Morphofunctional Study of the Therapeutic Efficacy of Human Mesenchymal and Neural Stem Cells in Rats with Diffuse Brain Injury

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We studied the effect of transplantation of human stem cells from various tissues on reparative processes in the brain of rats with closed craniocerebral injury. Combined treatment with standard drugs and systemic administration of xenogeneic stem cells had a neuroprotective effect. The morphology of neurons rapidly returned to normal after administration of fetal neural stem cells. Fetal mesenchymal stem cells produced a prolonged effect on proliferative activity of progenitor cells in the subventricular zone of neurogenesis. Adult mesenchymal stem cells had a strong effect on recovery of the vascular bed in ischemic regions.

Key Words: *brain trauma; mesenchymal stem cells; neural stem cells; xenotransplantation; neurogenesis*

Craniocerebral trauma (CCT) is characterized by high incidence of unfavorable outcomes with poscommotion syndrome, serious neurological deficit, and incapacity. These disorders are particular pronounced in children and young patients. The majority of clinical trials of the neuroprotective effect of pharmaceutical and metabolic maintenance of the brain failed to reveal a permanent improvement of neurological symptoms in patients with severe CCT [33]. The development of new efficient methods for medical rehabilitation and neuroprotection in brain injuries is an urgent problem of modern medicine.

Regenerative processes in the adult brain are associated with activity of neural stem cells (NSC). Pathological changes during nervous tissue injury and ischemic trauma stimulate neurogenesis in germinal zones of the brain [7,13,36]. Repopulations of stem cells (SC) can express the corresponding neuronal phenotype in zones of neurogenesis. However, these cells have a low ability to generate new neurons in response to injury. One of the approaches to cell replacement therapy suggests the evaluation of functional recovery of nervous tissues

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after transplantation of SC. These cells are obtained during cultivation of fetal and adult tissues. Biological studies of SC showed that NSC [4,16,24] and MSC of the bone marrow [28,34] are characterized by the required plasticity and therapeutic efficacy in the nervous system.

Not only autologous and allogeneic cells [3,6, 16,20-22], but also human SC of different tissue origin contribute to reparation of the nervous system and recovery of the neurological function in laboratory animals with experimental cerebral ischemia and CCT. Experiments on animals demonstrated the safety and efficacy of human SC. Some ethical problems in clinical medicine are related to the isolation of primary samples. Other problems are associated with a selection of the optimal cell source, availability of normal primary cells, and adequacy of the treatment procedure.

Here we review the results of preclinical studies with systemic transplantation of three types of human SC. The cells were administered individually or in combination with standard drugs. The effect of treatment on functional morphology of neurons and reparative processes in nervous tissue was studied on rats with experimental diffuse brain injury.

MATERIALS AND METHODS

The primary culture of adult bone marrow MSC and human fetal MSC and NSC (9-10 weeks gestation, medical abortion product) were obtained as described elsewhere [1,5]. Experiments were performed on male Wistar rats weighing 140-160 g. CCT was produced by a 50-g weight, which fell on the parietal region of the head from a height of 110 cm [6]. Fifty percent rats died over the first 5 min after the incidence of trauma. Survived animals were divided into groups with comparable severity of brain injury. It was estimated from the corresponding symptoms and tests over 30 min after trauma [3]. Group 1 consisted of animals with brain injury, which did not receive therapy. Group 2 specimens were treated with symptomatic and metabolic drugs (standard therapy). Adult MSC were administered to group 3 rats. Group 4-6 animals received combination therapy with standard drugs and SC (Table 1). The animals were not subjected to immunosuppression. The control group included sham-injured rats.

