Functional Properties of Mesenchymal Stem Cells Labeled with Magnetic Microparticles *in Vitro* and Analysis of Their Distribution after Transplantation

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Mesenchymal stem cells enzymatically isolated from human placenta were labeled with magnetic fluorescent microparticles (d=0.96 μ). We showed that microparticles in high doses (>10 μl stock suspension per 1 ml culture medium) significantly inhibited cell proliferation in culture. In our work we determined the optimal concentration of particles not affecting physiological properties of mesenchymal stem cells: it does not change cell proliferation, does not induce apoptosis, and does not modulate their transdifferentiation into neuronal cells. *In vivo* experiments showed that the chosen particles allow easy visualization of transplanted cells *ex vivo* on sections of different tissues.

Key Words: *mesenchymal stem cells; magnetic fluorescent microparticles; proliferation; differentiation*

Rapid development of new biomedical technologies in recent years made it possible to correct impaired functions of organs, tissues, and systems. Cell therapy is a new approach to the treatment of pathologies related to death of cell elements. Cell therapy is based on transplantation of different cells (embryonic stem cells, fetal cells, progenitor cells, autologous and allogeneic stem cells) directly into the damaged organ or intravenously (into systemic circulation) for replacement of damaged cells and tissues structures in the organism and stimulation of endogenous reparative processes [2,4]. In contrast to transplantation of donor organs, cell therapy is aimed at restoration of patient's own organs.

Visualization of administered cells is an important stage of preclinical and clinical trials for application of cell therapy. Non-invasive methods of visualization make it possible to determine the fate of transplanted cells without much inconvenience for the patient, trace migration of stem cells, evaluate their viability, and study the interaction between stem cells and damaged tissue. High-technology methods used for these purposes are safe for patient's organism, have no effect on transplanted cells, and can provide information (images) for a long time [7,9].

Here we propose a new approach to visualization of transplanted mesenchymal stem cells (MSC) labeled with magnetic microspheres. All possible adverse effects of microspheres on physiological characteristics of MSC are also studied in detail. The data on distribution of cells labeled with microspheres after their transplantation to rats are presented.

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MATERIALS AND METHODS

MSC were enzymatically isolated from human placenta as described earlier [1]. MSC were labeled with magnetic microparticles (d=0.96 μ) carrying Dragon Green fluorescent dye (λ_{ex}=480 nm, λ_{em}=520 nm; Bangs Laboratories) according to manufacturer's instructions. A suspension of microparticles was added to MSC culture when it attained 80-90% confluence (the concentration of microparticles varied in different experiments) and incubated for 24 h in a CO_2 incubator. After incubation, the medium was replaced. The efficiency of labeling was evaluated on an EPICS Coulter XL flow cytofluorometer. At least 10^4 events were recorded for each sample. The results were processed using WinMDI software.

For evaluation of possible effects of microparticles on cell proliferation, MSC were cultured (1000 cells per well of a 96-well flat-bottom plate) in 0.2 ml complete medium (CM; DMEM/F12 supplemented with 10% FCS, 200 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) with serial dilutions of particles (from 25 to 2.5 μl stock suspension per 1 ml CM). All experiments were performed in triplicates. The cells were incubated for 3 days, the supernatant was removed, and MTT (bromide 3-(4,5-dimethylthiasole-2-yl)-2,5-tetrasolium, 30 μl/well; initial concentration 5 mg/ml, Sigma) was added for evaluation of cell proliferation by the colorimetric method [9] based on MTT reduction to formazan. After incubation (2-4 h), dimethylsulfoxide (100 μl per well) was added to dissolve formazan crystals. Light absorption was measured 15 min after complete dissolution of crystals on Multiscan (Titertek) at λ=540 nm.

For evaluation of the effect of microparticles on spontaneous cell death in culture, MSC were labeled with magnetic particles (5 μl stock suspension per 1 ml culture medium) and cultured for 3 days. The cells were then harvested with trypsin/versene, washed twice with Hanks saline, fixed, and permeabilized with ice-cold 70% ethanol for at least 1 h at 4°C. The cells were then washed with PBS (2×5 min at 300g) and the pellet was resuspended in propidium iodide solution (PI, final concentration 25 μg/ml, Sigma) in PBS containing 10 μg/ml RNase (Sigma). Cell death was evaluated cytofluorometrically as described above.

