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Effect of Bioregulators Isolated from Rat Liver and Blood Serum on the State of Murine Liver in Roller Organotypic Culture after CCl_4-Induced Fibrosis

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We studied the protective effect of bioregulators isolated from the liver and blood serum of mammals under conditions of manifest fibrosis. Fibrosis was induced by CCl_4 administration for 30 days and then, the liver was cultured in a roller organotypic culture for 30 days in the presence of bioregulators. Hepatoprotective effect of bioregulators was evaluated on histological sections of the liver at different terms of culturing. Experiments with roller organotypic culture of the liver isolated from animals with *in vivo* CCl_4-induced fibrosis demonstrated the protective effect of bioregulator of the liver origin, while bioregulator isolated from the blood was ineffective.

Key Words: *bioregulators; liver; fibrosis; organotypic culturing*

Cell adhesion plays a fundamental role in the regulation of the key biological processes such as migration, proliferation, differentiation, apoptosis, gene expression, signal transduction, and morphogenesis [10,11]. Disturbances of cell–cell adhesion contacts serve as the initial step in various pathological processes. In particular, liver fibrosis represents a complex response to chronic exposure to harmful factors: tissue necrosis and inflammation promote regeneration, in this particular case, replacement regeneration accompanied by accumulation of extracellular matrix proteins in the liver and its dysfunction. Under conditions of fibrosis, extracellular matrix proteins are primarily produced by myofibroblast cells originating from different cell populations, the largest is Ito cell population [9].

It has been previously shown that cell adhesion to the substrate largely determines the state of cultured tissue. Tissue contact with the substrate triggers the mechanisms controlling cell differentiation, maintenance of their viability in the culture, apoptosis, *etc*. The absence of adhesion interactions between the tissue and the substrate leads to activation of cell sources of tissue regeneration, intensification of cell migration, accumulation of low differentiated cells and their proliferation [1,5]. Under conditions of roller culture, a certain group of bioregulators, membranotropic homeostatic tissue-specific bioregulators (MHTB), contributes to additional activation of the cell sources of regeneration in the cultured tissue and stimulates recovery and repair in pathologically altered tissues *in vivo* [6].

Our previous experiments on *in vivo* mouse model of CCl_4-induced fibrosis have demonstrated that MHTB isolated from mammalian liver produced a protective effect and decelerated fibrosis development [2].

It is currently accepted that MHTB are present in various tissues of animals, plants, and fungi. They were first found in the tissues as adhesion factors that modulate viscosity, elasticity, and permeability of the plasma membranes [7]. MHTB represent extracellular peptide–protein complexes [6]. In low doses, they affect the basic biological processes (adhesion, migration, differentiation, and apoptosis). Under conditions of organotypic culture, MHTB support tissue structure,

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cell viability, while *in vivo* they stimulate recovery and regeneration of pathologically altered tissue. Biological activity of MHTB is characterized by tissue specificity and the absence of species specificity.

We studied the protective effect MHTB isolated from mammalian liver and blood serum under conditions of manifest fibrosis and activation of cell sources of tissue regeneration during roller organic culturing.

MATERIALS AND METHODS

MHTB were isolated from mammalian liver and blood serum using a previously developed technique; their physicochemical properties were described in previous publications [2-4].

Experiments were carried out on male and female Wistar rats weighing 220-260 g. All procedures with animals were carried out in accordance with Directive 86/609/EEC. After decapitation under ether anesthesia, the liver was perfused through the portal vein with 10 ml of Ringer's solution (0.15 M NaCl, 1 mM $CaCl_2$, 5 mM KCl, and 1 mM HEPES) to remove the greater part of the blood. The liver was removed, cut into 1.5-2.0-cm^3 pieces and placed in Ringer solution for 2-2.5 h at 4-8°C. The tissue extract was centrifuged and the proteins were precipitated by ammonium sulfate (Reakhim). Bioregulator from the serum was obtained from a commercial product (BioloT).

Ammonium sulfate was added with stirring to blood serum and liver extract (780 g/liter); the solution was allowed to stay for 5 days at 4°C, then centrifuged (30 min at 12,000*g*), supernatants and precipitates were collected separately and dialyzed against water until complete removal of salt traces. Supernatant fractions were concentrated using a vacuum rotary evaporator at 40°C. In the obtained fractions, membranotropic activity was analyzed.

Liver fibrosis was induced by regular intraperitoneal injection of CCl_4 (Component Reactive) in a dose of 1 μl/g body weight in 30% peach oil (GalenoFarm) according to standard procedures [2,8]. The toxicant was injected twice a week over 30 days. Two-month-old male mice, (C57Bl/CBA)F_1 hybrids, were used in the experiment.

