Effect of Bioregulators Isolated from the Liver, Blood Serum, and Bile of Mammals on the State of Newt Liver Tissue in Organotypic Culture

V. P. Yamskova[1], A. V. Borisenko[1], M. S. Krasnov[1], A. P. Il'ina[2], E. Yu. Rybakova[1], D. I. Malcev[2], and I. A. Yamskov[2]

Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 3, pp. 156-164, July, 2010
Original article submitted March 24, 2010.

We developed models of *in vitro* organotypic culturing of newt liver tissue with and without adhesion to the substrate. The effects of bioregulators isolated from mammalian liver, blood serum, and bile were studied on the developed models and their specificity was demonstrated. The state of the liver was evaluated by the area of clusters of pigmented cells and by the number of mitoses in the connective tissue cells of the cortical layer. These bioregulators exhibited their biological effects only under conditions of roller organotypic culturing of newt liver tissue.

Key Words: *bioregulators; liver; newt; culturing; roller*

Organotypic culturing of tissues from adult newts can be used as an experimental model for evaluation of the effects of bioregulators responsible for normal morphology and function of these tissues [6,9], *e.g.* membranotropic homeostatic tissue-specific bioregulators (MHTB) identified previously in animal tissues [13,19,20]. These bioregulators were united in a group on the basis of their similar physicochemical properties and biological effects. MHTB are nanosized particles containing proteins, carbohydrates, and lipids [7,11]. It was found that the protein component is responsible for biological activity of MHTB and the major role is played by short peptides with a molecular weight of <6 kDa [7]. In some tissues, extracellular localization of some MHTB was shown [13,19]. Activity of MHTB is characterized by tissue specificity and the absence of species specificity [10,13,19]. Here we studied specific activity of MHTB previously identified in mammalian liver, blood serum, and bile [1,10]. These MHTB were synthesized in the liver; in light of this, it was interesting to evaluate their specific effects on liver tissue. Previous studies showed that MHTB isolated from blood serum stimulated proliferation of mammalian fibroblasts *in vitro* and improved wound healing by promoting recovery of the histological structure of the cornea and skin in mammals and amphibians *in vitro* and *in vivo* [4,19], while MHTB isolated from rat liver exhibited a hepatoprotective effect by modulating activity of cytochrome P-450 enzyme systems in rat liver [5]. The biological effects of MHTB isolated from cattle bile manifested in a decrease in the size of bile particles *in vitro* [1]. In the present study, specific activity of these MHTB was studied on the model of roller organotypic culturing of newt liver tissue *in vitro* developed by us for this purpose.

MATERIALS AND METHODS

Deionized water MilliQ 16 MΩ, medium 199 (M. P. Chumakov Institute of Polyomyelitis and Virus Encephalites), HEPES (Serva), FCS (M. P. Chumakov Institute of Polyomyelitis and Virus Encephalites), 4% gentamicin (Dal'khimfarm), cattle blood serum (Sverdlovsk Research Institute of Viral Infections),

[1]N. K. Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences; [2]A. N. Nesmeyanov Institute of Elementorganic Compounds, Russian Academy of Sciences, Moscow, Russia. ***Address for correspondence:*** embrmsk@mail.ru. M. S. Krasnov

0007-4888/10/1501-0140

cattle bile (oxgall, Samson-Med), ampholines (LKB), nitrocellulose filters (Gelman), and roller RM 5 (Assistant). All reagents were of chemically pure grade.

The study was performed on male and female Wistar rats weighing 210-230 g and on *Pleurodeles waltl* newts maintained in a vivarium and aquarium, respectively, of N. K. Kol'tsov Institute of Developmental Biology, Russian Academy of Sciences. Experiments were performed in September–October.

The nutrient medium for amphibians contained (per 100 ml): 64 ml medium 199, 27 ml boiled water, 7.14 mg HEPES, 9 ml FCS, and 200 µl 4% gentamicin sulfate.

MHTB were isolated from the liver of Wistar rats and from cattle blood serum (3 liters) and bile (2 liters) as described previously [10]. The tissue extract of the liver, blood serum, or bile were fractionated by the method including double protein sedimentation in saturated ammonium sulfate solution followed by isoelectrofocusing in sucrose density gradient at pH 3.5-10.0 using ampholines (96 h at 500-2000 V and 4°C) [8]. Fractions of acidic proteins (pH<3.0) of liver MHTB, blood serum MHTB, and bile MHTB were collected [8]. After dialysis against water (for removal of sucrose and ampholines), the fractions were lyophilized and dissolved in 1 ml water. Identification of MHTB was performed using adhesion biotesting method. The membranotropic effect was evaluated [8]. Molecular weight of the proteins was performed by MALDI-TOF mass-spectroscopy on a UltraFlex 2 time-of-flight mass spectrometer (Bruker Daltonic). Time-of-flight mass-spectra were recorded in forward and reflection mode. Before mass-spectrometry, the samples were evaporated to dryness, the residue was dissolved in 70% acetonitrile containing 0.1% trifluoroacetic acid to a final concentration of not less than 10 pmol/µl. The following matrices were used: sinapinic acid and 2-cyano-4-hydroxycinnamic acid.

For obtaining organotypic liver tissue cultures, newt were decapitated under urethane anesthesia, the liver was removed, 3×3×3-mm fragments were taken from the central lobe, transferred into a nutrient medium for amphibian, and cultured at 21-23°C in dark-glass vials. In each experiment, liver fragments from one newt were used for different experimental series and control.