Neuronal differentiation of MSC was performed in 24-well plates on coverslips. To this end, MSC were seeded in a concentration of 2500 cells per well, labeled with microparticles, and preinduced by replacing CM with a medium containing 1% FCS and 10 ng/ml basic FGF (bFGF). Control cells were cultured in the same medium without bFGF.

After 24-h incubation at 37°C and 5% CO_2 the induction medium was replaced with differentiation medium containing DMEM/F12, 1 μM RA (retinoic acid), 20 ng/ml bFGF, 20 ng/ml EGF, 100 ng/ml NGF, 10 ng/ml NT-3 (neurotrophin-3), 5 mg/ml insulin, 1 μM hydrocortisone, 1 μM progesterone (all reagents were from Sigma), penicillin, streptomycin, and L-glutamine. The cells were cultured for 4 weeks in a CO_2 incubator; the medium was replaced twice a week. Control cells were cultured in DMEM/F12 with penicillin, streptomycin, and L-glutamine, but without differentiation factors.

For evaluation of the expression of neuronal markers, MSC were twice washed with PBS (pH 7.4) and fixed in 4% paraformaldehyde (pH 7.4) for 20 min at room temperature. After fixation, the cells were washed with PBS, permeabilized with 0.6% saponin for 20 min at room temperature, and again washed with PBS (3×2 min). Nonspecific staining was blocked with PBS containing 1% FCS and 0.1% Tween-80

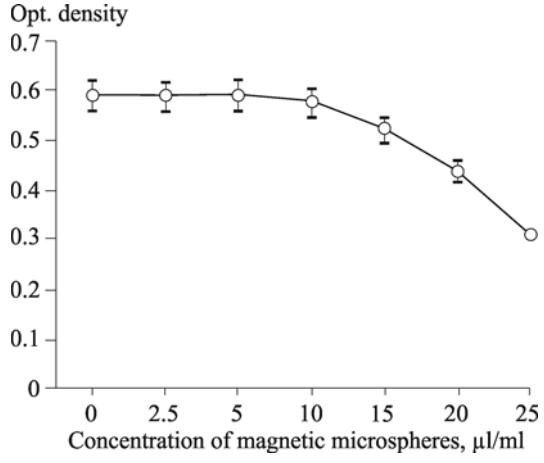

Fig. 1. Effect of different concentrations of magnetic microparticles on MSC proliferation.

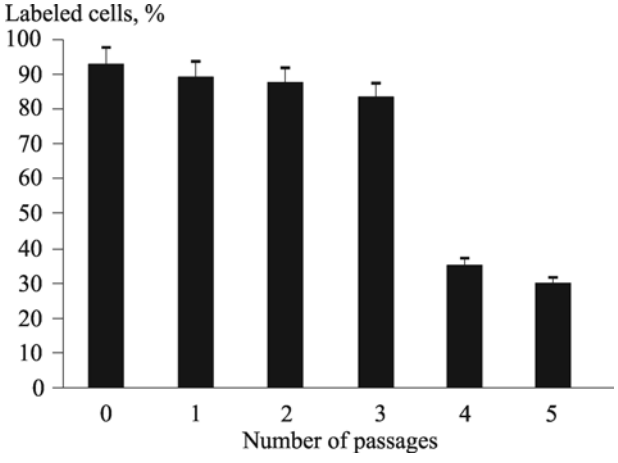

Fig. 2. The number of labeled cells in culture at different passages.

