Application of Multipotent Mesenchymal Stromal Cells from Human Adipose Tissue for Compensation of Neurological Deficiency Induced by 3-Nitropropionic Acid in Rats

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We evaluated possible therapeutic effect of multipotent mesenchymal stromal cells from human adipose tissue differentiated to neuronal phenotype with retinoic acid on Wistar rats subjected to toxic effect of 3-nitropropionic acid. Transplantation of mesenchymal stromal cells from human adipose tissue considerably decreased neurological symptoms, normalized exploratory activity (open field test) and long-term memory (Morris test), which correlated with normalization of pathomorphological manifestations in the brain. Destructive changes in the caudate nucleus caused by treatment with 3-nitropropionic acid (reduced size of neurons, changes in their shape, and cell edema) tended to decrease under the effect of multipotent mesenchymal stromal cells: the area of neurons increased 2-fold, the cells acquired typical round shape, cell edema decreased.

Key Words: *3-nitropropionic acid; multipotent mesenchymal cells; long-term memory; human adipose tissue*

Neurodegenerative diseases are not very prevalent, but have extremely complex etiology, therefore modern medicine have only limited recommendations for the treatment of these diseases. New approaches to the treatment of neurodegenerative diseases are related to cell technologies enabling transplantation of stem cells (SC) into the corresponding structures of the damaged brain. Considerable experience is now accumulated on the use of SC (primarily, fetal SC) in animals with experimental neurological pathologies and in clinical practice [4,12] for the treatment of Parkinson's disease, Huntington's disease, and multiple sclerosis. However, the results of these studies are insufficient for introduction of these methods into clinical practice.

The use of embryonic and fetal SC has a number of serious limitations, the most important of them are engrafting of the transplant, the possibility of its malignant transformation, and ethical problems. These limitations can be overcome by using autologous SC from adult body, but clinical efficiency of cell transplantation can be reduced in this case. Potential sources of SC for neurotransplantation in adult body are neural SC of the brain, multipotent mesenchymal stromal cells (MMSC) of the bone marrow, MMSC of the adipose tissue, and olfactory epithelial cells. Previous studies demonstrated the possibility of targeted differentiation of bone marrow and adipose tissue MMSC into cells

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with different phenotypes, including neuronal differentiation [8,14,15] using treatment with differentiation agents such as retinoic acid, β-mercaptoethanol, growth factors NGF and GDNF, *etc*. Some authors showed that proliferation and differentiation capacities of MMSC from the adipose tissue are higher than that of bone marrow MMSC [7]. The efficiency and appropriateness of preliminary neuronal differentiation of the transplanted cell culture are now actively discussed.

We focused on the problem, whether MMSC from human adipose tissue committed to neuronal phenotype with retinoic acid can improve general status and brain function in rats treated with neurotoxin 3-nitropropionic acid (3-NPA). The model of 3-NPA-induced neurotoxicity is a certain approximation to a clinical picture observed in Huntington's chorea, the pathology associated with progressive death of highly sensitive neurons located in basal ganglia [3]. Toxicity of 3-NPA leading to neurological abnormalities is determined by disturbances in mitochondrial processes induced by irreversible blockade of mitochondrial succinate dehydrogenase. Administration of 3-NPA to rats induced permanent oxidative stress causing massive oxidation of cell components followed by neuronal death [5]. There is no effective therapy for Huntington's chorea. At the same time, there are reports on successful transplantation of embryonic SC in this experimental pathology [13].

Here we studied the therapeutic effect of MMSC on neurological, behavioral, and neurohistological characteristics of animal brain on the model of 3-NPA-induced neurotoxicity in Wistar rats.

MATERIALS AND METHODS

Human adipose tissue MMSC were isolated from donor lipoaspirate material depleted of mature adipocytes. Stromal-vascular fraction was disaggregated with trypsin, the suspension was washed with DMEM/F12 medium and cultured in DMEM/F12 1:1 supplemented with bovine serum (final concentration 15%), L-glutamine (to a final concentration of 584 mg/liter) and amikacin (500 mg/liter). After passages 3-5, the cells were used for transplantation.

Neuronal differentiation was induced by 6-day culturing in the presence of 10 μM retinoic acid [9]. Differentiation was verified by the expression of specific neuronal markers nestin, GFAP, NMDA glutamate receptors on at least 30% cells in the population.

