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Age-Related Features of the Response of the Liver and Stem Cells during Modeling of Liver Cirrhosis

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We studied the age-related characteristics of the response of stem cells and liver in male Wistar rats to administration of carbon tetrachloride (CCl_4) and ethanol. It was shown that modeling of liver cirrhosis caused inflammation, fibrosis, damage to sinusoidal capillaries, necrosis, and disturbances in the functional activity of hepatocytes in young rats. These processes were accompanied by mobilization of profibrotic mesenchymal stem cells (MSC), proinflammatory hematopoietic stem cells (HSC) and lymphocytes ($CD45^{hi}CD133^{+}$) from the bone marrow into the blood and migration to the liver. On the other hand, the number of hepatocyte precursors expressing Sox9 (cells of Hering's canal), immature cholangiocytes, Ito cells, oval cells, and endothelial cells of the liver sinusoids) sharply increased in the liver. In young rats, mobilization and migration of MSC, HSC, and hepatocyte precursors against the background of liver cirrhosis were more intensive than in old animals. The higher resistance of old rats to exposure is associated with age-related changes in the niches as well as in mobilization and migration of cells.

Key Words: *liver cirrhosis; age-related features; mesenchymal stem cells; hematopoietic stem cells; precursors of hepatocytes*

More than 2 million deaths from liver diseases are registered annually; of these 1 million deaths are associated with liver cirrhosis or its complications [1]. Liver cirrhosis is the terminal stage of many liver diseases (viral hepatitis, non-alcoholic fatty liver disease) and metabolic diseases (Wilson—Konovalov disease) [15]. In 50% of cases, the development of liver cirrhosis is associated with the toxic effects of alcohol [2].

Aging is a natural process characterized by structural and functional changes in all organs and tissues [4]. Hepatic blood flow and the content of hepatocytes decrease with age, while hepatocyte apoptosis increases. At the intracellular level, the area of the endoplasmic reticulum and the number of mitochondria in hepatocytes decrease and lipofuscin granules accumulate. In the endothelial cells lining the sinusoidal capillaries, the number of fenestrae decreases (defenestration), endocytosis is impaired [9]. Some authors point to age-related differences in the activity of liver regeneration [12] and its sensitivity to damaging agents (ethanol, thioacetamide) [8]. The formation of cirrhosis in adulthood is accompanied by more pronounced metabolic disorders (a decrease in the level

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of albumin, an increase in the activity of aminotransferases) than at an early age [10].

Liver regeneration is a complex process involving oval cells [13], diploid hepatocytes [14], mature hepatocytes and cholangiocytes [11], and endothelial cells of sinusoidal capillaries [5]. During aging, activity of liver cells changes [3], which affects the regeneration processes. There is an opinion that restoration of the liver structure proceeds with the participation of hepatocyte precursors [7]; it is possible that bone marrow mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC) are involved in this process. However, the absence of complete picture of age-related differences in stem cells in liver pathology impedes the development of personalized protocols for the treatment of liver diseases.

The objective of this study was to evaluate the characteristics of stem cell response in rats of different age groups during simulation of liver cirrhosis.

MATERIALS AND METHODS

The experiments were performed on male Wistar rats obtained from the nursery of the Department of Experimental Biological Models of the E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine (veterinary certificate is available). All manipulations were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The study was approved by the Ethics Committee of the E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine.

The animals were divided into 4 groups (5 rats per group): intact young rats (group 1), intact old rats (group 2), young rats with liver cirrhosis (group 3), and old rats with liver cirrhosis (group 4). At the beginning of the experiment, the age of young and old rats was 3 and 9 months, respectively.

Liver cirrhosis was simulated by oral administration of 40% CCl_4 oil solution (0.2 ml per 100 g body weight once a week for 3 months) and 5% ethanol solution that was freely available. The rats were euthanized CO_2 overdose after 6 (groups 1 and 3) or 12 months (groups 2 and 4).

The plasma concentrations of total and direct bilirubin were measured by photometry, activities of alkaline phosphatase, AST, and ALT were assessed by the kinetic colorimetric method, the concentrations of cholesterol and triglycerides were measured by the colorimetric enzymatic method, and glucose was assayed by the hexokinase method using Beckman Coulter diagnostic kits.