For roller culturing, tissue fragments (n=5) were placed on a roller and rotation velocity was set at 35 rpm.

For culturing on a substrate, the tissue fragments were placed on nitrocellulose filters into vials and incubated stationary without medium stirring.

In the beginning of the experiment, the test MHTB was once added to vials to a final concentration of 10^{-12} mg peptide/ml. After 3-day culturing, the tissues were fixed in Bouin fixative, embedded in paraffin blocks, and 3-4-µ histological sections were prepared. The sections were stained with hematoxylin and eosin; the state of the tissue was evaluated by light microscopy, the area of pigmented cell clusters was measured morphometrically. Mitoses in connective tissue cells of the cortical layer were counted. For each experimental point, 150 sections were performed. At least 500 cells in each section were analyzed.

The data were processed by methods of variation statistics. The means and errors of the means were calculated. The significance of differences between the means was evaluated using Student t test.

RESULTS

Fractions of MHTB isolated from mammalian liver, blood serum, and bile and exhibiting membranotropic activity in ultralow doses were studied [1,8]. We previously showed that these fractions mainly consist of small peptides <10 kDa [1]. MALDI-TOF analysis showed that the studied fractions contained peptides with a molecular weight of 3625 and 5025 Da (liver), 1789, 2548, and 3104 Da (blood serum), and 2155, 2488, 3156, and 3281 Da (bile).

The tissues of tailed amphibians are characterized by higher regeneration capacity than mammalian tissues [18]. During organotypic culturing, liver cells from adult amphibians were characterized by higher viability compared to liver cells of adult mammals. Previous studies showed that organotypic cultures of adult amphibian liver can be maintained for a long time [15]. During roller culturing of amphibian tissues, tissue culture remains viable for a long time due to activation of cell sources of regeneration [3,9].

Figure 1 shows histological sections of intact liver of *Pl. waltl* newt. The liver is coated with a fibrous capsule; a cortical layer presented by multilayer structures of connective tissue cells is located under the capsule. These cells have low cytoplasm volume and large nuclei of varying shape; mitoses were sometimes seen among them. The liver in adult tailed amphibians differs from mammalian liver by preserved hemopoietic function [2]. The liver parenchyma in newt is compact and is structurally similar to that in mammals. Parenchymal cells have irregular polygonal shape, sometimes rounded angles and larger size. The vessels are surrounded by a well-developed area of connective tissue cells containing bile ducts. The lumens of blood vessels contain erythrocytes. Newt liver contains many pigmented cells often forming groups in both the cortical layer and parenchyma.

Pigmented cells of amphibian liver are analogous to mammalian Kupffer cells and play a role of macrophages (absorb various particles, *e.g.* microorganisms

and viruses, and detoxify xenobiotics) [16]. These cells originate from hemopoietic stem cells [17] and differ from other phagocytes of vertebrates by their capacity to synthesize melanin in melanosomes, but at the same time they differ from melanocytes originating from the neural crest [16]. According to published data, pigment distribution in these cells of newt liver varies in different seasons: it decreases by the second half of winter and increases in spring and summer [2].

Organotypic culturing of newt liver for 3 days led to appreciable changes in the liver tissue, which considerably differed in roller and stationary culturing.

We examined the marginal zone, parenchyma, and perivascular zones differing by the types of cells and their functions in the liver [2]. The state of liver tissue was evaluated by measuring the area of pigmented cell clusters and number of mitoses in the cortical zone, which according to published data reflects the capacity of amphibian liver tissue to respond to damaging influences [14].

Examination of histological sections of roller organotypic cultures revealed normal histological structure of the liver, adhesion interactions between parenchymal cells were preserved, although the processes of cell lysis and death were also noted (Fig. 2). Pyknotic nuclei were seen in many parenchymal cells; in pigmented cells, pigment precipitation was noted. Large associates of pigmented cells were found in the parenchyma and perivascular zones. In the cortical zone we found low number of cell layers, mitoses were seen in some of these cells. A narrow zone of connective tissue cells around blood vessels contained several types of hemopoietic cells.

Culturing of newt liver on the substrate was associated with parenchymal cell degradation in the central part of the tissue fragment, whereas in the marginal zones both the parenchymal and connective tissue cells were viable despite the presence of cells with pyknotic nuclei (Fig. 3). Disturbed adhesion interactions and lysis of parenchymal cells were seen. Clusters of pigmented cells were primarily located in the cortical and perivascular zones. These clusters were unevenly distributed in these zones and were less dense than cluster of pigmented cells identified in liver tissue after roller culturing (Fig. 3). In the central zone of tissue fragment, pigment release from cells was noted (Fig. 3). No mitoses of connective tissue cells were seen.