Experimental 3-NPA-induced neurotoxicity was reproduced on 18 mature Wistar rats weighing 250-300 g maintained under standard vivarium conditions. The animals received intraperitoneal injections of 3-NPA in a dose of 30 mg/kg body weight/day for 7 days. Then, suspension of MMSC from the human adipose tissue (300,000-500,000 cells) was stereotactically injected into the right cerebral ventricle (experimental group, 9 rats). The controls (9 rats) received an equivalent volume of Hanks solution. For evaluation of the dynamics of neurological disturbances caused by 3-NPA, the state of animals was daily (for 20 days starting from the day of MMSC transplantation) scored using a 5-point scale [6].

Exploratory activity of animals was tested in the open field test and in Morris water maze. Open field testing was performed on days 8 and 28 of the experiment: locomotor and exploratory activities of animals were evaluated. Morris water maze was used on day 30 of the experiment: the path in the maze and the time of finding the platform by trained animals were determined [10].

For morphometry, the brain of Wistar rats was fixed in 10% neutral formalin, embedded in paraffin, and sagittal sections (7 μ) of the right hemisphere were prepared and stained with 1% cresyl violet [1]. Neurohistological preparations were examined and morphometried under a Leica DMLB microscope equipped with a Leica DC-300 digital camera (resolving power 3.2 Mpixel) and Leica Qwin software for processing of video images.

Area of neurons and neuroglia (in μ^2), number of nervous and glial cells per field of view (per 0.09 mm^2), and the ratio of cell axes (AR) were analyzed [2]. The letter parameter is the ratio of the long axis of the ellipse circumscribing the neuron to its short axis, *i. e.* characterizes elongation of the nerve cell.

The data were processed using Statistica 6.0 software; the differences between the groups were analyzed using ANOVA Kruskal—Wallis test and nonparametric Mann—Whitney test.

RESULTS

In contrast to control group animals, the rats treated with 3-NPA demonstrated neurological symptoms (Fig. 1). In animals receiving MMSC these symptoms were less pronounced and more rapidly disappear after 3-NPA withdrawal. On day 21 after transplantation of differentiated MMSC from human adipose tissue, the neurological score in animals receiving 3-NPA and 3-NPA+MMSC differed significantly (by almost 2 times, Fig. 1; $p<0.05$).

Testing in Morris water maze showed that 3-NPA to animals considerably impaired their learning capacity (the path of the rat in the maze in-

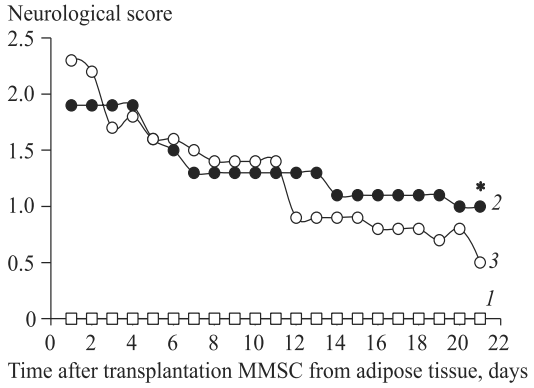

Fig. 1. Dynamics of neurological symptomatics in rats. *1*) control (intact animals); *2*) after 7-day intraperitoneal treatment with neurotoxin 3-NPA; *3*) after intracerebroventricular transplantation of differentiated MMSC from the adipose tissue against the background of 7-day treatment with 3-NPA.

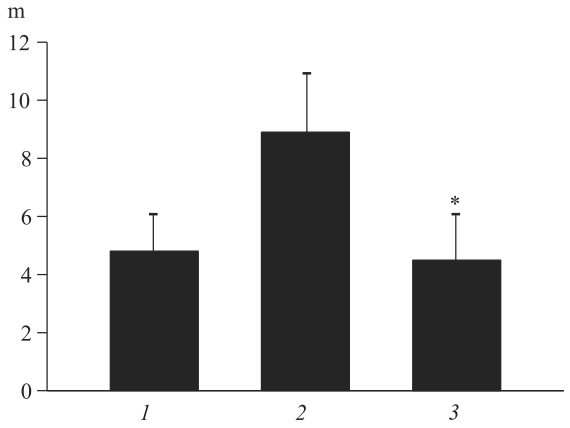

Fig. 2. Results of Morris test: Search for the platform on day 3 of learning (path length). *$p<0.05$ compared to rats treated with 3-NPA (on day 21).

creased from 5 to 9 m; Fig. 2), whereas transplantation of MMSC almost completely restored learning. The animals receiving MMSC swam more rapidly. The results obtained in this test suggest that reparation processes in the brain were more effective in rats receiving MMSC.