In standard histological preparations of the liver stained with hematoxylin and eosin, inflammation, fibrosis, and degree of liver damage were evaluated. Sinusoidal capillaries were counted using the ImageJ program and a 500×500 μm grid (10 vertical and 10 horizontal equidistant lines) placed onto the micrograph of the histological section. The relative density of sinusoidal capillaries was calculated by the formula: $Vv=(P1/Pc)\times100\%$, where Vv is relative density of capillaries, P1 is the number of grid nodes hitting the element, Pc is the total number of grid nodes.

Mononuclear cells in the bone marrow, blood, and liver parenchyma were obtained by the standard methods. To evaluate the content of stem cells in the tissues, the expression of surface markers CD45, CD90, Sox9 (Becton Dickinson), CD133, CD326 (Abcam) on mononuclear cells was evaluated using a FACSCanto II flow cytometer with FACSDiva software (BD Biosciences).

The results were processed by methods of variational statistics using the SPSS 12.0 software. The arithmetic mean (M), error of the mean (m), and the probability value (p) were calculated. Significance of differences was evaluated using the parametric Student's t test or nonparametric Mann—Whitney U test. The difference between the two compared values was significant at $p<0.05$.

RESULTS

Irrespective of the age of experimental animals, administration of CCl_4 and alcohol induced similar changes in the liver: fatty and hydropic degeneration of hepatocytes, fibrosis with the formation of false lobules, lympho-histiocytic infiltration of the portal tracts, piecemeal necrosis of hepatocytes along the periphery of the lobules, signs of cholestasis (Fig. 1). Massive destruction of hepatocytes under the influence of alcohol and CCl_4 naturally led to an increase in the levels of alkaline phosphatase, AST, ALT, bilirubin, cholesterol, triglycerides, and glucose in the blood plasma of rats of both age groups in comparison with intact controls of the corresponding age (Fig. 2). Comparison of the biochemical and histological parameters allowed us to identify age-related features of toxic liver cirrhosis. In old rats, activity of inflammation and fibrosis was lower, and damage to blood vessels and hepatocytes was less pronounced than in young rats (Fig. 1). The consequence of significant damage to hepatocytes in young animals was more pronounced violations of the biochemical parameters of plasma (alkaline phosphatase, AST, ALT, bilirubin, and glucose) in comparison with old animals.

According to modern concepts, MSC and HSC maintain inflammation and fibroplastic process [6]. Our data attested to the release of actively proliferating $Sox9^+/CD45^{hi}CD133^+$ lymphocytes and