The rats of groups 2 and 4-6 received standard drugs with the antioxidant, antihypoxic, nootropic, and anxiolytic properties. The therapy started 1 day after trauma and continued for 2 days. Actovegin (5.0 mg/kg) and mexidol (5.0 mg/kg) were given twice daily at a 6-h interval. Cavinton and piracetam in single doses of 5.0 and 200 mg/kg, respectively, were administered on day 2 after CCT. All drugs were injected intraperitoneally (0.1 ml per 100 g body weight). Group 3-6 animals received a single intravenous injection of human SC (2×10^6 cells) on day 1 after trauma. The cells were suspended in 0.5 ml physiological saline. Neurospheres were obtained during cultivation of NSC/progenitor cells from the periventricular area of human fetal brain and dissociated by mechanical disintegration. Proliferative activity of SC suspension was estimated by staining of acetone-fixed smears for proliferating cell nuclear antigen (PCNA).

The animals were killed 3 days (subgroup A) or 2 weeks after CCT (subgroup B). A synthetic analogue of thymidine bromodeoxyuridine (BrdU, Aldrich) was injected intraperitoneally for *in vivo* labeling of replicating cells. Crystalline BrdU was dissolved in physiological saline and 0.007 M NaOH at 37°C. A pulse-label approach was used to reveal the cells formed in brain zones of neurogenesis. BrdU (100 mg/kg, 40 mg/ml) was administered to rats of the control (n=3) and treatment groups (n=3) on day 1 after CCT. The animals received 3 injections of BrdU at 1-h intervals. The samples were

TABLE 1. General Characteristics of Samples to Study the Effect of Human SC on Rats with CCT

Group	Number of animals	
	day 3 (subgroup A)	day 14 (subgroup B)
Control (sham-injured)	15	12
1 (trauma, without therapy)	15	15
2 (trauma+standard therapy)	12	12
3 (trauma (trauma+adult MSC)	12	12
4 (trauma+standard therapy+adult MSC)	15	15
5 (trauma+standard therapy+fetal MSC)	15	15
6 (trauma+standard therapy+fetal NSC)	15	11

taken 1 h after the last injection of BrdU. A prolonged pulse of proliferation marker was used to study the migration of labeled cells. Three animals of each group (subgroup A) received BrdU (50 mg/kg, 20 mg/ml) over the first 2 days after CCT. BrdU was injected twice daily at 6-h intervals. The brain was removed 1 day after the last injection of BrdU. Three rats of subgroup B received BrdU in a dose of 50 mg/kg for 5 days (beginning from the 7th day after CCT). BrdU was injected twice daily at 8-h intervals. The samples were taken 2 days after the last injection of BrdU.

The brain was taken from rats after decapitation under nembutal anesthesia. The brain from rats of each subgroup was immersed in 10% neutral formalin (*n*=5-7), acid Bouin's fluid (*n*=2-3), or acid alcohol (96% ethanol and glacial acetic acid in the 3:1 ratio, *n*=4-5) for 24 h. Two frontal segments were cut off from the rostral part of the lateral ventricles (0.7-0.2 mm anterior to the bregma) and dorsal hippocampus (2.5-3.3 mm posterior to the bregma). After BrdU injection, the samples of some animals were taken for parasagittal orientation of histological sections. Tissue samples were dehydrated and embedded in Paraplast Plus (Kendall). For a histological study, microtome sections (5 m) were stained with hematoxylin and eosin. The sections fixed in acid alcohol and refixed in acid alcohol after fixation in Bouin's fluid were stained with 0.1% cresyl violet and thionin (Fluka) by the method of Nissl. Histotopographic mapping of the damaged zone and accurate determination of the level of sections were performed by the stereotaxic atlas of rat brain [26].