The area of pigmented cell clusters in newt liver tissue was considerably larger in fragments subjected to roller culturing compared to that in fragments cultured on the substrate and even in intact tissue (Table 1). The number of mitoses in the cortical zone was significantly lower than in intact liver, but higher than in the liver tissue cultured on the substrate. These fin-

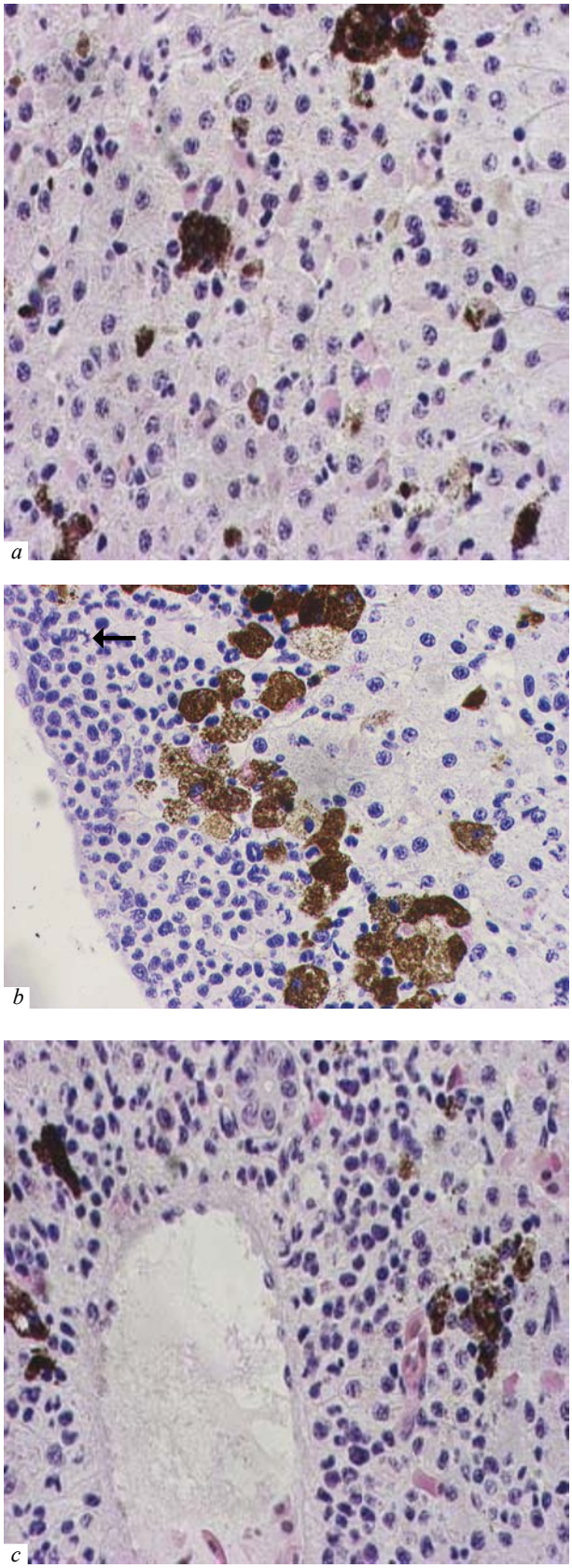

Fig. 1. Intact newt liver, ×200. Here and on Fig. 5: Hematoxylin and eosin staining. *a*) parenchyma; *b*) marginal zone (arrow shows mitoses); *c*) perivascular zone.