Quantitative evaluation of exploratory (Fig. 3, *a*) and locomotor (Fig. 3, *b*) activities in the open field test on day 28 of the experiment (day 21 after transplantation of MMSC) showed that treatment with 3-NPA more markedly impaired exploratory activity (suppressed it by 4-6 times). Transplantation of adipose tissue cells had a normalizing effect: exploratory activity increased 2-fold, but this effect was practically insignificant due to individual differences. Locomotor activity did not recover under the effect of MMSC.

Neurohistological study showed that injection of 3-NPA induces pronounced pathomorphological changes in the brain of Wistar rats. The neurons in the caudate nucleus in intact rats had rather round than ellipsoid shape (Fig. 4, *a*). Under the effect of 3-NPA, the neuronal bodies acquired an elongated shape and their size decreased by almost 2-fold (Fig. 4, *b*). The neuronal cytoplasm was sharply thinned and looked pale, while the karyoplasm was more intensively stained, therefore the nuclei of nerve cells were dark. The nuclear membrane was thickened due to accumulation of chromatin granules along its perimeter. The nucleus contained 2-3 small nucleoli. Zones of edema were seen around most neuronal somas and blood capillaries. The neuropil looked like a loose network probably due to edema around neuronal processes. The cells of neuroglia were intensively stained and polymorphic

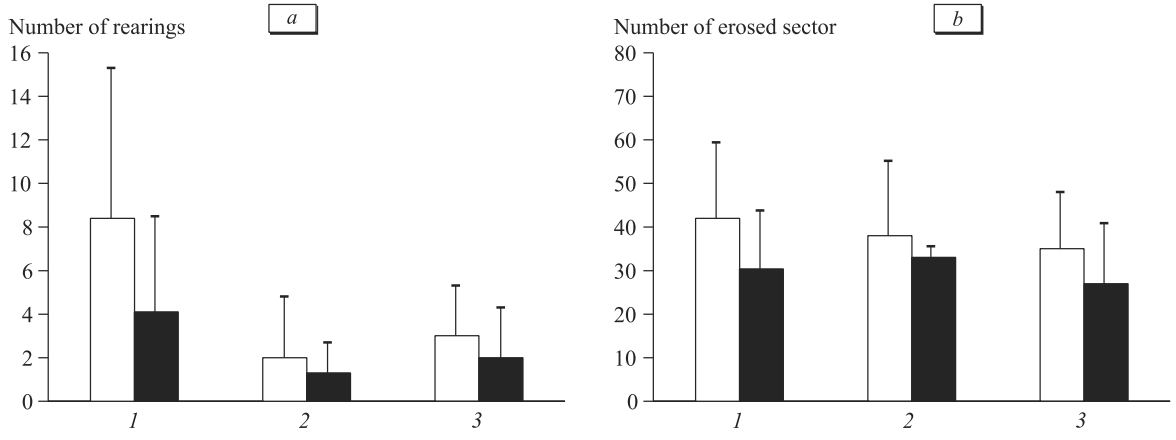

Fig. 3. Dynamics of behavioral characteristics of rats in the open field test before (day 8) and on day 21 after transplantation of differentiated MMSC from human adipose tissue (day 28 of the experiment). *a*) exploratory activity; *b*) locomotor activity. Light bars: before cell transplantation; dark bars: after cell transplantation. *1*) intact animals; *2*) rats treated with 3-NPA; *3*) rats treated with 3-NPA and receiving MMSC from the adipose tissue.