Immunohistochemical studies were performed with mouse monoclonal antibodies to PCNA (PC10, DAKO, 1:200), BrdU (Bu2a, DAKO, 1:50), and vascular endothelial growth factor (VEGF, C-1, Santa Cruz, 1:50) and rabbit polyclonal antibodies to neurotrophin-3 (NT-3, N-20, Santa Cruz, 1:30). Before application of antibodies to BrdU, VEGF, and NT-3, deparaffinized sections were immersed in citrate buffer (pH 6.0) and heated in a microwave at 720 W for 5 min. The samples were incubated in a solution of primary antibodies at 4°C for a night (humid atmosphere). They were washed in phosphate buffered saline (PBS) and treated with secondary antibodies in accordance with the manufacturer's recommendations. Biotinylated horse antibodies to mouse IgG (BA-2000, Vector Lab., 1:250) and streptavidin-peroxidase complex (SA-5004, Vector Lab., 1:250) were used with monoclonal antibodies. The biotin-streptavidin peroxidase kit for rabbit Ig (MP Biomedicals) was used with polyclonal antibodies.

Histological characteristics of cerebral microvessels were visualized with the immunoperoxidase kit (MP Biomedicals), which consisted of biotinylated goat antibodies to mouse IgG and streptavidin-peroxidase reagent. Our previous experiments showed that after fixation of rat brain in acid alcohol, the capillary network is selectively stained by anti-mouse Ig antibodies of various manufacturer's (MP Biomedicals, DAKO, Santa Cruz, and Vector). A positive reaction product is not only found in the lumen of vessels, but also contours the vascular wall. A positive vascular reaction was not observed after addition of normal mouse or rat serum/plasma into the blocking solution. Treatment of brain tissue with an acid fixative is probably followed by opening of antigenic determinants. They are common for the proteins belonging to a family of mouse and rat Ig. It cannot be excluded that a positive reaction is related to the presence of cell adhesion molecules of the Ig protein superfamily with Ig-like fragments in the extracellular domain. We found no data on the selective staining of rat brain vessels without administration of special antibodies to the endothelium.

Antigens in histological sections were detected according to the main principles of immunoperoxidase studies. All antibodies and kits were tested with a positive control of experimental samples. After fixation of the brain in formalin and Bouin's fluid, sections (7 () were subjected to immunostaining for PCNA. The samples were immunostained for BrdU (fixation in formalin), NT-3 (fixation in Bouin's fluid), VEGF (postfixation in Bouin's fluid after fixation in formalin), and vessels (fixation in acid alcohol) using 9-m sections. Immunohistochemical solutions were prepared in PBS (pH 7.4). Endogenous peroxidase was blocked by 3% H_2O_2. The blocking solution consisted of 2% normal serum from animals (donors of secondary antibodies), 1% BSA, and 0.1% Triton X-100. Normal rat serum (2%) was added to mouse Ig in the solution of biotinylated antibodies to reduce the nonspecific reaction during a study of monoclonal antibodies. The substrate enzyme (peroxidase) was developed with diaminobenzidine (Liquid DAB+, DAKO) or aminoethylcarbazole (AEC+, DAKO). When required, the cell nuclei were additionally stained with hematoxylin. The samples with aminoethylcarbazole were embedded in a water-soluble medium (Faramount, DAKO).

Histological preparations were examined under an Olympus CX41 microscope. Photomicrography was performed using a Nikon CoolPix 4500 digital camera. Quantitative studies were conducted with a computerized system for the analysis of micro-

scopic images (license software Morphostar-2 (IMSTAR) and AnalySIS 5.0 (Soft Imaging System GmbH) according to the main principles of stereology in morphometry. The following stereological parameters were evaluated: AT, total area tested; volume density (integral criterion for the amount of tissue structures in reference to the total area of structures per AT); average cross-sectional area of the structure; numerical density (number of cross-sectional structures per unit sectional area); linear density (total length of structures per unit area); and optical density of staining of structures in the section. The number of structures in the right and left hemispheres of each animal was evaluated in test fields of three brain sections.