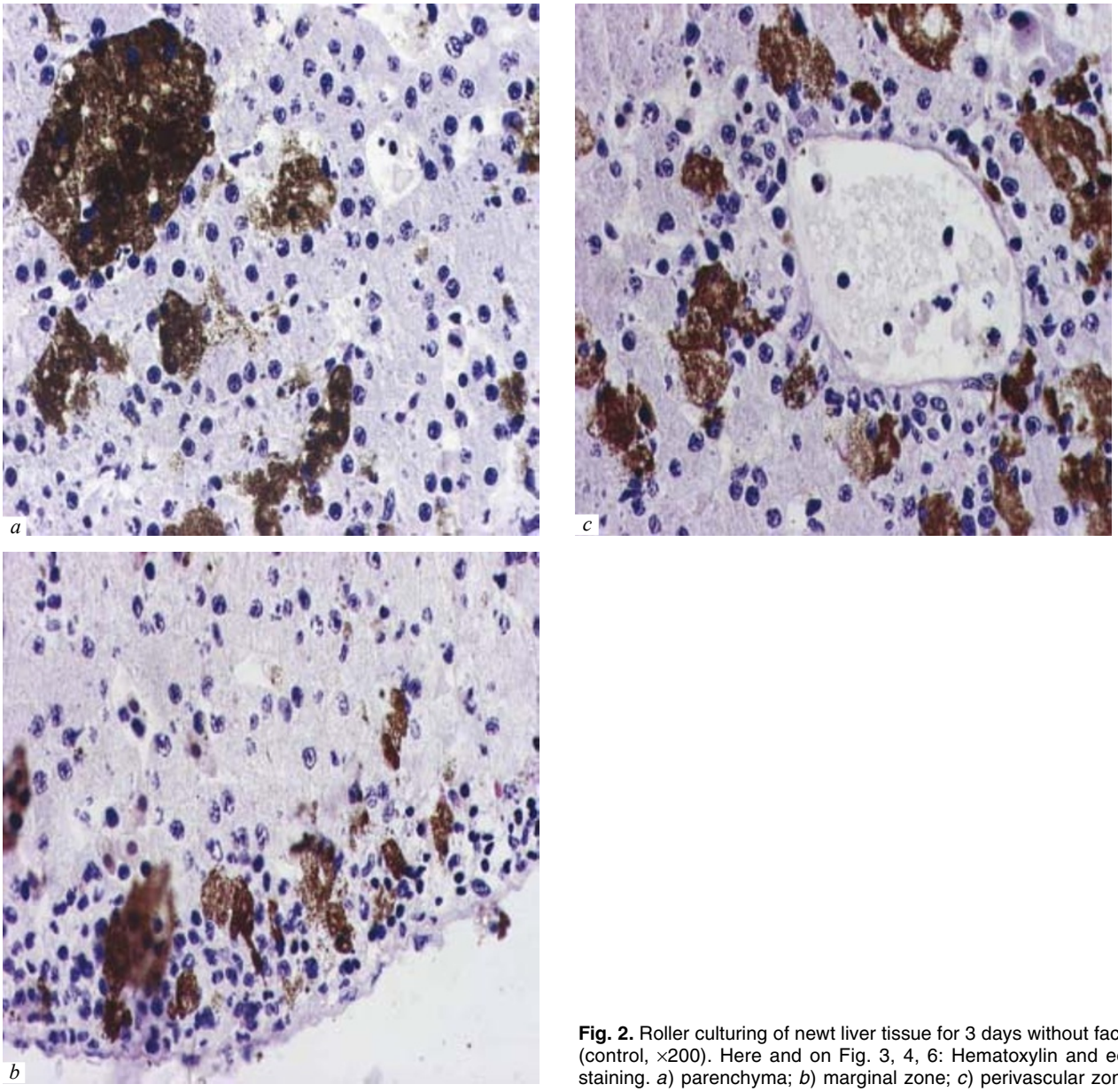

Fig. 2. Roller culturing of newt liver tissue for 3 days without factors (control, ×200). Here and on Fig. 3, 4, 6: Hematoxylin and eosin staining. *a*) parenchyma; *b*) marginal zone; *c*) perivascular zone.

dings suggest that the viability and histological structure of amphibian liver during roller culturing is preserved better than during culturing on the substrate. It cannot be excluded that the amount of pigment in liver melanomacrophages increases during roller culturing due to stimulation of metabolic activity of these cells. These processes are suppressed during culturing of amphibian liver tissue on the substrate, which leads to massive cell death in the tissue (Fig. 3).

Evaluation of specific biological activity of regulatory proteins isolated from mammalian liver, blood serum and bile on experimental models of organotypic cultures of newt liver tissue showed that these proteins produce different effects on the state of this tissue (Tables 1 and 2).

Addition of MHTB from mammalian liver to roller culture of newt liver fragments considerably increased the area of pigmented cell clusters (Table 1). Histological examination showed that the parenchyma retained compact structure, but cells with changed nucleus morphology (pyknotic nuclei) were somewhere seen. Pigment cell clusters were distributed unequally and primarily concentrated around large vessels; they also formed cords going from the marginal zone to the center of the liver (Fig. 4). The cortical zone was well preserved, solitary mitoses of connective tissue cells were seen in it (Table 2).

Addition of MHTB from blood serum to roller culture of newt liver tissue significantly reduced the area of pigmented cell clusters compared to the control