The animals were sacrificed by cervical dislocation on day 30 of the experiment. The isolated liver was placed into sterile 3.5-cm Petri dishes in medium 199 (PanEco) supplemented with antibiotic–antimycotic (Gibco). Then, the edges of the liver lobe (2-3 mm from each side) were removed with a blade, and the rest material was cut into pieces of 5×5 mm.

Organotypic roller culturing of mouse liver was performed under the following conditions: 79% medium 199, 20% fetal calf serum (PanEco), 1% antibiotic–antimycotic. Before addition to vials, the medium was filtered through 0.22-μ Millex-GV (Millipore) sterilizing membrane filters. The liver fragments were placed in 20-ml dark-glass culture flasks. In each flask, 4 ml medium and 40 μl MHTB (concentration 10^{-10} mg protein/ml) were added. Three experimental groups were formed, in which the fragments were cultured in normal medium (group 1; control) or in the presence of MHTB isolated from the liver (group 2) or MHTB isolated from the serum. All flasks were closed with sterile stoppers, wrapped with Parafilm M (Parafilm), and placed in a thermostat. Mouse liver was cultured in a RM5 roller (Assitent) in the dark at 37±1°C; rotation speed 35 rpm. The culture medium was not changed throughout the culturing period.

Liver fragments were fixed in formalin:alcohol: acetic acid 9:6:1 mixture (Serva) and then processed routinely. These sections were stained using complex multicolor Mallory technique (Serva) that specifically visualizes collagen and with hematoxylin and eosin (Serva). The histological preparations were analyzed under a Leica DM RXA2 light microscope.

RESULTS

On day 14 of culturing, no pronounced changes in the liver tissue were observed in the control group, mild degeneration was revealed in the zone of porto-portal tracts. Staining for collagen showed loose (diffuse) porto-portal septa (Fig. 1, *a*, *b*). In group 2, degenerative changes were almost complete absent and infiltration around the vessels was clearly seen. Staining for collagen also showed loose (diffuse) porto-portal septa (Fig. 1, *c*, *d*). In group 3, pronounced degeneration of the liver parenchyma with preservation of the cord structure was observed. Staining for collagen also showed loose (diffuse) porto-portal septa (Fig. 1, *e*, *f*).

On day 21 of culturing, preserved parenchyma cells with distinct nuclei were seen at the periphery of liver fragments, cord structure around the portal tracts was preserved, infiltration foci were found around the portal tracts. In the central zone of cultured fragments, degenerative changes, violation of the cord structure of the tissue, as well as minor cell death of liver parenchyma were observed. Staining for collagen showed diffuse porto-portal septa (Fig. 2, *a*, *b*).

In group 2, mild periportal degeneration was observed. Staining for collagen showed loose (diffuse) porto-portal septa (Fig. 2, *c*, *d*). In group 3, we observed pronounced periportal degeneration with disorganization of the cord structure, and intensive perivascular infiltration. Viable parenchymal liver cells were seen everywhere in the tissue fragment, but minor cell death was visualized in the central region. Staining for collagen showed loose (diffuse) porto-portal septa (Fig. 2, *e*, *f*).

