Morphofunctional Study of the Therapeutic Effect of Autologous Mesenchymal Stem Cells in Experimental Diffuse Brain Injury in Rats

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Translated from *Kletochnye Tekhnologii v Biologii i Medicine*, No. 3, pp. 157-165, August, 2006
Original article submitted March 6, 2006

Effects of systemic transplantation of mesenchymal stem cells obtained by culturing of autologous bone marrow on proliferative activity of cells and functional morphology of neurons after diffuse brain injury were studied in Wistar rats. Comparative analysis of the results indicated that systemic injection of mesenchymal stem cells in a syngeneic organism produced proliferotropic, angiogenic, and, presumably, neurotrophic effects. The therapeutic effect visually manifested on day 2 after intravenous injection of mesenchymal stem cells during the early period of reparative regeneration of ischemic cell and tissue structures of the brain. The neuroprotective effect of mesenchymal stem cells was more pronounced against the background of basic therapy.

Key Words: *mesenchymal stem cells; brain injury; neurons; proliferation; PCNA*

The possibility of using mesenchymal stem cells (MSC) for the treatment of some severe pathologies, including neurodegenerative diseases, is substantiated by recent data of molecular and cell biology on plasticity of stem cells and their differentiation into specialized somatic cells of various types and on their capacity to activate proliferation of own resident stem cells in tissues of adults [7,14]. Today the use of adult bone marrow MSC for transplantation attracts special attention [1,16]. According to published reports, MSC transplantation promotes repair of damaged nervous system and recovery of the neurological status in focal cerebral ischemia and local brain injury [5,8,12,13,16].

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In countries with well-developed industry brain injuries, particularly in children and young people, are associated with high mortality and disability of victims and remain a pressing problems of public health having serious socioeconomic consequences. Patients with brain injury can suffer from diffuse damage to the brain secondary with respect to the trauma. It is assumed that this type of injury is one of the most prevalent causes of the post-commotion syndrome and serious neurological disability. For this reason the development of new methods for the treatment of cerebral injuries using stem cell transplantation becomes an important task.

We studied the effects of transplanted MSC, obtained by culturing bone marrow cells from adult Wistar rats, used alone and in combination with basic therapy, on reparative processes in the nervous tissue after closed brain injury causing diffuse damage to the brain in laboratory animals.

0007-4888/06/1421-0140

MATERIALS AND METHODS

The study was carried out on the brain of 2-month-old male Wistar rats (n=60) weighing 140-150 g. The animals were divided into 5 groups. Control group consisted of sham-injured rats. Experimental group 1 consisted of animals with brain injury receiving no treatment, group 2 were animals with injury receiving basic therapy, group 3 injury+MSC transplantation, group 4 injury+combined treatment (Table 1). Direct dynamic brain trauma was inflicted to 46 rats by a falling weight [4]. Narcotized animals were fixed in a special device with a cushion to rule out mandibular fracture during the impact. A 50-g weight fell free from a height of 110 cm in a hollow tube (1.2 cm in diameter) onto the parietal area of animal head. The impact area was 0.5 cm^2. About 45% animals died within 4 min after the impact. Group 1 survivors received no treatment. Group 2 rats received basic therapy, including antiedematous, antihypoxic, and antiinflammatory drugs: single injection of 25% magnesium sulfate (0.6 ml/kg) on day 1; 40% glucose (1.2 ml/kg) with mexidol and actovegin (3.3 mg/kg each in 0.2 ml solvent) daily during the first 3 days. Group 3 animals received a single intravenous injection of 2×10^6 MSC suspended in 0.5 ml saline 24 h after the injury. Primary MSC culture from the bone marrow of adult rats was prepared as described previously [1]. Group 4 rats received combined treatment, consisting of basic therapy and MSC injection in accordance with the protocols for groups 2 and 3. Control group consisted of 14 sham-injured animals, narcotized and fixed in the device without inflicting the injury.

The rats were narcotized with nembutal 1 h, 1, 3, and 14 days after the injury; the brain was isolated, placed into 10% buffered formalin or 70% ethanol for 24 h and then cut into two saggittal blocks or 4 frontal fragments at the level of the rostral compartment of the lateral ventricles, hippocampus, midbrain, and cerebellum. Tissue blocks were dehydrated and embedded in Paraplast Plus (Kendall). Microtome sections (6-7 μ) were placed onto Histo-Bond adhesive slides (Marienfeld). The sections were stained with hematoxylin and eosin for histological analysis. Sections fixed in ethanol were stained with cresyl violet and with thionine after Nissl. Histotopographic mapping of damaged areas and precise determination of the levels of coronary sections were carried out using stereotaxic atlas of the rat brain [15].