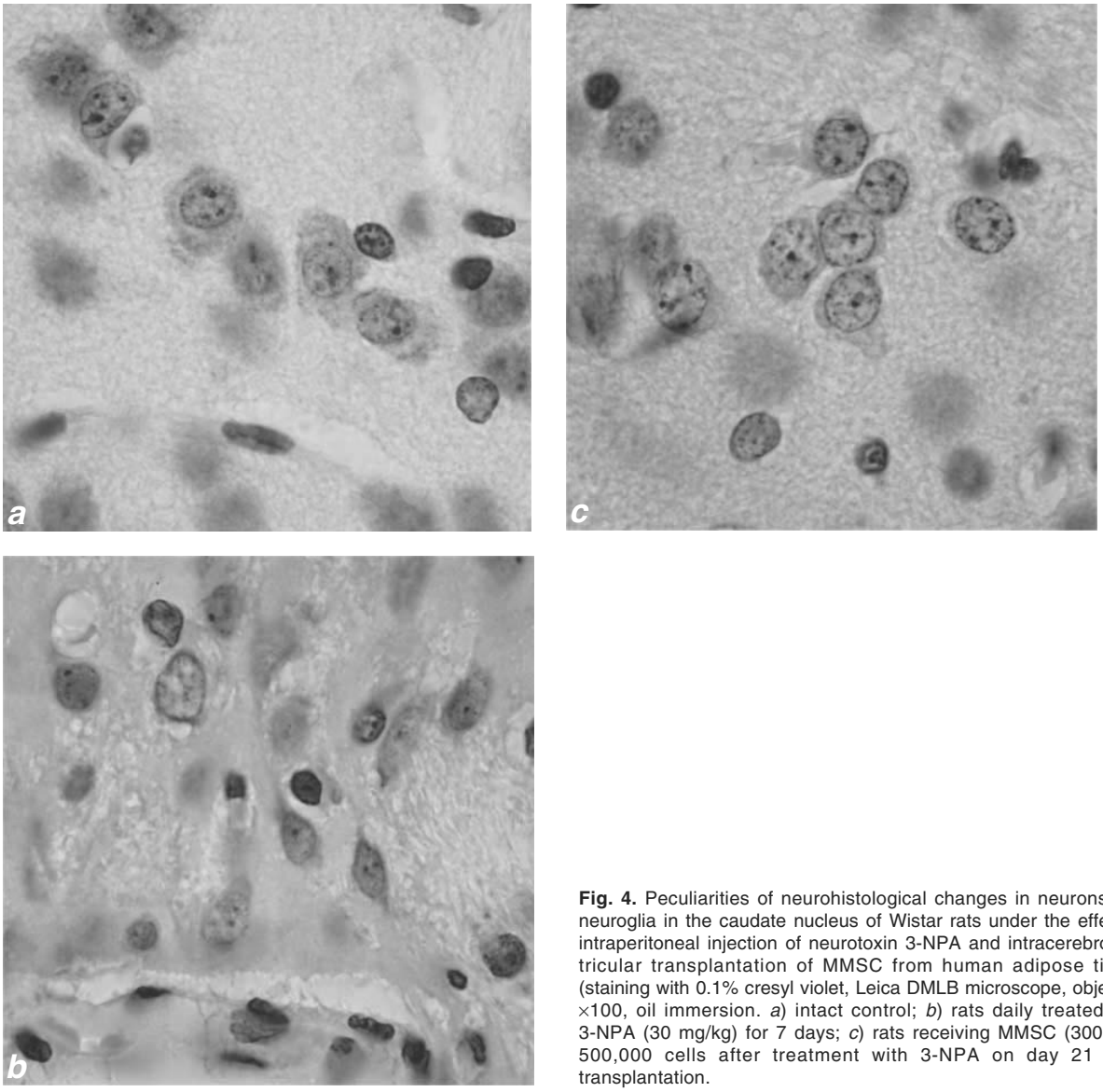

Fig. 4. Peculiarities of neurohistological changes in neurons and neuroglia in the caudate nucleus of Wistar rats under the effect of intraperitoneal injection of neurotoxin 3-NPA and intracerebroventricular transplantation of MMSC from human adipose tissue (staining with 0.1% cresyl violet, Leica DMLB microscope, objective ×100, oil immersion. *a*) intact control; *b*) rats daily treated with 3-NPA (30 mg/kg) for 7 days; *c*) rats receiving MMSC (300,000-500,000 cells after treatment with 3-NPA on day 21 after transplantation.

shape. Hence, 3-NPA induced destructive changes in the nervous tissue of the caudate nucleus.