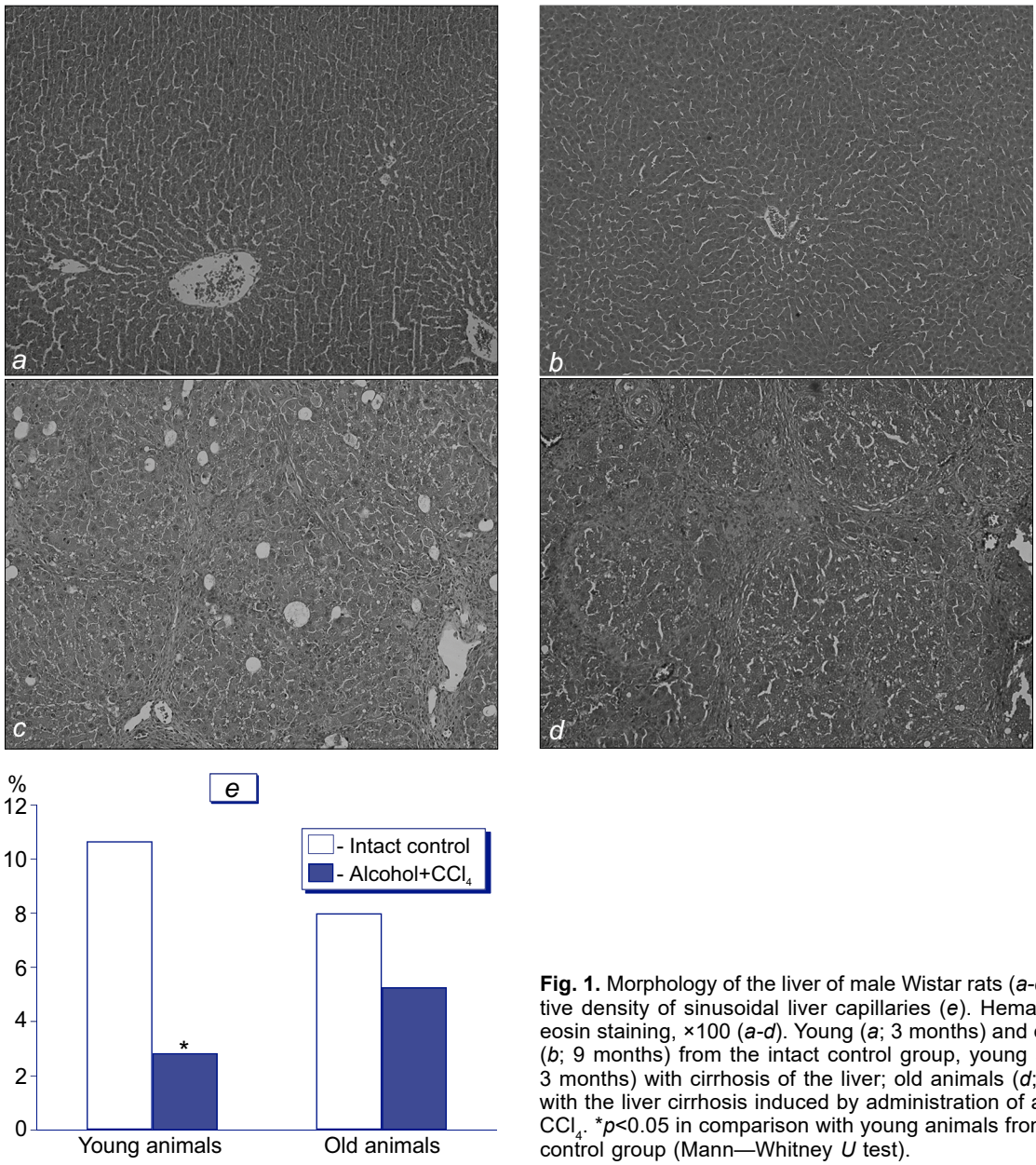

Fig. 1. Morphology of the liver of male Wistar rats (*a-d*) and relative density of sinusoidal liver capillaries (*e*). Hematoxylin and eosin staining, ×100 (*a-d*). Young (*a*; 3 months) and old animals (*b*; 9 months) from the intact control group, young animals (*c*; 3 months) with cirrhosis of the liver; old animals (*d*; 9 months) with the liver cirrhosis induced by administration of alcohol and CCl_4. *$p<0.05$ in comparison with young animals from the intact control group (Mann—Whitney *U* test).

$Sox9^{+/}CD45^{-}CD326^{-}CD133^{+}CD90^{+}$ HSC into circulation, and their recruitment into the liver parenchyma of old and young animals with cirrhosis induced by CCl_4 and alcohol (Fig. 3). At the same time, we observed an increase in the number of MSC ($CD45^{-}CD326^{-}CD133^{-}CD90^{+}$) in the liver. Detailed analysis of cytometry results drew us to a conclusion that activity of mobilization and migration of MSC, HSC, and lymphocytes in young rats was significantly higher than that in old rats with liver cirrhosis (Table 1). This largely explains the age-related differences in activity of inflammation, fibrosis, and liver destruction during cirrhosis development. In this context, MSC and HSC can be considered as markers of progression of toxic liver cirrhosis.

Many cell types are involved in liver regeneration: hepatocytes and their true and facultative progenitors [7]. The contribution of each population to recovery of liver structure and function is not clearly understood.

When evaluating progenitor cells expressing Sox9 in control rats and animals with cirrhosis of the liver of different ages, we found a number of regularities. In old intact rats, the number of various precursors (cells of Hering's canal, immature cholangiocytes, Ito cells, oval cells, and endothelial cells of liver sinusoids) was higher than in young animals. The exception was $Sox9^{+}$ ductal hepatocytes: their content decreased by 82% in comparison with that in young animals. Diploid hepatocytes with high proliferative activity were revealed in young animals with experimental cirrhosis