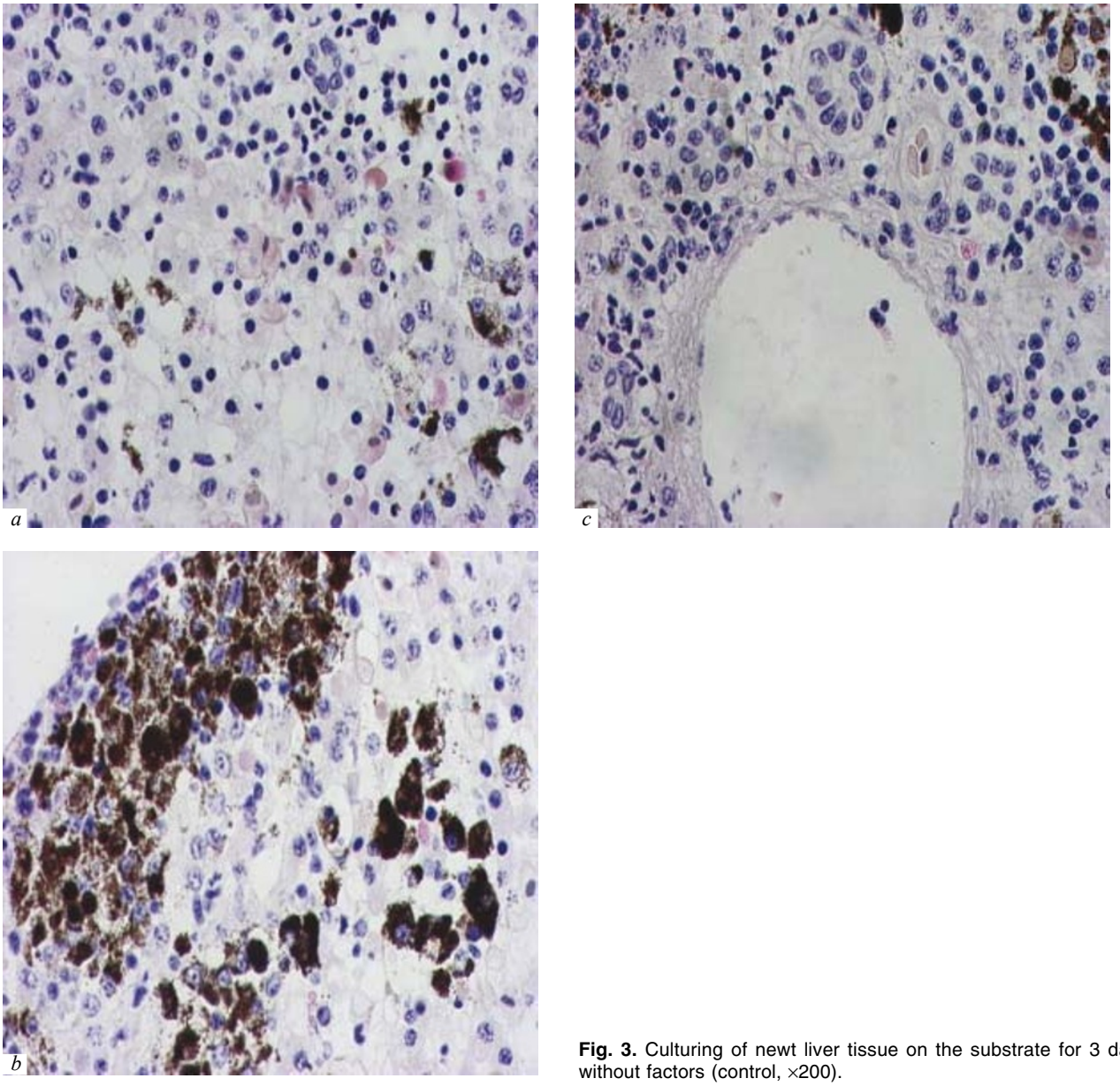

Fig. 3. Culturing of newt liver tissue on the substrate for 3 days without factors (control, ×200).

(Table 1). Pigmented cells formed dense solitary clusters located in both the cortical zone and parenchyma. No signs of cell lysis and destructions were seen in the parenchyma, cell-cell contacts were preserved (Fig. 5). The number of mitoses of connective tissue cells in the cortical zone increased compared to the control (Table 2). The number of cell layers in the cortical zone was higher than in the control and intact liver.

Addition of MHTB from the bile to roller culture of newt liver tissue significantly reduced the area of pigmented cell clusters compared to the control (Table 1). The clusters were primarily located at the boundary between the cortical zone and parenchyma and formed prolate associates. In the parenchyma, signs of tissue degradation e.g. lysis of hepatocytes, broken adhesion interactions between cells, and formation of cavities were seen (Fig. 6). Mitoses of connective tissue cells in the cortical zone were less numerous than in the control (Table 2).

Evaluation of the effects of MHTB from the liver, blood serum, and bile on organotypic culture of newt liver on the substrate yielded different results (Tables 1 and 2). No mitoses of connective tissue cells of the marginal zones were found in control and experimental organotypic cultures grown on the substrate. These findings attest to suppression of hemopoietic cells. MHTB from blood serum considerably increasing the number of mitoses in these cells during roller culturing was biologically inactive in adhesion culture. It also had no effect on the state of pigment cell clusters in the liver under these culturing conditions. These findings suggest that tissue adhesion to the substrate is a