Murine monoclonal antibodies to PCNA (proliferating cell nuclear antigen) diluted 1:50 (clone PC10, Calbiochem) and biotin-streptavidin-peroxidase kit for detection of mouse immunoglobulins (MP Biomedicals) were used for immunostaining of proliferating cells. The substrate enzyme was developed with diaminobenzidine (Liquid DAB+Substrate Chromogen System, DAKO).

RESULTS

In sham-injured animals the morphology of the parietal cortical (Fig. 1, *e*) and brain stem neurons (Fig. 2, *a*) did not differ from normal. Immunohistochemical study showed that nuclei of ependymal cells, epithelial cells of vascular plexuses, and glial cell were PCNA-positive. Intensive PCNA-immunopositive staining of cell nuclei was observed in the paraventricular zone (PVZ) of the lateral ventricles and migration tracts (Fig. 3, *a*) and in the gray matter neurons of the midbrain and pons. Labeled glial cells were seen in the diencephalon tissue and white matter lamina in the cerebellar gyri. PCNA-positive cell nuclei looked like chains between nerve fibers in the corpus callosus, pons, and medulla oblongata conduction tracts. The least number of labeled glial nuclei was detected in the cerebral cortex. No positive reaction to PCNA was detected in the subgranular zone of the hippocampal dentate gyrus.

TABLE 1. Characteristics of the Material for Studies of MSC Effect after Brain Injury

Group	Number of animals per group after different periods after the injury			
	1 h	1 day	3 days	14 days
Control	3	3	3	5
Experiment				
1 (injury without treatment)	3	3	5	5
2 (injury+basic therapy)	—	—	5	5
3 (injury+MSC)	—	—	5	5
4 (injury+combined treatment)	—	—	5	5

Note. Combined treatment included basic therapy and injection of MSC.