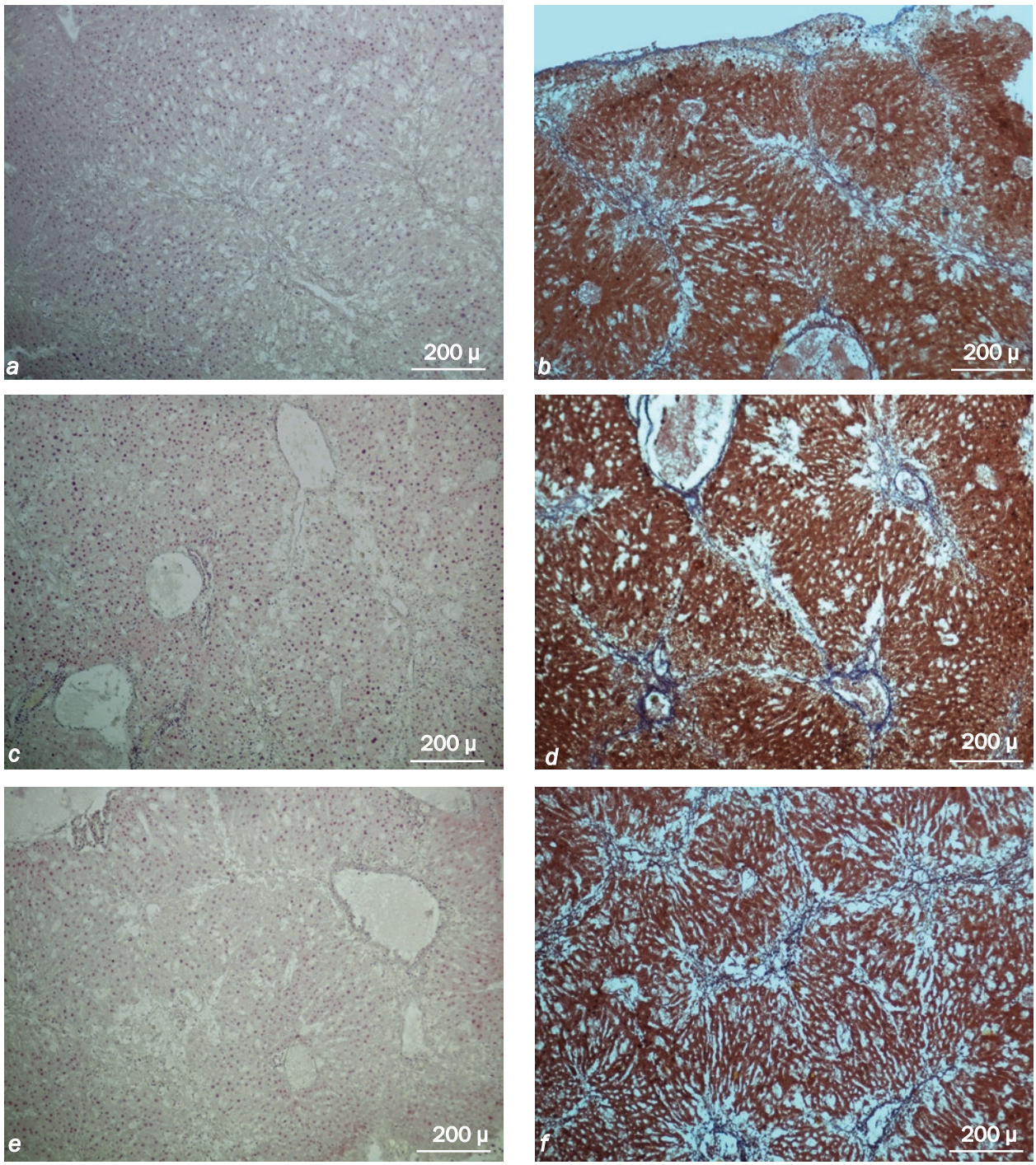

Fig. 1. Mouse liver tissue after 14-day culturing. *a*, *b*) Group 1 (fibrotic liver); *c*, *d*) group 2 (fibrotic liver+liver-derived MHTB); *e*, *f*) group 3 (fibrotic liver+serum-derived MHTB). Here and in Figs. 2, 3: staining with hematoxylin and eosin (*a*, *c*, *f*), after Mallori (*b*, *d*, *f*).

On day 28 of culturing, almost all parenchymal liver cells died in the control group. Staining for collagen showed pronounced (dense) porto-portal septa (Fig. 3, *a*, *b*). In group 2, preserved parenchyma cells with distinct nuclei were seen at the periphery of the liver fragments, cord structure around the portal tracts was preserved, periportal infiltration foci were found. Degenerative changes and disruption of the cord structure of the tissue were seen in the central area of cultured fragments. Specific staining for collagen revealed porto-portal septa; collagen in these septa did not form dense bundles, but was diffusely distributed (Fig. 3, *c*, *d*). In group 3, almost complete cell death was seen. Viable cells were present in the periportal zone in the form of infiltrations. In the central region, death of

