.6-2.0)	0.45* (0.4-0.5)	1.4 (1.25-1.40)
	CCl_4 and nuclei	2.05* (2.0-2.1)	0.475* (0.40-0.55)	1.4 (1.25-1.50)

nation and glycolysis than transplantation of neonatal liver cells. This was associated with lower intensity of peroxidation processes and hepatocyte degeneration.

This more pronounced protective effect is probably determined by prolonged induction of cell self-renewal program and intracellular adaptation mechanisms related to more balanced and adequate production of proapoptotic proteins, modulators of the proliferative process, and inducible stress-proteins. Moreover, these findings attest to a possible direct or indirect influence of transplanted nuclei on cell genome in the damaged organ of the recipient. It can be hypothesized that transplantation of neonatal liver cell nuclei led to expression of genes responsible for production of regulatory peptides providing better preservation of the target organ under the action of pathogenic factors. However, this hypothesis requires additional experimental verification with the use of modern methods.

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