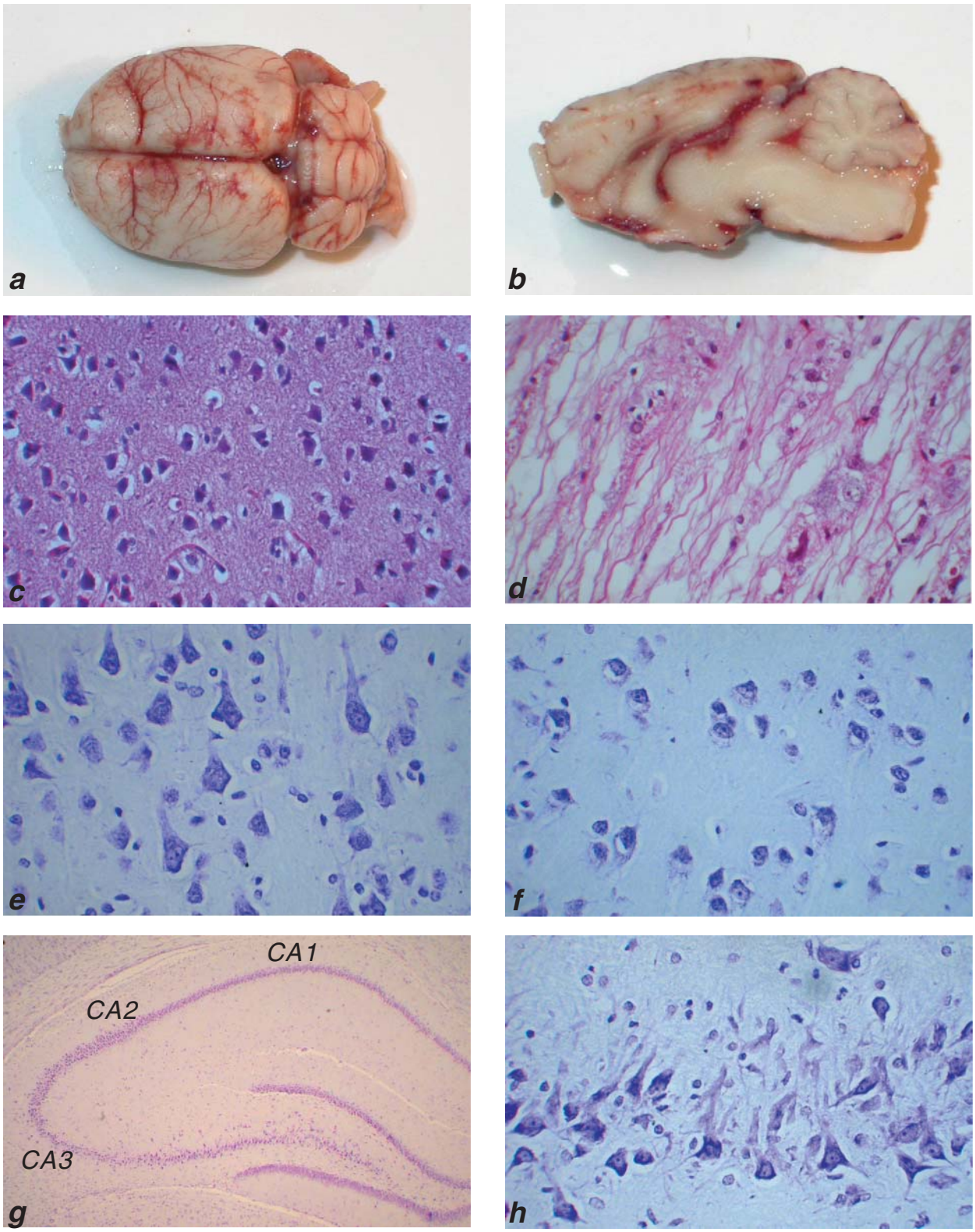

Fig. 1. Pathomorphology of diffuse traumatic injury to rat brain. *a*, *b*) macropreparations 1 h after the trauma; *c*) ischemia of cortical polymorphic layer after 24 h; *d*) edema of pons conduction tracts after 24 h; *e*) parietal cortical pyramidal layer in the control; *f*) cortical pyramidal neuron "prolapse" and polymorphism on day 3 after injury; *g*, *h*) neurodegenerative changes in hippocampal pyramidal CA2-CA3 field cells after 2 weeks (*h*: fragment of *g*); *c*, *d*: hematoxylin and eosin staining; *e-h*: Nissl staining. ×250 (*c-f*, *h*), ×25 (*g*).

One hour after the trauma diffuse hemorrhages and dilated plethoric vessels were seen in the frontoparietal pia mater (Fig. 1, *a*). Vast hemorrhages into ventricles and basal cistern and punctate hemorrhages between the cortex and corpus callosus were detected on saggittal sections (Fig. 1, *b*). Histological study showed paretic dilatation of vessels in the cortex and edema of the corpus callosus. The hippocampal neurons were spindle-shaped and hyperchromatic. Foci of the neuropile edema and cords of hyperchromatic neurons with pericellular edema appeared in the thalamus. Neurons with acute swelling were seen along with collapsed cells.

Histological study 24 h after trauma revealed edema and ischemic injuries to the nerve tissue in the zone of impact. Neurons with apical spiral-shaped dendrites with microscopic signs of their ruptures were seen in the pyramidal layer. According to pathomorphological criteria, the nerve cells were in a state of ischemic shock with characteristic condensation of the nuclei and shrinkage of the perikaryon (Fig. 1, *c*). Edema of the white matter was the most pronounced in the cerebellum, pons, and medulla oblongata. The conduction tract fibers were loosely arranged, axons could be visually characterized as sharply eosinophilic, swollen, and twisted (Fig. 1, *d*). The intensity of cell immunostaining for PCNA decreased in foci of edema and ischemia. On day 3, sites of the neuronal loosening appeared in the cortical pyramidal layer (Fig. 1, *f*) with less intensive (compared to control animals) staining of the basophilic substance in the cytoplasm. The content of Nissl substance in diencephalic neurons also decreased (Fig. 2, *b*). Despite the severity of nerve tissue ischemia, no local foci of necrotic cell death were detected 1 and 3 days after trauma. On day 3, cell proliferation increased in virtually all regions of the brain, especially in the ventricular subependymal region (Fig. 2, *e*; 3, *b*), and PCNA-positive cell nuclei with characteristic linear orientation appeared in the subgranular layer of the hippocampal dentate gyrus (Fig. 3, *e*). Their number in the hippocampal neurogenesis zone somewhat increased later (Fig. 3, *f*).