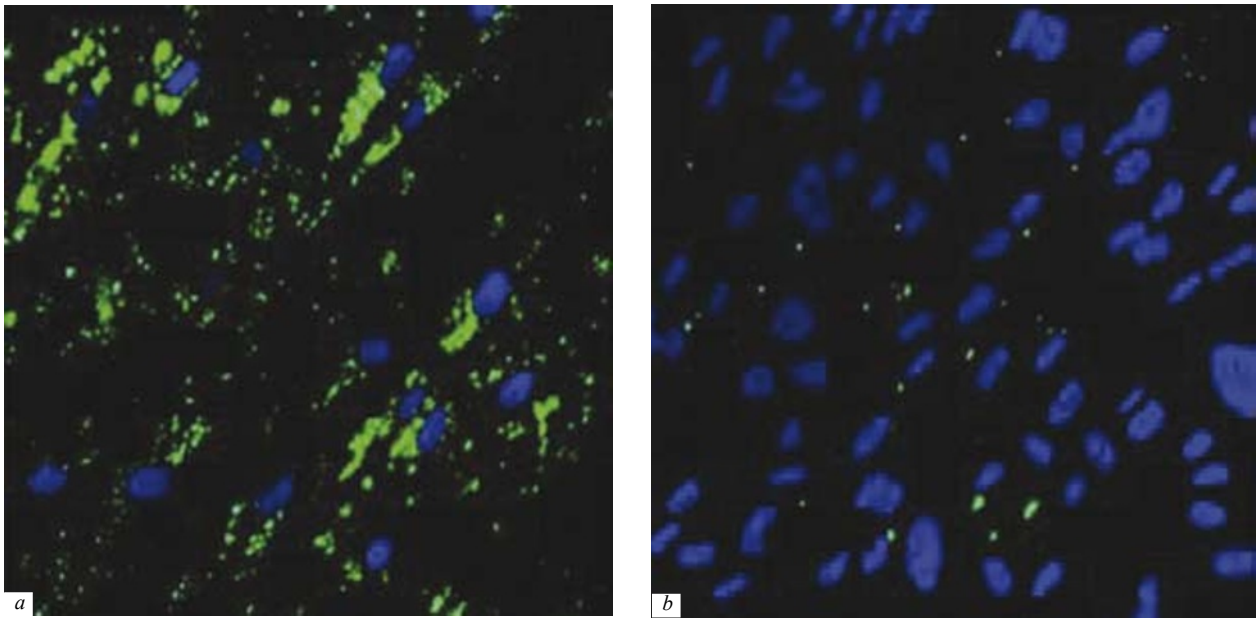

Fig. 3. MSC labeled with magnetic fluorescent microparticles in culture during passage 0 (*a*) and labeled MSC during passage 5 (*b*). Nuclei were poststained with DAPI, ×400.

(30 min at room temperature). The cells were then incubated with mouse antibodies against human neuronal markers NSE (neuron-specific enolase) and GFAP (glial fibrillary acidic protein, 1:100, Chemicon) for 1 h at room temperature, washed twice, incubated with antimouse antibodies (1:100, Chemicon) labeled with rhodamine (λ_{ex}=550 nm, λ_{em}=570 nm) for 40-60 min at room temperature, and 2-fold washed with PBS containing 1% FBS and 0.1% Tween-20. Cell nuclei were poststained with DAPI (1μg/ml PBS) and the preparations were then embedded into a medium for fluorescence protection.

In vivo experiment was carried out on albino male Wistar rats with initial body weight of 260-300 g. MSC labeled with microparticles (2×10^6 cells per 1 ml physiological saline) were injected intravenously (n=3); each rat received 1 ml cell suspension. The experimental animals were sacrificed 7 days after injection of labeled MSC, the spleen, liver, kidneys, and lungs were isolated, frozen in liquid nitrogen vapors