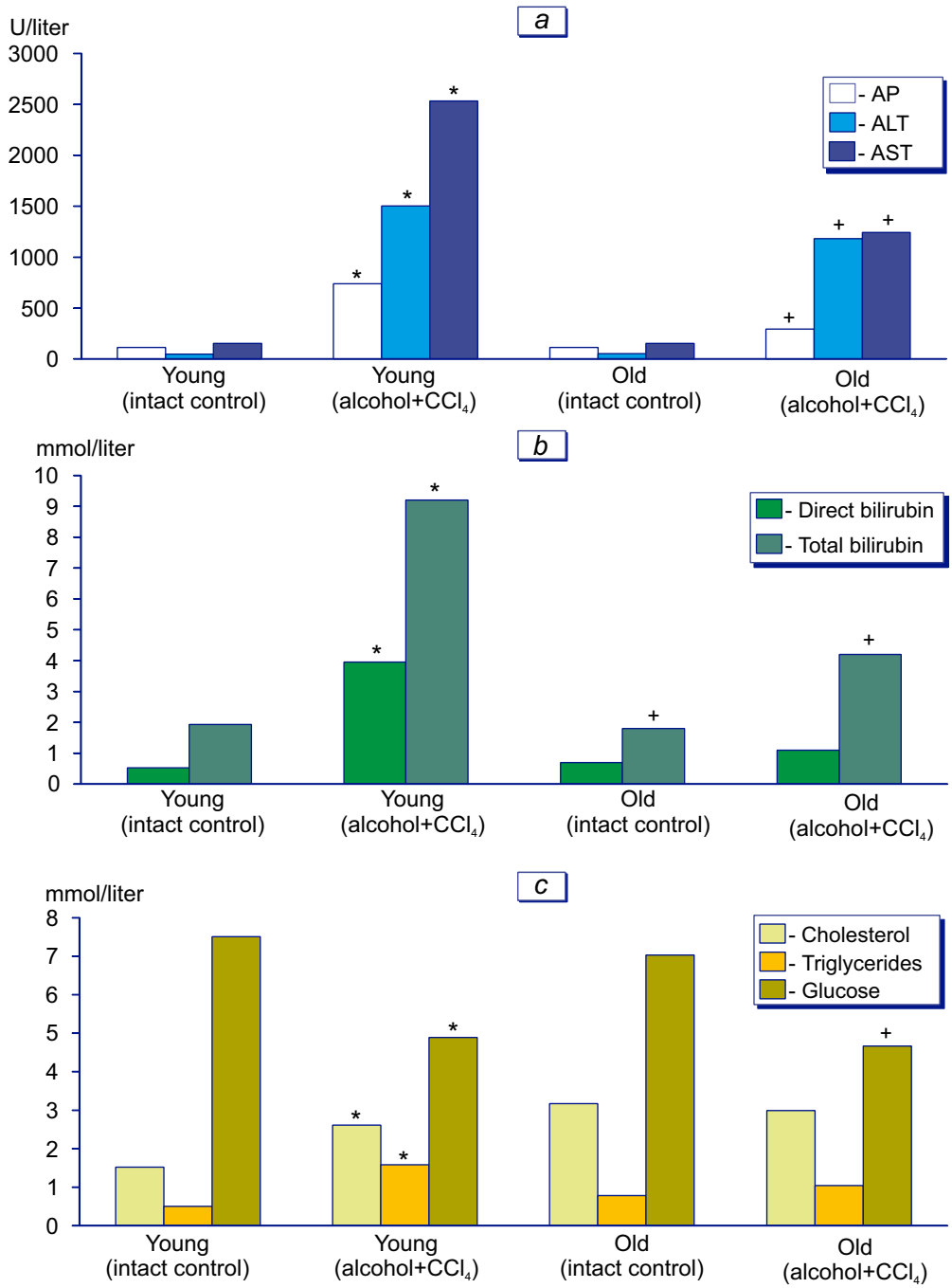

Fig. 2. Concentration of alkaline phosphatase (AP), AST, ALT (*a*), total and direct bilirubin (*b*), cholesterol, triglycerides, and glucose (*c*) in the blood serum of young and old male Wistar rats with cirrhosis of the liver caused by administration of alcohol and CCl_4. $p<0.05$ in comparison with *young and $^+$old animals from the intact control group (Mann—Whitney *U* test).

of the liver (Table 2). Necrosis of hepatocytes in old rats with cirrhosis was less pronounced than in young animals. This explains the age-related differences in proliferative activity of bipotent cells of Hering's canal and oval liver cells (Table 2). It is noteworthy that in the damaged liver of old rats, the content of $Sox9^+$ hepatocytes and cholangiocytes with a niche in the ducts was significantly lower than in the liver of healthy old rats. It can be assumed that the decrease in the population of proliferating true and facultative progenitor cells in the liver parenchyma of old rats is associated with age-related changes in niches. This assumption does not contradict the results of other authors [3]. The observed difference can be determined by more intensive differentiation of precursors into mature liver cells.