After 2 weeks a trend to normalization of the microscopic organization of the brain and neuron morphology by the content of Nissl substance, particularly for large neurons of the diencephalon and ponticular nuclear formations, could be seen. However, discharged and prolapsed neurons were retained in some sites of the sensorimotor cortex and neurodegenerative changes were seen in the pyramidal layer of hippocampal CA2-CA3 fields (Fig. 1, *g*, *h*). These changes were described previously in experimental diffuse injury to the brain in mice [17]. Selective neuronal loss in the hippocampal CA2 and CA3 fields was noted [17]. Characteristic signs of diffuse injury to the brain in mice were short-term neurophysiological depression, short period of brain edema development, and long-term mnestic deficit.

According to morphofunctional data, the regeneration phase in ischemic nerve tissue was not over 2 weeks after the injury. Large areas of proliferating cells were seen in the cortex, corpus callosus, and diencephalon. The zones of thalamic and hypothalamic nucli, along with proliferating glia, contained PCNA-positive nuclei of new capillary endothelium (Fig. 2, *g*).

Hence, we see that closed brain injury causes diffuse damage to the brain in rats. Primary trauma is associated with vascular disorders and traumatic edema peaking on day 1. The formation of edema leads to ischemia of the cortical and brain stem neurons. The reactive and compensatory-regeneratory mechanisms with initiation of cell proliferation in neurogenesis zones are triggered during the earliest periods after the injury. The regeneration phase is prolonged with slowly running reparative processes in damaged tissue and cellular structures of the brain. According to published data, proliferation of resident oligodendrocyte precursor cells is essential for regeneration of damaged brain; these cells divide without any therapy and differentiate mainly into astrocytes, later filling the zone of residual lesions [18].

In rats receiving basic therapy cerebrocortical and subcortical capillaries on day 3 after injury were moderately plethoric and edematous foci were less pronounced. Accumulations of basophilic cells appeared in the ventricular subependymal zones. Ependymal cells were enlarged and sometimes formed a multilayer lining. Proliferative activity of cells in vascular plexuses, microglia, and, particularly, ependymal and subependymal layers (Fig. 2, *f*) was higher than in experimental group 1. The effect of basic therapy was evaluated only for this term after the trauma. After 2 weeks no appreciable changes in the brain of group 2 rats were seen in comparison with group 1.

The content of Nissl substance in the cortical and thalamic neuron cytoplasm increased on day 2 after injection of MSC in group 3 rats (Fig. 2, *c*). Analysis of preparations immunostained for PCNA showed intensive proliferation of vascular endothelium in zones of restoration of functional morphology. Clearly discernible zones of intensive cell proliferation were seen in the cortex, corpus callosus, and olfactory tract. Visually the zone of neurogenesis in PVZ of the lateral ventricles was acti-