At the level of the hippocampus, we examined the sensorimotor cortex, dorsolateral thalamic area, and hippocampal dentate gyrus (HDG, formation area). A quantitative study of the vascular bed was performed in cortical layers I-V and thalamic nuclei (AT=33 mm^2 for each zone, objective ×10). A morphometric study of Nissl-stained neurons was performed in layer V of the sensorimotor cortex and LVDL region of the thalamus (objective ×10, 10 test fields, AT=0.2 mm^2). During image analysis, we contoured and took into account only the sections of neuronal bodies that passed through the nucleus. Studying the HDG region (AT=1.28 mm^2, 8 test fields) suggested the evaluation of the numerical density of PCNA-positive cell nuclei and relative content of proliferating cell nuclei in the subgranular layer of the dentate gyrus. The total area of proliferative regions in the subventricular zone of the lateral ventricles in the right and left hemispheres was estimated in the rostral part of the brain (PCNA-immunostained samples). The average density of PCNA-positive nuclei was measured in these structures. The results were analyzed by nonparametric Mann—Whitney *U* test.

RESULTS

Stratification of the cerebral cortex and morphological characteristics of neurons (Fig. 1, *a*) in the cortex and diencephalon of sham-injured animals did not differ from the control. The total number of neurons in the pyramidal layer of the sensorimotor cortex was 17.4-17.0%. The numerical density was 890-850 neurons per mm^2. The average cross-sectional area of bodies was 200 m^2. These parameters for neurons of dorsolateral thalamic nuclei were 11.8-11.0%, 568-538 cells/mm^2, and 208-204 m^2, respectively (Table 2). The capillary network was regular in the cerebral cortex and dorsolateral thalamic area. An immunopositive reaction product was found in the lumen of vessels and contoured the vascular wall (Fig. 2, *a*). Table 3 shows quantitative parameters of the microcirculatory bed. Cells of the outer granular layer and outer and inner pyramidal layers of the sensorimotor cortex (Fig. 3, *a*) and some nuclei of the diencephalon exhibited a positive reaction for VEGF. A strong reaction for VEGF was typical of the neuronal cytoplasm in hippocampal pyramidal layers. A small number of NT-3-positive cells with moderate expression of the immunopositive reaction were visualized in layers III-VI of the sensorimotor cortex (Fig. 4, *a*) and reticular and caudate nuclei of the diencephalon.

The layer of small dark cells with PCNA-positive nuclei was present in the subventricular space of the lateral ventricles (Fig. 5, *a*). Cell bundles were spread laterally in frontal sections (Fig. 5, *b*). The total area of proliferating cells in subventricular zones of the right and left hemispheres was approximately 0.09 mm^2 per section (level of the rostral part of the brain). The density of PCNA-positive nuclei was 17.8-15.7% (Table 4). A small number of proliferating nuclear antigen-positive cells were found in HDG (42-43 cells per mm^2). Not less than 40% of these cells were located in the subgranular zone. Labeled cells were located in the subependymal layer 1 h after the BrdU pulse (Fig. 5, *c*). A study of parasagittal sections showed that the distribution of repopulating cells has a rostrocaudal gradient. The concentration of PCNA-positive and BrdU-labeled cells was highest in the dorsolateral angle of the rostral ventricle, but decreased in a caudal direction. The number of labeled cells decreased in the subependymal space, but increased in the migration path on day 1 day after 2-day administration of BrdU. BrdU-positive cells were located over the migration path and in cell layers of the olfactory bulb on day 2 after 5-day labeling. BrdU-labeled cells were rarely found in the subgranular layer of HDG from control specimens.

Pathomorphology of CCT. A histological study of brain sections was performed 3 days after trauma. Nervous tissue injury was revealed in the projection of stroke. The parietal region of the cortex and thalamus included diffuse focal zones of edema and swelling of the neuropil (spongy substance), bundles of ischemic and wrinkled neurons with pericellular edema and, sometimes, local necrotic areas and typical "rose" neurons. A few petechiae were visualized between the cortex and *corpus callosum*. In some regions of hippocampal pyramidal layers, the neurons were fusiform and hyperchromic. Histopathological changes were absent or insignificant in some survived animals. The degree