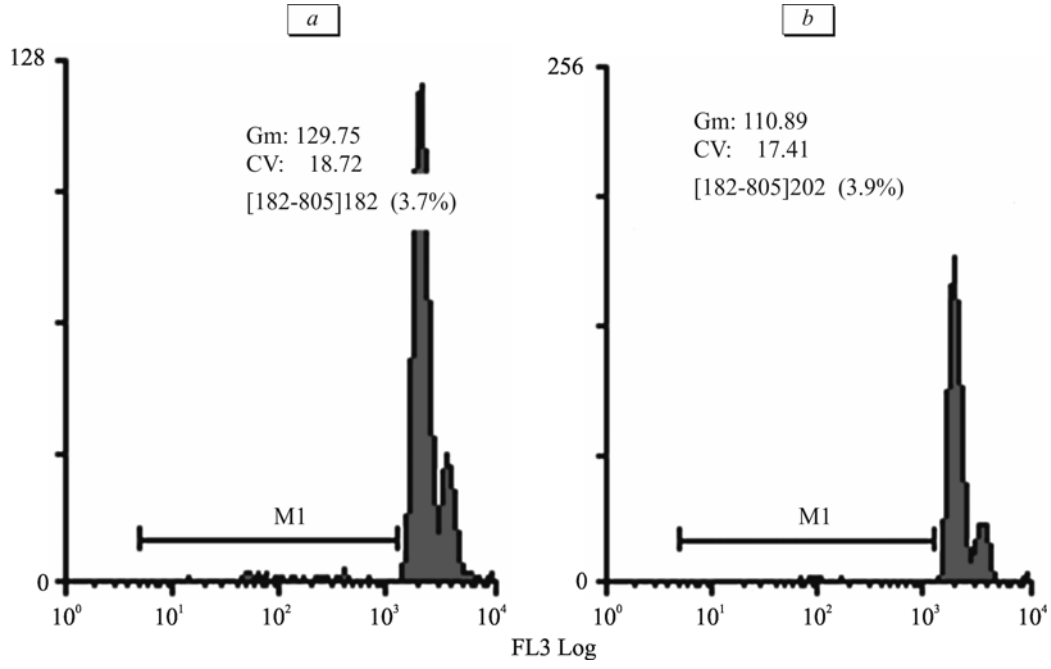

Fig. 4. Spontaneous cell death in culture. *a*) control unlabeled MSC; *b*) MSC labeled with microspheres.

for 10 sec, and placed in liquid nitrogen for 1 h and then into a freezer at -70°C. Sections (10 μ) were prepared on a Microm HM560 cryotome (Zeiss). The sections were mounted on slides and fixed with 4% paraformaldehyde (pH 7.4) for 20 min at room temperature. After fixation, the sections were twice washed with PBS, poststained with DAPI (1 μg/ml PBS), and the distribution of labeled MSC was analyzed under a fluorescent microscope.

RESULTS

For evaluation of possible effects of microparticles on cell proliferation, MSC were cultured in CM with serial dilutions of microparticles (from 25 to 2.5 μl stock suspension per 1 ml CM, Fig. 1). Considerable inhibition of cell proliferation was observed after addition of 15 μl stock suspension to the culture. These results agree with published data on dose-dependent inhibition of proliferation of bone marrow stromal cells at high concentration of microspheres [6]. The maximum concentration of microspheres not affecting proliferation of MSC from human placenta was 5 μl/ml. This concentration was used in further experiments for cell labeling.

The method of cell labeling used by us ensures effective cell loading. The efficiency of MSC labeling with magnetic particles according to flow cytofluorometry data was ~90% (Fig. 2).

We monitored the presence of microparticles in cells during their subculturing. To this end, labeled MSC were cultured for 1 month under standard conditions (5 passages). After each passage, the number of labeled cells was determined cytofluorometrically (Fig. 2) and visually under a fluorescent microscope (Fig. 3). We found that the number of labeled cells in 4-passage cultures considerably decreased (they constituted no more than 35% of the total cell number in culture). Moreover, the content of labeled particles per cell also decreased after subculturing, because during cell division the label is distributed between the two daughter cells.

Apart from proliferative potential, spontaneous cell death in culture is also an important physiological characteristic. For evaluation of the effect of microspheres on this process, we cultured MSC labeled with microparticles (5 μl/ml) for 3 days and recorded cell death using PI test (Fig. 4).

The chosen concentration of microspheres had no effect on the level of spontaneous cell death in culture. Moreover, microparticles did not induce death of MSC. Thus, microspheres used in our study did not affect physiological characteristics of MSC, their proliferation capacity and viability.

Apart from high proliferative potential, MSC from human placenta are characterized by the capacity to differentiate in different types of mature specialized cells of the mesodermal lineage [5]. There are published data that microspheres do not affect differentiation of MSC from human placenta into osteocytes and adipocytes [3]. Therefore we studied possible effect of microspheres on transdifferentiation of placental MSC. To this end, MSC labeled with microspheres were induced towards the neuronal differentiation pathway.