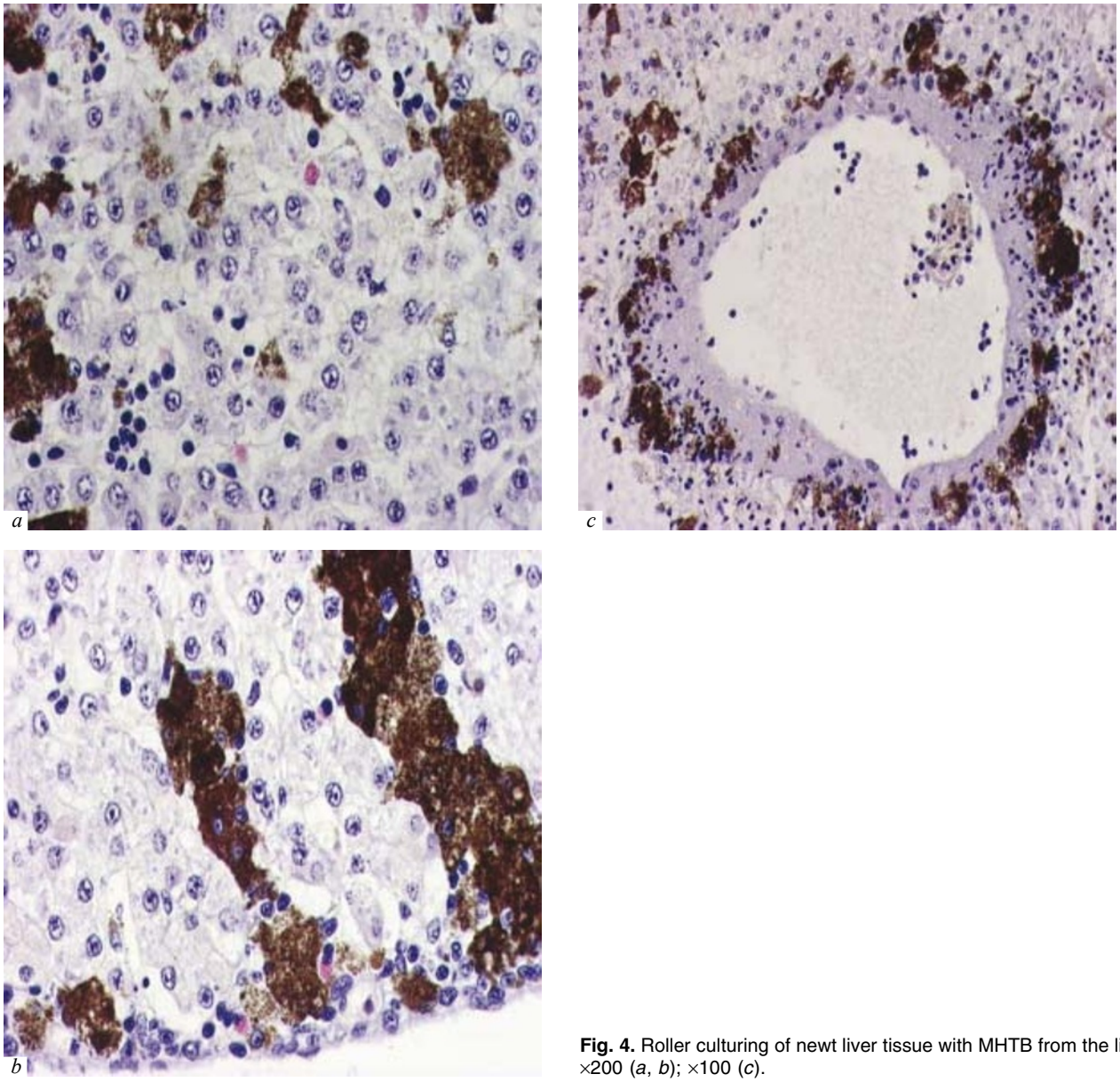

Fig. 4. Roller culturing of newt liver tissue with MHTB from the liver. ×200 (*a*, *b*); ×100 (*c*).

crucial factor for manifestation of biological activity of the studied MHTB. The method of roller culturing of newt liver tissue is an adequate experimental model for evaluation of the biological effects of substances regulating the morphofunctional properties of liver tissue.

Thus, MHTB isolated from mammalian liver, blood serum, and bile differed by their biological effects (Tables 1 and 2, Figs. 1-6).

MHTB from the liver significantly increased the area of pigmented cluster cells, but suppressed mitoses of connective tissue cells of the cortical zone. We hypothesized that MHTB from the liver manifests activates melanomacrophages and suppresses processes of hemopoiesis in amphibian liver. Liver MHTB can stimulate migration of melanomacrophages in the liver inducing the formation of large clusters. It can also be assumed that liver MHTB stimulate biosynthesis of melanin under these culturing conditions thus facilitating identification of melanin-containing cells. Another possible explanation is that enlargement of pigmented cell clusters is related to increased phagocytic activity of melanomacrophages and accumulation of products of degradation of hemoglobin, ferritin, and hemosiderin. Moreover, MHTB from the liver can also stimulate differentiation of macrophage precursor cells into mature melanomacrophages. According to published data, these cells are recruited macrophages belonging to mononuclear phagocyte system and can synthesize melanin independently [16]. In poikilothermic animals, the hepatocyte plasma membrane is enriched with unsaturated fatty acids determining its high fluidity. It is now established that melanins exhibit the

TABLE 1. Effect of MHTB on the Area of Pigmented Cell Clusters in Organotypic Tissue Culture of Newt Liver *in Vitro* under Different Culturing Conditions (%, *M*±*m*)

Culture	Culturing	
	roller	substrate
Intact liver	8.8±0.3	8.8±0.3
Control	11.3±0.4	4.4±0.1
MHTB from the liver	16.2±1.1*	4.5±0.2
MHTB from blood serum	9.4±0.3*	2.6±0.1*
MHTB from bile	7.7±0.3*	2.6±0.1*

Note. Here and in Table 2: *$p<0.05$ compared to the control.

TABLE 2. Effect of MHTB on the Number of Mitoses in Capsular Zone Cells in Organotypic Tissue Culture of Newt Liver *in Vitro* under Different Culturing Conditions (%, *M*±*m*)

Culture	Culturing	
	roller	substrate
Intact liver	3.42±0.38	3.42±0.38
Control	0.33±0.07	0
MHTB from the liver	0.01±0.01*	0
MHTB from the blood serum	4.17±0.35*	0
MHTB from bile	0.16±0.04*	0

properties of antioxidants acting as highly effective trappers of O_2^- ions [14]. Summarizing published data and the results of our experiments we can conclude that MHTB from the liver in ultralow doses exhibit hepatoprotective effects.

MHTB from blood serum significantly increased the number of mitoses of connective tissue cells in the cortical zone and maintained viability of parenchymal cells. Stimulation of mitotic activity of connective tissue cells under the effect of serum MHTB can be explained by the capacity of this MHTB to maintain hemopoietic processes in the capsular area of amphibian liver under these culturing conditions. It should be noted in this connection that our previous experiments demonstrated stimulation of proliferative activity of mammalian connective tissue cells *in vitro* in the presence of serum MHTB [10].