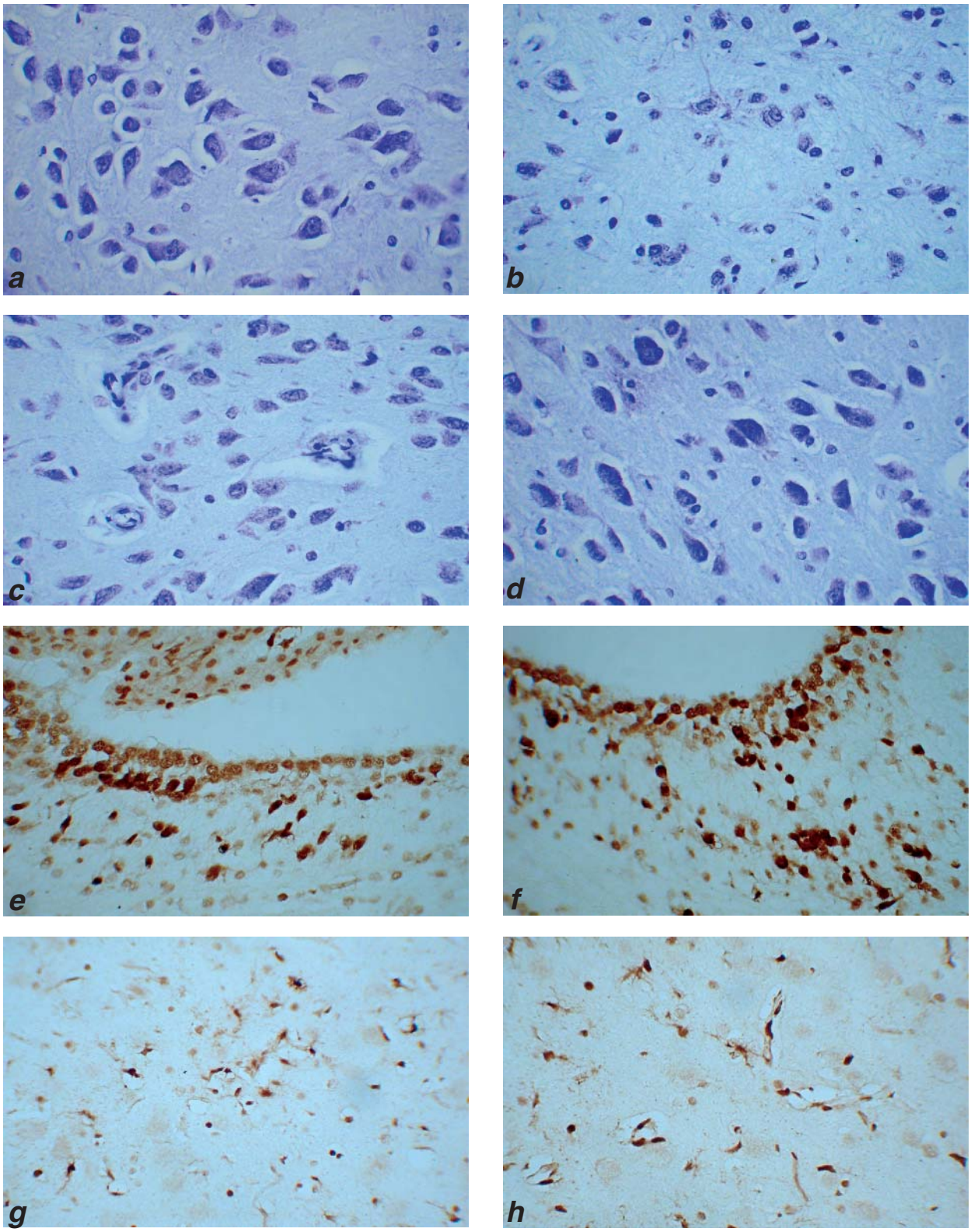

Fig. 2. Morphology of neurons in the thalamic medial nuclei (*a-d*) and cell proliferative activity (*e-h*) in control (*a*), on days 3 (*b*, *e*) and 14 (*g*) after injury, on day 3 during basic therapy (*f*), on days 3 (*c*) and 14 (*d*) after injection of MSC, and on day 3 (*h*) after combined treatment. *a-d*) Nissl staining, ×500; *e*, *f*) proliferative activity of ependymal cells; *g*, *h*) angiogenesis in the thalamus; *e-h*) immunohistochemical reaction of cell nuclei with PCNA antibodies. Biotin-streptavidin-peroxidase method, diaminobenzidine, ×250.