TABLE 1. The Content of MSC, HSC, Total Lymphocyte Population, and Lymphocytes with the Inflammatory Phenotype in the Arterial Blood (Hepatic Artery), Liver, and Bone Marrow of Young and Old Male Wistar Rats with Liver Cirrhosis Induced by Alcohol and CCl_4 (% of stained mononuclear cells; *M±m*)

Localization	Young animals		Old animals	
	intact control	alcohol+CCl_4	intact control	alcohol+CCl_4
MSC ($CD45^-CD326^-CD133^-CD90^+$)				
Arterial blood	13.0628±5.1366	2.4301±2.0247*	13.1592±10.7573	15.2181±9.0006
Liver	3.3840±1.5918	18.1077±0.4001*	0.1358±0.0061	0.3656±0.2555$^+$
Bone marrow	14.6209±0.5179	12.1878±4.6076	5.8289±2.3236	3,3102±0,2306$^+$
HSC ($CD45^-CD326^-CD133^+CD90^+$)				
Arterial blood	0.0379±0.0062	0.0010±0.0010*	0	0.0492±0.0222$^+$
Liver	0.0068±0.0450	0.5697±0.3810*	0	0.0073±0.0037$^+$
Bone marrow	4.7070±0.5394	0.0373±0.0238*	0.0193±0.0126	0.0779±0.0215$^+$
Lymphocytes ($CD45^+CD90^+CD133^-$)				
Arterial blood	29.5209±2.2541	2.7651±1.9420*	15.4327±3.0332	4.1539±2.6251$^+$
Liver	9.6915±3.4876	14.7533±9,6819*	0.2715±0.1828	9.4516±8.0639$^+$
Bone marrow	39.9018±4.6306	48.7044±5.9216	24.0011±1.2291	22.2639±9.1843
Lymphocytes with an inflammatory phenotype ($CD45^{hi}CD133^+$)				
Arterial blood	0.5081±0.2103	0.0851±0.0390*	0.9851±0.5475	1.7243±1.4610$^+$
Liver	0.1719±0.0523	0.6644±0.2804*	0.1111±0.0636	2.1525±1.9469$^+$
Bone marrow	0.1666±0.0378	0.3988±0.1738*	0.3077±0.0499	0.2724±0.0540

Note. $p<0.05$ in comparison with *young and $^+$old animals from the group of intact control (Mann—Whitney *U* test).

TABLE 2. Contents of Different Populations of Hepatocyte Precursors in the Parenchymal Fraction of the Liver in Young and Old Male Wistar Rats with Liver Cirrhosis Induced by Alcohol and CCl_4 (% of stained mononuclear cells; *M±m*)

Cells (immunophenotype)	Young animals		Old animals	
	intact control	alcohol+CCl_4	intact control	alcohol+CCl_4
Diploid hepatocytes of the periportal zone ($Sox9^+CD45^-$)	0.2200±0.0336	6.4759±2.0436*	3.0111±1.4376	0.3256±0.0188$^+$
Cells of Hering's canal	0.0165±0.0061	0.3767±0.1030*	0.2586±0.0177	0.7366±0.0277$^+$
Immature cholangiocytes ($CD45^-CD326^+CD133^-Sox9^+$)	0	0.6620±0.0820*	0.0976±0.0053	0.0084±0.0084$^+$
Intermediate hepatocytes ($CD45^-CD326^+CD133^-Sox9^-$)	4.8107±2.3770	0.8528±0.1332*	0.5241±0.0936	0.2508±0.1397$^+$
Total population of hepatocytes and cholangiocytes ($CD45^-CD326^-CD133^-Sox9^-$)	61.2186±1.8131	63.2155±13.4914	81.0505±3.9169	76.8252±0.0107
Hepatic stellate cell ($CD45^-CD133^+$)	0.0127±0.0043	3.3979±0.3225*	0.2432±0.0331	0.1336±0.0114$^+$
Endothelial cells of liver sinusoids ($CD133^+CD45^+$)	0.1341±0.0421	3.2522±0.0882*	0.7890±0.2406	0.3534±0.0466$^+$
Oval cells ($CD326^+CD133^+$)	13.1790±3.4103	69.5828±3.6206*	15.8902±8.3417	23.8150±0.2235$^+$

Note. $p<0.05$ in comparison with *young and $^+$old animals from the group of intact control (Mann—Whitney *U* test).