TABLE 2. Quantitative Characteristics of Nissl-Stained Neurons in the Pyramidal Layer (V) of the Sensorimotor Cortex and Dorsolateral Thalamic Nuclei in Rats with CCT ($M \pm m$)

Brain region, parameter	Period	Control	Trauma					
			without therapy	+ST	+MSC	+ST+MSC	+ST+fetal MSC	+ST+fetal NSC
Cortex								
optical density of staining, arb. units	3 days	0.085±0.002	0.074±0.001*	0.080±0.001	0.083±0.003	0.084±0.005	0.088±0.005	0.087±0.004
	2 weeks	0.086±0.004	0.077±0.005	0.081±0.003	0.083±0.004	0.089±0.009^{+}	0.085±0.003	0.084±0.004
volume density of neurons, %	3 days	17.4±0.4	10.4±0.6*	11.9±0.3	11.8±0.8	13.4±0.8^{+}	13.0±0.4^{+}	13.9±0.7^{+g}
	2 weeks	17.0±0.4	10.8±0.5*	12.4±0.3	13.1±0.6*	14.3±0.6^{+x}	14.2±0.7^{+}	13.4±0.5^{+}
cross-sectional area of neurons, m^2	3 days	196±2	174±5*	181±2	187±5	191±5^{+}	184±6	194±4^{+x}
	2 weeks	199±4	174±4*	180±5	178±4	185±5^{+}	189±6	189±5^{+}
number of neurons per mm^2	3 days	890±15	600±31*	659±17	631±36	699±41	708±21^{+}	718±29
	2 weeks	850±11	623±27*	690±27	736±35	768±22^{+}	757±47^{+}	708±27
Thalamus								
optical density of staining, arb. units	3 days	0.099±0.003	0.072±0.006*	0.087±0.008	0.084±0.004	0.090±0.006	0.087±0.006	0.101±0.005^{+}
	2 weeks	0.101±0.003	0.082±0.003	0.095±0.007	0.090±0.006	0.098±0.005^{+}	0.087±0.002^{+}	0.097±0.007^{+}
volume density of neurons, %	3 days	11.8±0.3	7.2±0.5*	7.3±0.3	8.2±0.6	9.7±0.7^{+x}	9.8±0.3^{+x}	9.8±0.7^{+x}
	2 weeks	11.0±0.4	6.6±0.3*	8.3±0.6	8.6±0.5^{+}	9.6±0.7^{+}	10.1±0.9^{+}	8.9±05^{+}
cross-sectional area of neurons, m^2	3 days	208±6	179±3*	175±4	190±11	193±5^{+x}	207±3^{+x}	203±9
	2 weeks	204±4	177±4*	182±6	186±4^{+}	196±6^{+}	204±12	189±4
number of neurons per mm^2	3 days	568±15	405±24	418±21	440±39	499±37^{+}	474±13	481±19
	2 weeks	538±15	375±14*	459±32	458±23^{+}	488±27^{+}	490±25^{+}	468±18^{+}

Note. Each group consists of 7-8 specimens. Here and in Tables 3 and 4: ST, standard therapy. $p<0.5$: *compared to the control; $^{+}$compared to trauma with no therapy; ^{x}compared to animals of the ST group.

of staining for basophilic Nissl substance became less pronounced after trauma (Fig. 1, *b*). It reflects the total content of ribonucleoproteins in the neuronal cytoplasm. A morphometric study revealed that the volume density, number, and size of neurons in the pyramidal layer decreased by 1.6 times, 1.4 times, and 13%, respectively. Similar changes were found in thalamic neurons (Table 2). Emptying of vessels and presence of paretically widened or collapsed profiles of the vascular section were detected in the sensorimotor cortex. In the zone of ischemic injuries, the vessels were weakly stained for immunoglobulins (Fig. 2, *b*). Therefore, the number of visualized vessels was reduced by 1.4 times ($p<0.05$). Qualitative and quantitative changes in the vascular network were not observed in the thalamus. Table 3 shows the quantitative characteristics of the capillary bed in the cortex and thalamus.