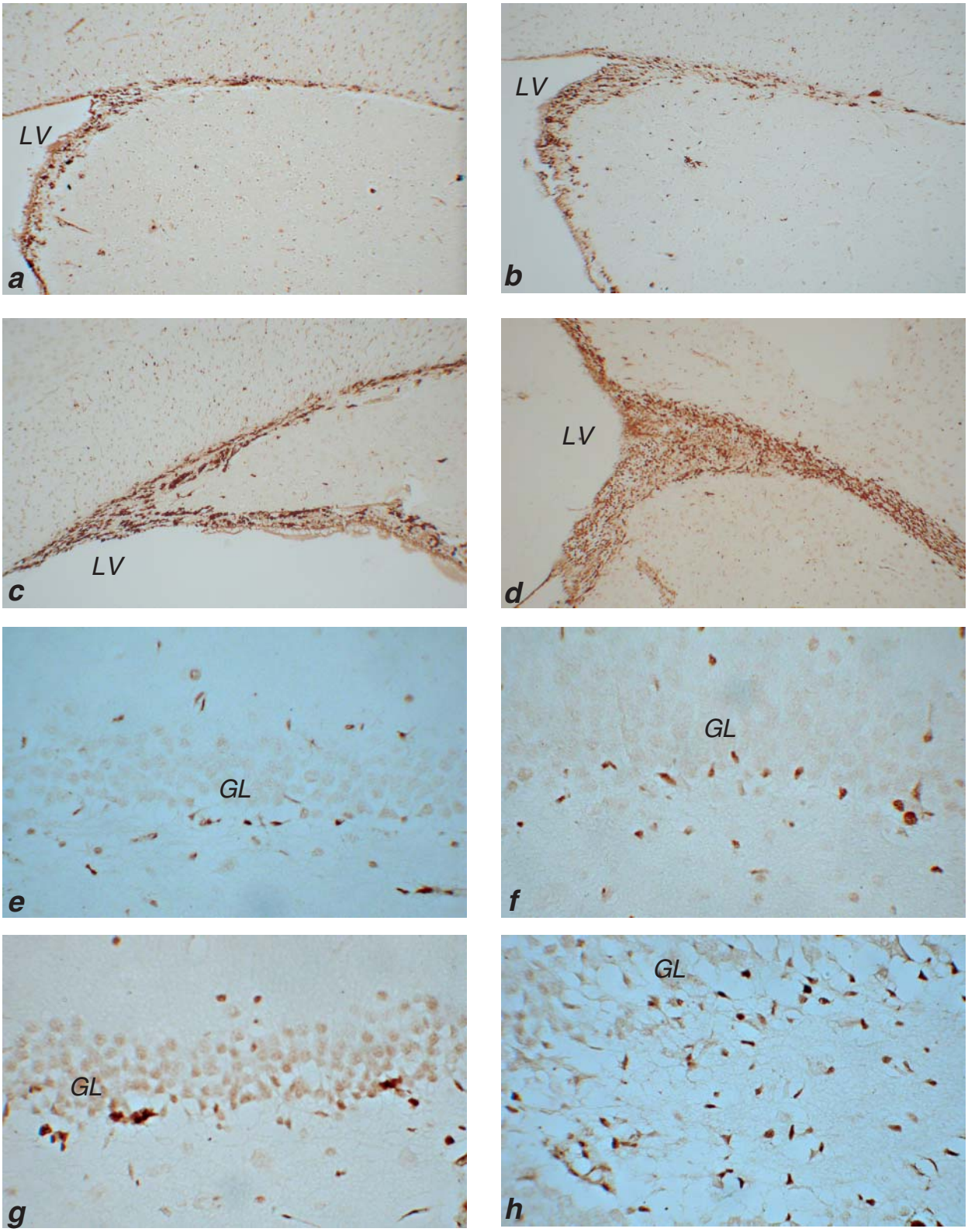

Fig. 3. Proliferative activity of cells in the lateral ventricle rostral compartment PVZ (*a-d*) and subgranular layer of the hippocampal dentate gyrus (*e-h*) in the control (*a*), on days 3 (*b*, *e*) and 14 (*f*) after injury, on day 2 after injection of MSC (*c*, *g*), and on day 2 after combined treatment (*d*, *h*). Immunohistochemical staining of cell nuclei with antibodies to PCNA. Biotin-streptavidin-peroxidase method, diaminobenzidine. ×125 (*a-d*), ×250 (*e-h*). LV: lateral ventricles; GL: granular layer.

vated on day 2 after injection of MSC (Fig. 3, *c*), and small groups of PCNA-positive cells appeared in the hippocampal subgranular zone (Fig. 3, *g*). Two weeks after injection of MSC the parietal cortex of traumatized animals little differed from the normal by histological picture and content of Nissl substance in neurons. The content and distribution of basophilic granules in the cytoplasm and the morphology of thalamic, hypothalamic, ponticular, and medulla oblongata neurons returned to normal by this period (Fig. 2, *d*). "Loosened" nuclear layers with small hyperchromatic neurons were still observed only in the hippocampus. According to immunohistochemical findings, foci of cell proliferation were seen in the cortex, corpus callosus, neurogenesis zones, and brain stem parts.

In rats receiving combined treatment only local areas with swollen nerve fibers were seen in conductive tracts of the corpus callosus, pons, and medulla oblongata on day 3 after injury. A characteristic feature of this term was intensive cell proliferation in the neurogenesis zones (Fig. 3, *d*, *h*), ischemic cortex, olfactory tract, thalamus, and basal part of the midbrain. In one rat of this group cell proliferation was maximum in the migration tract of the rostral compartment and in the olfactory bulb. After 2 weeks the histological picture of the cortex and other brain compartments in experimental group 4 virtually did not differ from normal variants in the control group. However, despite recovery of the neuron morphology, there were still some local foci of cell proliferation and elevated activity of neurogenesis zones in the cortex, corpus callosus, and diencephalon. Hence, by this term no complete recovery of damaged cell and tissue structures of the brain was attained even in animals receiving combined treatment.