Four weeks after induction of differentiation, the expression of neuronal markers by placental MSC was evaluated by immunocytochemical methods. It was found that mesenchymal cells (both loaded and not loaded with microspheres) induced towards neuronal differentiation were positively stained with antibodies to neurospecific proteins NSE and GFAP (Fig. 5). MSC cultured under standard conditions in the absence of differentiation factors expressed neither NSE, nor GFAP.

MSC induced towards neuronal differentiation expressed markers specific for the nervous tissue. The presence of microparticles in cells did not modulate differentiation and the level of protein expression. This was seen from comparison of cells cultured in the presence and absence of microparticles. The microparticles were present in cells throughout the experiment (4 weeks), which suggests that they can be used for clinical trials, because they enable detection of transplanted cells in tissues and organs of the recipient over a rather long period.

The experimental animals were sacrificed 7 days after intravenous injection of labeled MSC by intraperitoneal injection of 1 g/kg chloral hydrate. The liver, spleen, kidneys, and lungs were removed and cryostat sections were prepared for evaluation of distribution of MSC labeled with microparticles in the organism of the recipients (Fig. 6). Labeled cells were detected in all tissue samples.

The use of fluorescent particles makes it possible to visualize transplanted cells by the method of fluorescent microscopy on cryostat sections of animal tissues *ex vivo*, while the use of magnetic microspheres carrying a fluorescent label allows tracing them *in vivo* by magnetic resonance imaging [1]. Thus, application of microspheres for labeling of MSC in culture does not change their physiological characteristics and their capacities as stem cells. Moreover, cell loading with microparticles essentially facilitates the search for transplanted cell in the organism both *in vivo* and *ex vivo*.

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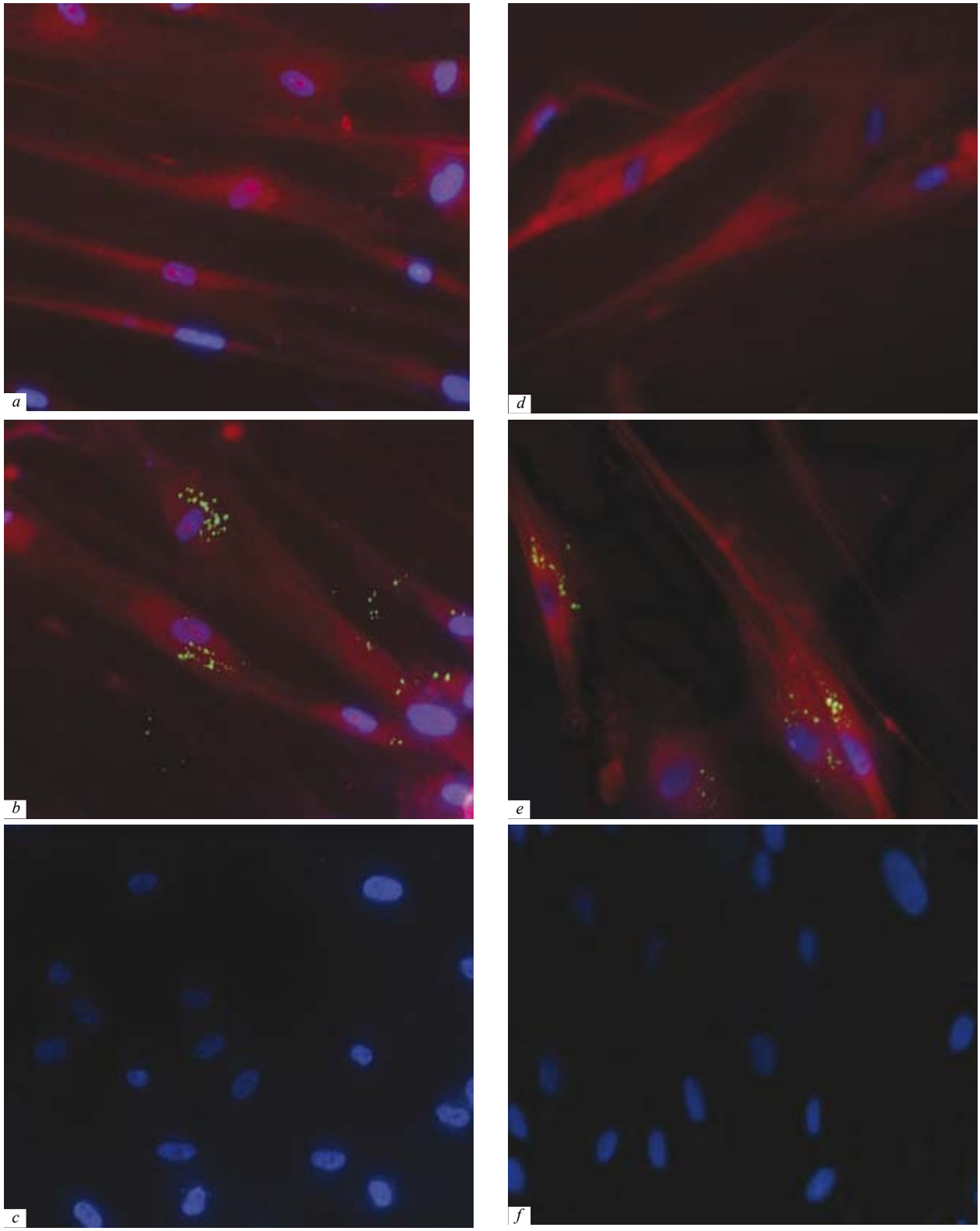