In our experiments, MHTB from the bile significantly reduced the area of pigmented cells in newt

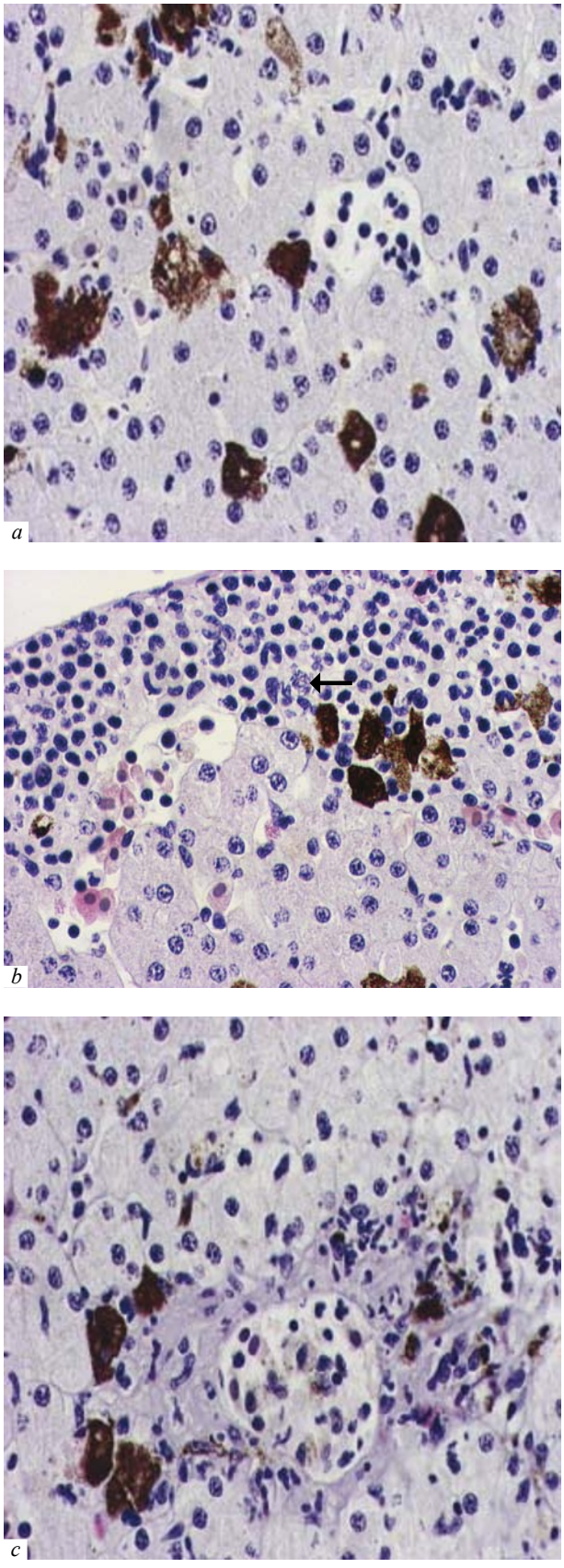

Fig. 5. Roller culturing of newt liver tissue with MHTB from blood serum, ×200.