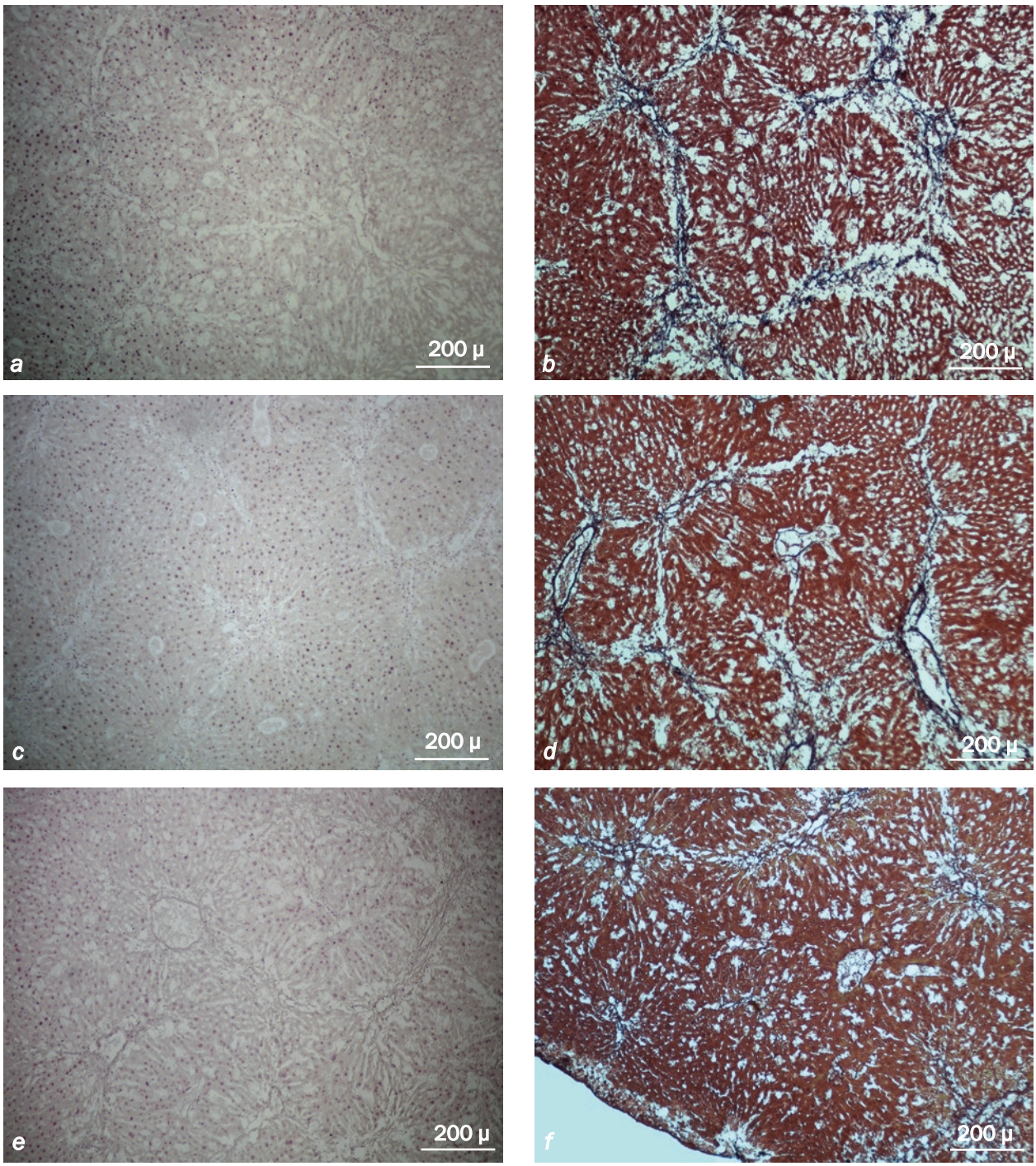

Fig. 2. Mouse liver tissue after 21-day culturing. *a*, *b*) Group 1 (fibrotic liver); *c*, *d*) group 2 (fibrotic liver+liver-derived MHTB); *e*, *f*) group 3 (fibrotic liver+serum-derived MHTB).

parenchymal cells and degeneration were observed. Specific staining for collagen revealed porto-portal septa with thick collagen bundles, while collagen distribution in the periportal zone was denser than in the control (Fig. 3, *e*, *f*).

Thus, liver-derived MHTB produced a protective effect on fibrotic liver tissue during its roller culturing in comparison with the control and serum-derived MHTB. These effects were most pronounced at late terms (days 21 and 28 in culture).

These results can be attributed to the effect of liver-derived MHTB on cellular sources of regeneration in the liver tissue. It should be noted that stimulation of of recovery and repair processes in damaged tissue via additional activation of regeneration sources was also demonstrated for MHTB isolated from other tissues [6].