Fig. 5. Immunohistochemical staining of MSC induced towards neuronal differentiation *in vitro*. *a*, *b*) labeled with microparticles (*a*) and unlabeled (*b*) MSC stained with antibodies to NSE 4 weeks after induction of neuronal differentiation; *c*) unlabeled cells not induced to neuronal differentiation stained with antibodies to NSE; *d*, *e*) labeled with microparticles (*d*) and unlabeled (*e*) MSC stained with antibodies to GFAP 4 weeks after induction of neuronal differentiation; *f*) unlabeled cells not induced to neuronal differentiation stained with antibodies to GFAP. Nuclei were poststained with DAPI, ×400.

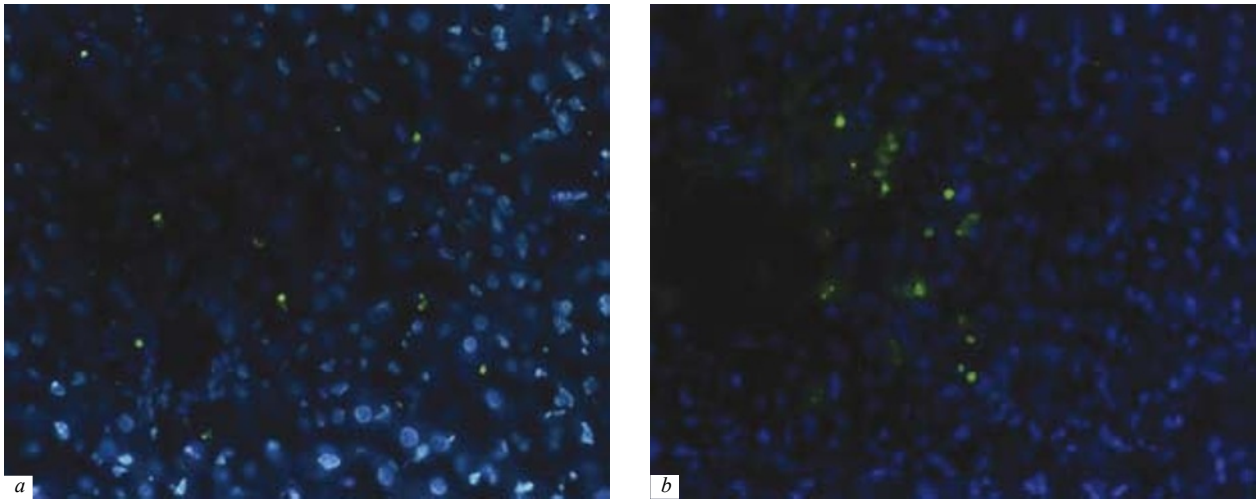

Fig. 6. Cryostat sections of the liver (*a*) and lungs (*b*) 7 days after intravenous injection of MSC labeled with magnetic fluorescent microparticles. Green fluorescence: cells labeled with microparticles. Nuclei were poststained with DAPI, ×100.

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