Under the effect of MMSC (Fig. 4, *b*) the neurons of the caudate nucleus became larger and acquired round shape. The area of the cytoplasm also increased. Several large nucleoli and chromatin threads were seen in the nucleus. The karyoplasm was weekly stained. The cytoplasm of neurons contained granules of a chromatophilic substance. The color and shape of neuroglial cells indicated weakened reactivity. Thus, MMSC transplantation reduced pathomorphological changes in the caudate nucleus of rat brain induced by 3-NPA.

Evaluation of the shape of neurons in intact cells by methods of computer morphometry revealed (Fig. 5, *a*) that the ratio of cell axes (AR) for the majority of neurons of the caudate nucleus (60%) did not exceed 1.4, *i.e*. they had round shape, and only 3% cells were elongated. The AR median for caudate nucleus neurons in intact rats was 1.34 (1.23-1.50 quartile range).

Under the effect of 3-NPA, caudate nucleus neurons acquired an elongated shape (Fig. 5, *b*). The ratio between different groups of cells changed and the diagram of cell distribution by AR was shifted to the right. After injection of the toxin, the number of round neurons (AR<1.4) decreased, the number of elongated neurons (AR from 1.6 to 2.0) increased. At the same time, cells with sharply elongated bodies (AR>2) appeared. The AR me-

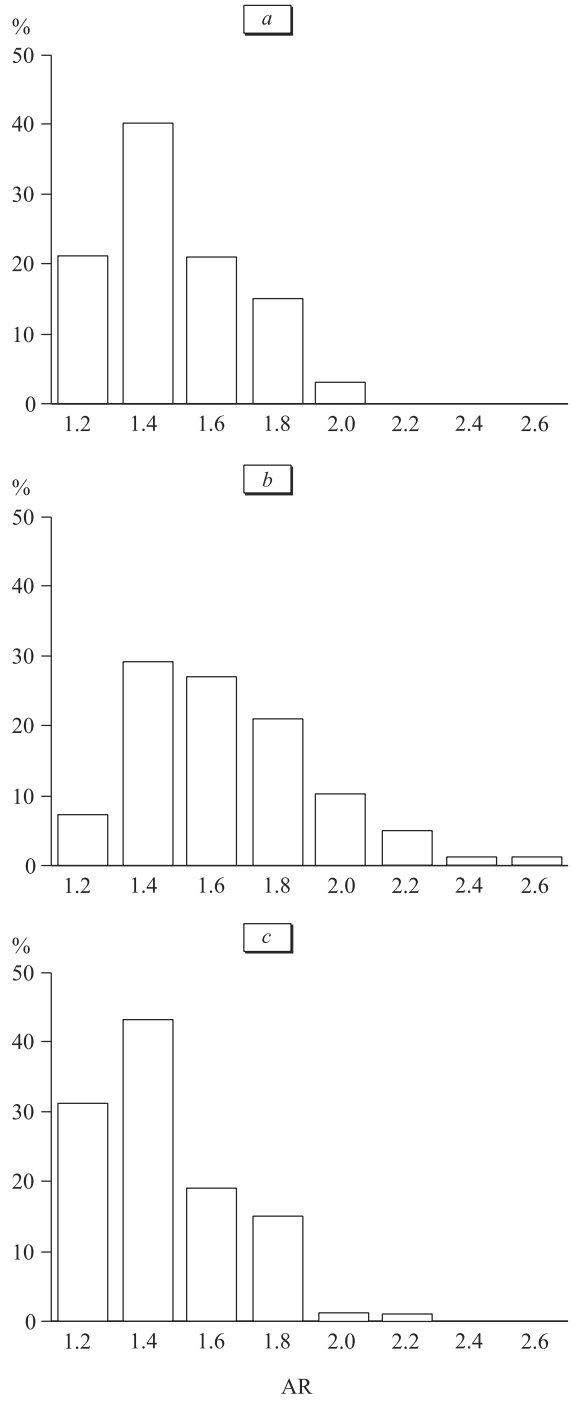

Fig. 5. Dynamics of morphological parameters of caudate nucleus neurons in Wisrat rats receiving daily intraperitoneal injection of toxin 3-NPA (30 mg/kg) for 7 days and intracerebroventricular injection of MMSC. *a*) control rats; *b*) rats receiving 3-NPA; *c*) rats receiving MMSC.

dian for caudate nucleus neurons in animals receiving 3-NPA was 1.50 (interquartile range 1.32-1.70).