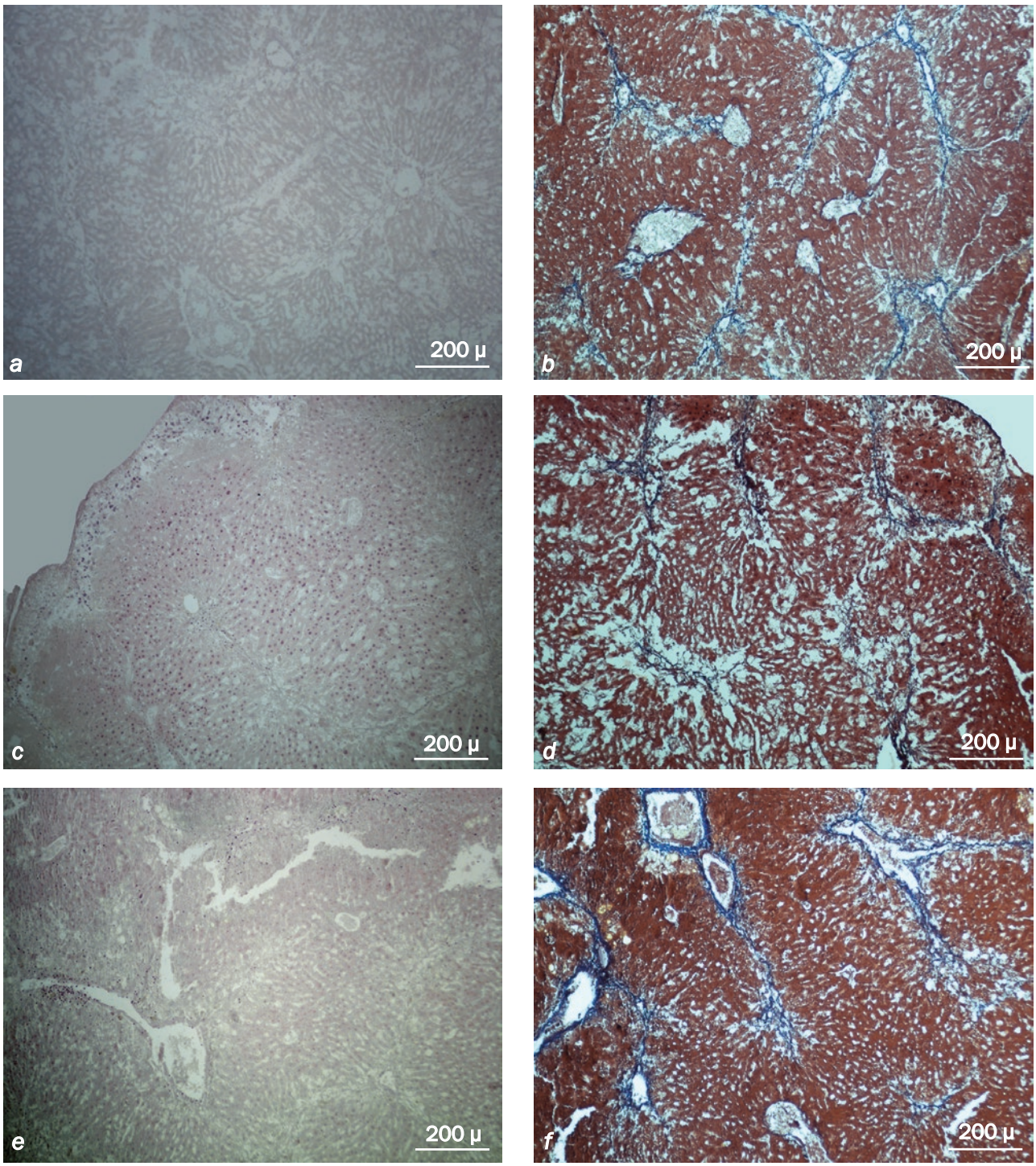

Fig. 3. Mouse liver tissue after 28-day culturing. *a*, *b*) Group 1 (fibrotic liver); *c*, *d*) group 2 (fibrotic liver+liver-derived MHTB); *e*, *f*) group 3 (fibrotic liver+serum-derived MHTB).

The study also revealed clear-cut tissue-specific nature of the protective effect of liver-derived MHTB and its significant difference from the action of serum-derived MHTB. It should be also noted that MHTB isolated from blood serum also exhibited a tissue-specific protective effect manifested in the maintenance of cell viability around the portal tracts.

It was previously assumed that Ito cells, hepatocytes, portal fibroblasts and bone marrow-derived fibroblasts serve as cell sources for replacement regeneration (during fibrosis) [10]. It can be hypothesized that bioregulator isolated from the whole liver produces a protective effect on both Ito cells and hepatocytes, while bioregulator isolated from the blood

serum affects only connective tissue cells (portal and bone marrow-derived fibroblasts). Alternation of the effects of these bioregulators will probably provide the protective effect for all cell populations in the liver.

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