Our results are in line with the previous data indicating that pathological events forming in traumatized adult brain and ischemic injury stimulate neurogenesis in cerebral germinative zones [6,10, 19]. It was recently found that the repair processes in adult nervous tissue were realized at the expense of neural stem cells, including oligodendrocyte precursor cells. According to modern notions, neural stem cells in adult organism are undifferentiated cells of the neuroectodermal origin with high proliferative activity; they can differentiate into neuronal or macroglial cells (astrocytes and oligodendrocytes) [6]. These cells, neuronal and glial precursors, are located at least in three germinative zones: in the ventricular wall and adjacent subventricular zone, in the subgranular zone of the hippocampal dentate gyrus, and in the olfactory bulb [6,10,11].

It was shown that though the repopulating nerve stem cells are present in adult brain and express the respective neuronal phenotype, their potentialities for regeneration of new functional neurons in response to injury are limited [12]. For this reason the possibility of repair of the nervous system with transplanted cells, which could replace dead cells, attracts special interest. It was demonstrated that bone marrow stromal cells significantly reduced manifestations of motor and neurological deficit in animals with trauma [12]. Bromodeoxyuridine-labeled cells migrated into the damaged brain parenchyma and expressed NeuN (neuronal marker) and GFAR (astrocyte marker). Labeled MSC were detected also in other organs, primarily in vascular structures, without side effects. These data suggest that intravenous injection of MSC can be useful in the treatment of brain injury aftereffects. Subsequent studies confirmed the plasticity and therapeutic potential of MSC in the nervous system not once [16].

According to our data, the neuroprotective effect of MSC in diffuse brain injury consisted not only in stimulation of reparative processes during the regeneration phase in general (rapid restoration of Nissl substance in neurons, acceleration of gliogenesis and angiogenesis), but also, presumably, activation of the germinative zones of neurogenesis. Analysis of published data suggests that the therapeutic effect of MSC in nerve tissue damage can be realized by several pathways. First, some data indicate that after migration into damaged nervous tissue MSC start expressing neuronal and astrocytic markers and the possibility cannot be excluded that they replace dead cells [12]. Second, according to previous findings, stem cell factors stimulate neurogenesis *in vitro* and *in vivo* [9]. It seems that MSC expression products stimulate not only nervous stem cells, but also vascular endothelium. Moreover, MSC activate cells additionally expressing growth factors in the brain. This viewpoint is confirmed by the data according to which intravenous injection of MSC after local brain injury increases the expression of nerve growth factor and brain-derived neurotrophic factor [13]. Moreover, it was found that the neurotrophic factors expressed by glial cells promote the survival of neurons and their recovery after injuries or injury-induced degeneration [2]. Subependymal microglia is rapidly activated and migrates to an appreciable distance into damaged brain parenchyma [13]. According to our data, trauma-induced proliferation of glial cells is potentiated by basic therapy and activated by MSC injection. Presumably, by reducing the development of edema and restoring the microcirculation, basic ther-

apy promotes more rapid delivery, accumulation, and/or migration of MSC to the damaged brain areas.

Hence, our experiments demonstrated proliferotropic, angiogenic, and, presumably, neurotrophic effects of MSC. Indirect data indicate that during nervous tissue regeneration MSC activate cell proliferation in neurogenesis zones. Visually the therapeutic effect is detected on day 2 after intravenous injection of MSC. The activating effect of MSC is potentiated by basic therapy. Presumably, combined treatment of even total diffuse brain injury, including basic therapy and intravenous injection of MSC, will create prerequisites for activation of the mechanisms of compensatory adaptive processes during the reparative regeneration period and for improvement of nervous tissue trophics, essential for restoration of functional morphology of damaged neurons.

These results are in line with the conclusions on the positive therapeutic effect of MSC in brain injury. Immanent functions of MSC obtained by adult bone marrow culturing observed in model experiments can serve as an experimental basis for limited pilot clinical trials on the use of autologous MSC in combined therapy of human brain injuries.

The authors are grateful to Prof. E. F. Lushnikov, Head, Clinical Morphological Department of Medical Radiology Research Center, and Prof. E. V. Loseva, Head, Laboratory of Functional Morphology, Institute of Higher Nervous Activity and Neurophysiology, for consultations.

The study was supported by Diod Company.

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