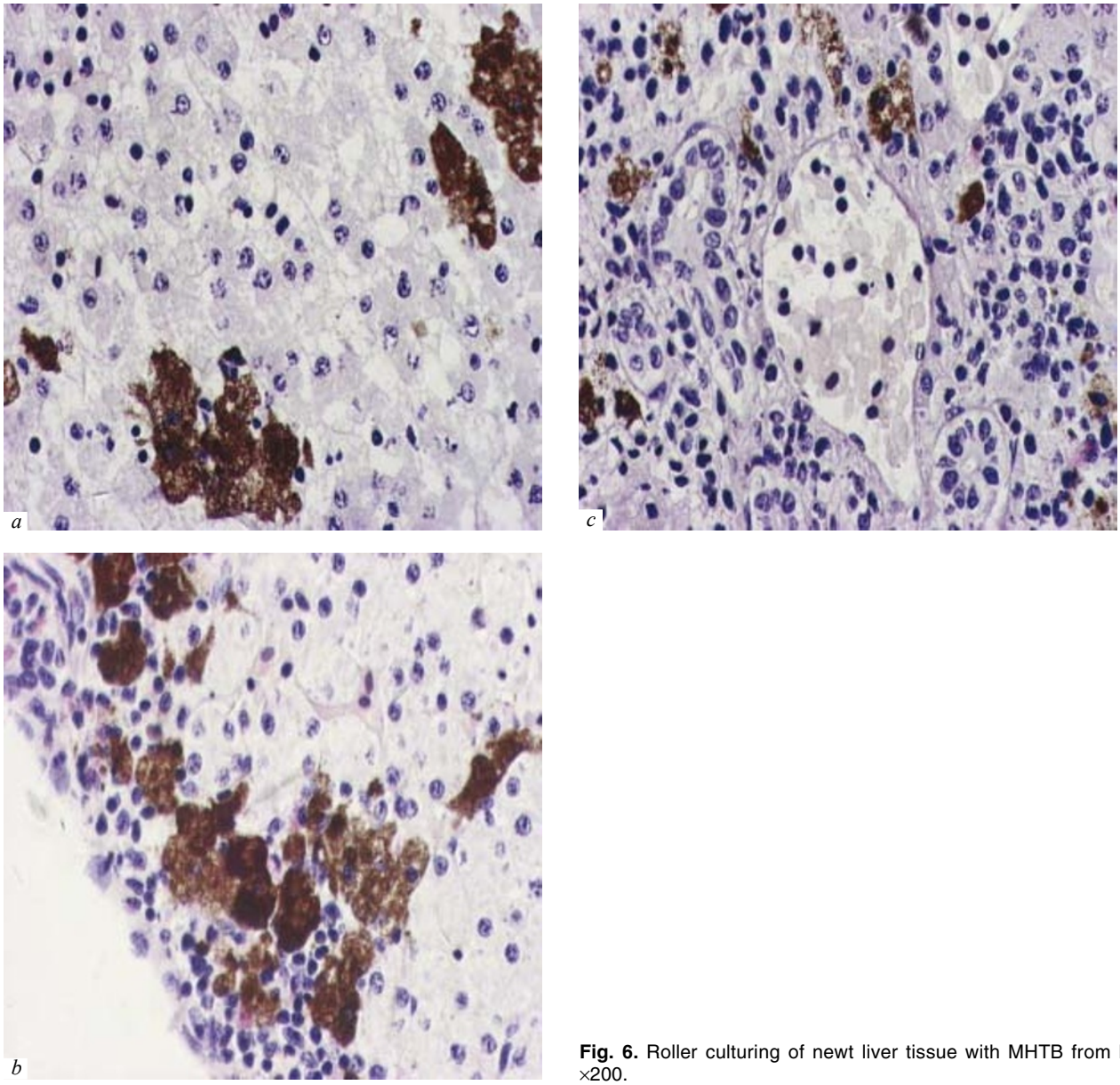

Fig. 6. Roller culturing of newt liver tissue with MHTB from bile, ×200.

liver in both roller and stationary cultures. It also reduced the number of mitoses of connective tissue cells compared to the control. It can be hypothesized that this MHTB produced a suppressive effect on melanomacrophages and cells of the marginal zone of newt liver *in vitro*. We now cannot propose a mechanism underlying this biological effect of MHTB from the bile.

Thus, MHTB isolated from the liver, blood serum, and bile exhibit different biological activity despite the fact that all these substances are synthesized by liver cells.

Our findings suggest the roller culturing maintains the tissue structure, adhesion interactions, and cell viability. The effects of MHTB isolated from the liver, blood serum, and bile were also observed only in roller cultures. These findings agree with previously reported properties of MHTB isolated from bovine cornea [6]. Assuming that MHTB additionally activate cell sources of regeneration in the tissue during roller culturing we can hypothesize that different biological effects of MHTB isolated from the liver and serum is related to their capacity to activate different cell sources of regeneration in the newt liver.

REFERENCES

1. A. V. Borisenko, V. P. Yamskova, I. V. Blagodatskikh, *et al., Biol. Membrany*, **24**, No. 3, 227-233 (2007).
2. M. A. Vorontsova, L. D. Liozner, I. V. Markelova, and E. Ch. Pukhal'skaya, Newt and Axolotl [in Russian], Moscow (1952).
3. E. N. Grigoryan, M. S. Krasnov, K. S. Aleinikova, *et al., Dokl. Akad. Nauk*, **405**, No 4, 566-570 (2005).

4. R. A. Gundorova, I. P. Khoroshilova-Maslova, E. V. Chencova, *et al., Vopr. Oftal'mol*., **113**, No. 2, 12-15 (1997).
5. S. V. Kotelevtsev, V. P. Yamskova, I. A. Yamskov, *et al., Ontogenez*, **31**, No. 4, 274-276 (2000).
6. D. V. Margasyuk, M. S. Krasnov, I. A. Yamskov, and V. P. Yamskova, *Izvl. Akad. Nauk. Ser. Biol*, No 6, 736-745 (2008).
7. I. A. Yamskov, A. A. Vinogradov, A. N. Danilenko, *et al., Priklad. Biokhim. Mikrobiol.*, **37**, No. 1, 36-42 (2001).
8. I. A. Yamskov, V. P. Yamskova, A. N. Danilenko, *et al., Ross. Khim. Zh*., **43**, No. 5, 34-39 (1999).
9. V. P. Yamskova, M. S. Krasnov, D. V. Margasyuk, and I. A. Yamskov, *Izv. Akad. Nauk. Ser, Biol*, No 1, 11-17 (2009).
10. V. P. Yamskova and M. M. Reznikova, *Zh. Obshch. Biol*., **52**, No. 2, 181-191 (1991).
11. V. P. Yamskova, E. Yu. Rybakova, A. A. Vinogradov, *et al., Priklad. Biokhim. Mikrobiol*., **40**, No. 4, 407-413 (2004).
12. V. P. Yamskova, V. S. Skripnikova, A. A. Molyavka, *et al., Biokhimiya*, **74**, No. 9, 1195-1203 (2009).
13. A. V. Borisenko, V. P. Yamskova, M. S. Krasnov, *et al.,* Biochemical Physics Frontal Research, Eds. S. D. Varfolomeev, *et al*., New York (2007), pp. 35-45.
14. G. Frangioni, G. Borgioli, and S. Bianchi, *J. Exp. Zool. A Comp. Exp. Biol.,* **296**, No. 2, 125-136 (2003).
15. M. A. Monnickendam and M. Balls, *Experientia*, **29**, No. 1, 1-17 (1973).
16. G. Sichel, M. Scalia, and C. Corsaro, *Microsc. Res. Tech.,* **57**, No. 6, 477-490 (2002).
17. G. Sichel, M. Scalia, F. Mondio, and C. Corsaro, *Pigment Cell Res*., **10**, No. 5, 271-287 (1997).
18. D. L. Stocum, *Wound Repair, Regeneration and Artificial Tissues,* Austin, Texas (1995). pp. 7-50.
19. V. P. Yamskova, M. S. Krasnov, E. Yu. Rybakova, *et al., Biochemical Physics Frontal Research*, Eds. S. D. Varfolomeev, *et al*., New York (2007), pp. 71-78.
20. V. P. Yamskova, E. Yu. Rybakova, V. V. Vecherkin, *et al., Ibid*, pp. 61-69.