The intensity of neuronal immunostaining for VEGF decreased in the zone of traumatic injury, but increased in the perifocal area and intact tissue (Fig. 3, *b*). Neurotrophin-3-expressing cells were found in pyramidal layers of the hippocampus (Fig. 4, *b*). The density of PCNA-positive cell nuclei in the subventricular space increased by 1.15 times on day 3 after trauma ($p<0.05$). The numerical density of proliferating cells increased by 1.6 times in the dentate gyrus. The relative number of these cells was shown to increase in the subgranular layer. These cells were characterized by linear orientation. PCNA-positive microglial cells were found in the zone of traumatic injury. A BrdU labeling study showed that endogenous neural stem/progenitor cells are activated on day 1 after CCT. After the BrdU pulse, labeled cells were concentrated over the boundary of the lateral ventricles. Replicating cells of the subependymal space were arranged in small groups or chains and migrated in a rostral direction (Fig. 5, *e*). BrdU accumulation in cell nuclei was significant in connective tissue of the pia mater and perivascular regions (particularly in the basal part of the brain). They probably belong to the population of cambial cells and are strongly predis-

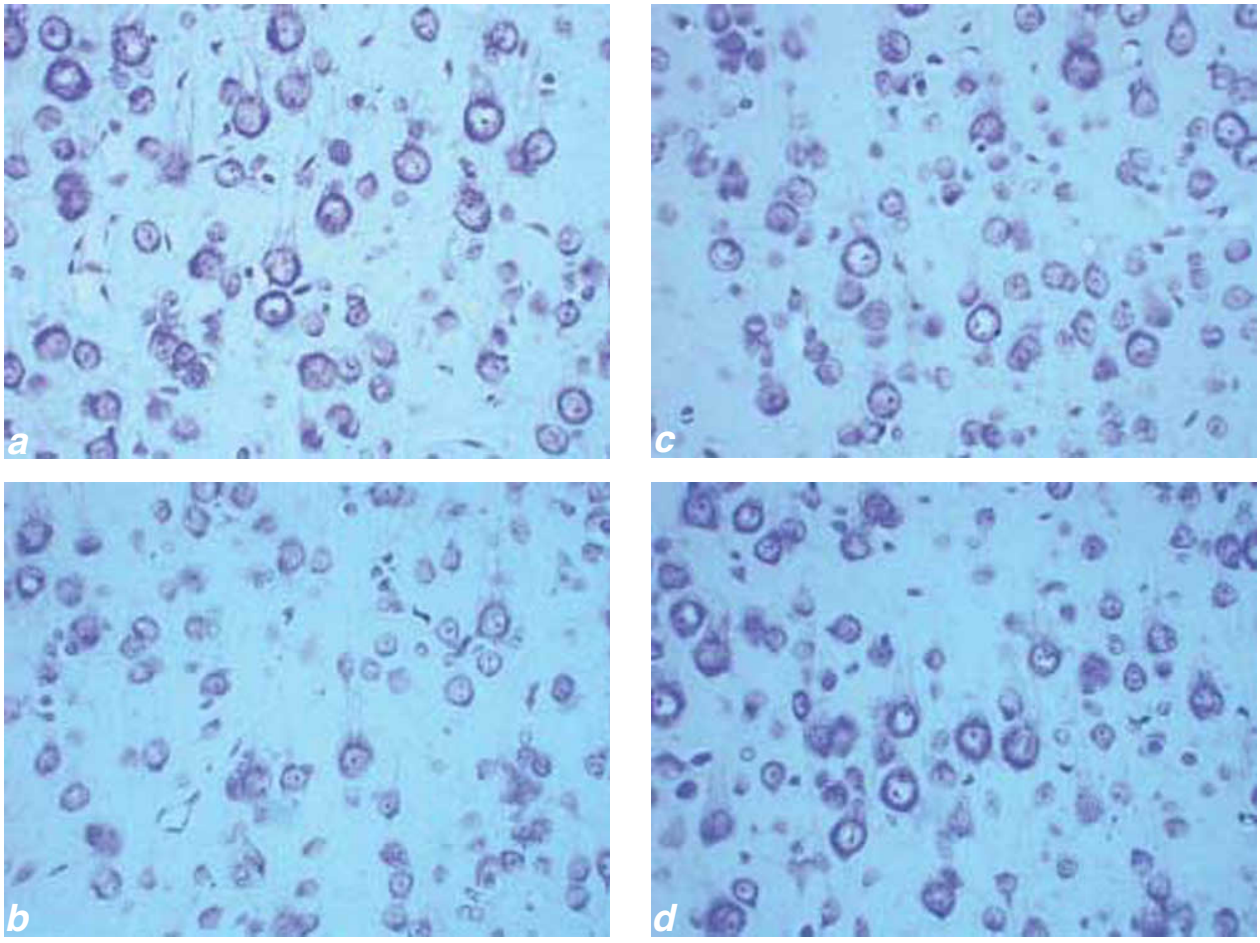

Fig. 1. Morphology of neurons in the inner pyramidal layer of the sensorimotor cortex in rats with CCT (*280). Control (*a*); day 3 after trauma (*b*); day 14 after administration of adult MSC (*c*); day 3 after standard therapy and administration of fetal NSC (*d*). Nissl staining.

posed to posttraumatic disorders. After 2-day administration of BrdU, labeled cells were concentrated in the zone of nervous tissue injury (Fig. 5, *f*).