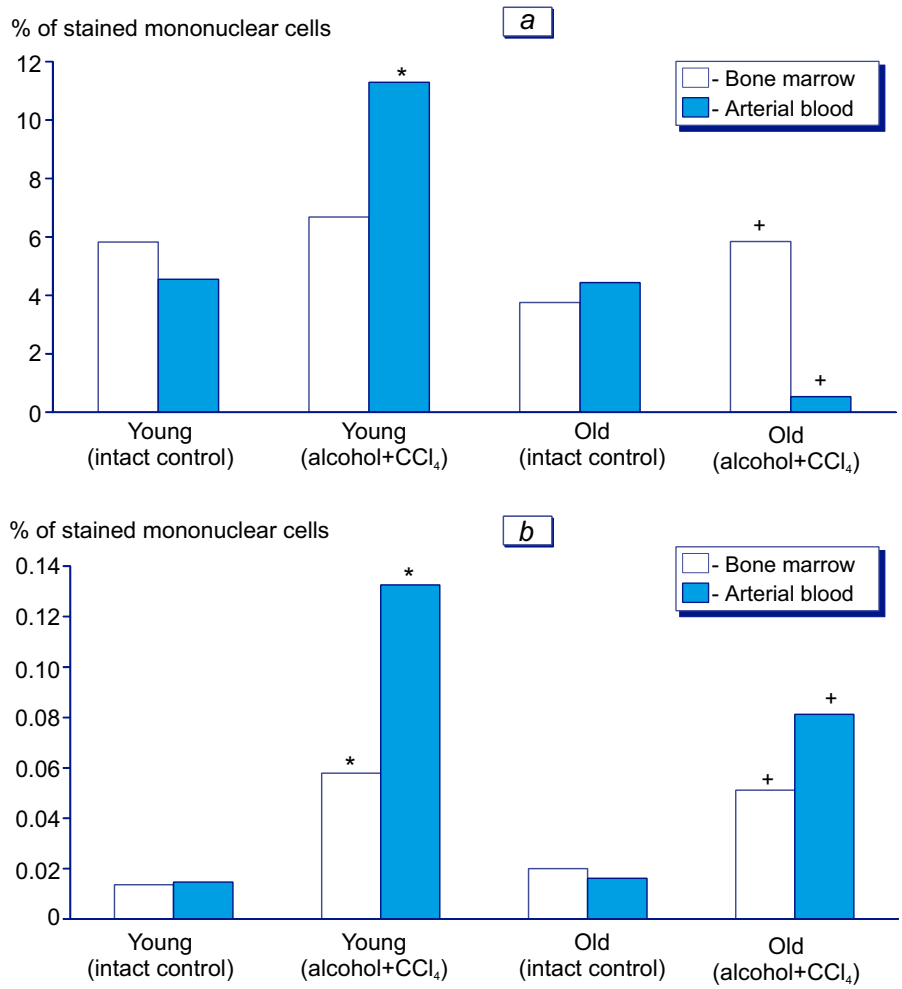

Fig. 3. Content of Sox9$^+$ HSC/CD45$^-$CD326$^-$CD133$^+$CD90$^+$ (*a*) and lymphocytes with the inflammatory phenotype/CD45^{hi}CD133$^+$ (*b*) in bone marrow and arterial blood (hepatic artery) of young and old male Wistar rats with cirrhosis of the liver caused by administration of alcohol and CCl_4. $p<0.05$ in comparison with *young and $^+$old animals from the intact control group (Mann—Whitney *U* test).

The obtained results show that induction of liver cirrhosis with CCl_4 and alcohol in young rats is accompanied by a more pronounced cell response than in old animals: liver infiltration with inflammatory cells, necrosis of hepatocytes, endothelial damage, mobilization and migration of MSC and HSC, proliferative activity of hepatocytes precursors. At the same time, the increase in the number of hepatocytes precursors is not a guarantee of liver regeneration. On the contrary, age-related changes in the niche, cell mobilization, and migration processes observed in old animals can be considered as protective factors improving resistance of the liver to the effects of damaging agents such as CCl_4 and alcohol.

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