After transplantation of MMSC (Fig. 5, *c*) the neurons with sharply elongated shape (AR>2.4) were not found or were scanty (AR from 2.0 to 2.4). The number of neurons in the medium group (AR from 1.6 to 1.8) slightly decreased. At the same time, the number of cells with AR~1 sharply increased. About 70% nerve cells in this group had AR<1.5. The pattern of cell distribution by the shape of the cell body in animals receiving MMSC was close to that in the control group. The AR median in the group receiving MMSC were 1.28 (interquartile range 1.18-1.40).

Evaluation of AR with the median test revealed significant differences between the caudate nucleus neurons in rats treated with 3-NPA and animals receiving MMSC after 3-NPA treatment. Computer morphometry showed that after transplantation of MMSC (against the background of 3-NPA treatment, the size of neurons in the caudate nucleus increased 2-fold compared to this parameter in animals receiving 3-NPA alone (Fig. 6, *a*). Analysis of the density distribution of neurons and neuroglia revealed no appreciable changes in rats receiving MMSC injection compared to animals treated with 3-NPA (Fig. 6, *b*). Hence, morphometry data are consistent with the results of neurohistological analysis and confirm the positive effect of MMSC on the structural parameters of the caudate nucleus in the brain of Wistar rats exposed to destructive effects of 3-NPA toxin.

Thus, the symptoms of Huntington disease modeled in Wistar rats by treatment with 3-NPA normalized after intracerebroventricular administration of MMSC differentiated towards neuronal phenotype with retinoic acid. Transplantation of MMSC not only reduced clinical manifestations of experimental pathology, but also restored learning capacity. Normalization of clinical and physiological parameters was accompanied by normalization of neurohistological parameters of the caudate nucleus, a component of nigrostriatal complex of the brain. Morphometry revealed not only normalization of the size, but also the shape of neuronal bodies in the caudate nucleus. This attests to the efficiency of MMSC transplantation. The described model is promising for the study of the mechanisms of SC effects *in vivo*. The efficiency of MMSC transplantation can be explained by the fact that MMSC of the adipose tissue are capable of neoangiogenesis and can improve microcirculation via production of growth factors [11]. However, this hypothesis requires further verification.

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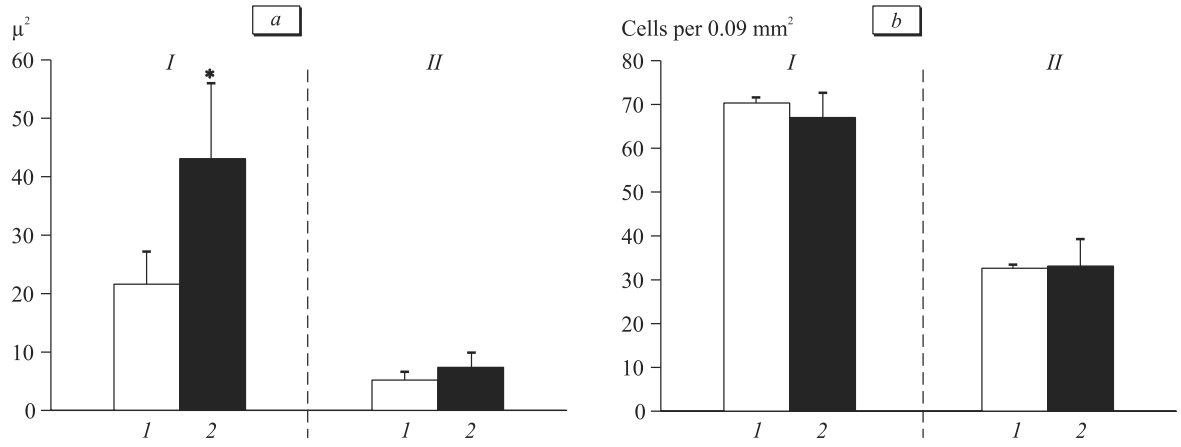

Fig. 6. Dynamics of the size (*a*) of neurons (*I*) and neuroglia (*II*) and their densities (*b*) under the effect of 3-NPA (*1*) and MMSC (*2*).*$p<0.05$ compared to group 1.

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