Microscopic signs of the brain, morphology of neurons, and state of cerebral vessels were slightly improved after 2 weeks. However, quantitative characteristics of neurons and microcirculatory bed remained practically unchanged in this period (as compared to the previous period; Tables 2 and 3). The parietal cortex was characterized by loosening or disappearance of neurons. The development of neurodegenerative changes in hippocampal pyramidal layers was accompanied by the loss of VEGF-immunostained damaged neurons. Proliferative activity of cells was high in the dentate gurus. Migration of labeled cells from the subependymal layer was observed.

Therefore, closed CCT in rats is followed by vascular disturbances and diffuse ischemic injury to neurons of the cortex and brainstem. The early posttraumatic period is characterized by activation of regenerative processes and proliferation of endogenous NSC/progenitor cells. Reparative regeneration of damaged cells and tissue structures in the brain occurs over a prolonged period. Our results are consistent with published data on the location of endogenous NSC/progenitor cells and features of neurogenesis under conditions of adult brain injury. According to the present view [7,13], the subependymal zone of the lateral ventricles and subgranular zone of HDG are the major germinal regions in the adult brain. The identity of SC is a disputable problem. A growing body of evidence indicates that these cells may have a phenotype of astrocytes [36]. The majority of adult brain cells are in the dormant state. The exceptions are neurogenic zones with slow division of stem-like cells. As differentiated from these cells, progenitor cells (precursor cells) have a short cycle of division, retain the proliferative activity, and differentiate into nerve cells. However, progenitor cells are not capable of self-renewal. They are more committed than NSC.

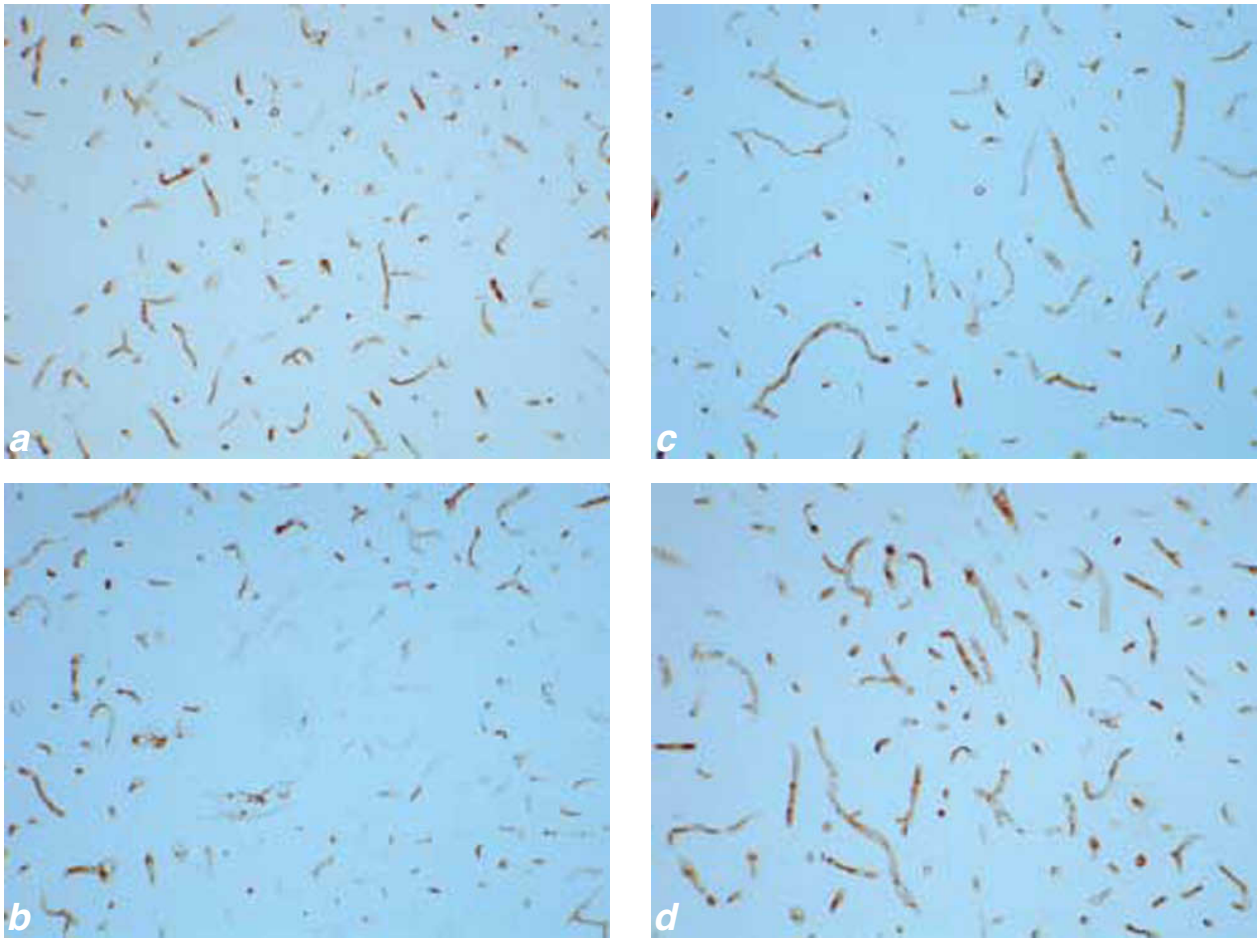

Fig. 2. Histological pattern of the vascular bed in the sensorimotor cortex of rats with CCT (*140). Control (*a*); day 3 after trauma (*b*); day 3 after standard therapy (*c*); 2 weeks after combination therapy with adult MSC (*d*). Staining for immunoglobulins. Method of the biotin-streptavidin peroxidase complex, diaminobenzidine.

It remains unclear whether these cells have the property of pluripotency. The subventricular zone extends over a whole length of the wall of the lateral ventricles and includes the largest population of dividing cells in the adult mammalian brain. Cells migrate from the subventricular zone, pass through the rostral migration path, and reach the olfactory bulb. In the olfactory bulb, these cells differentiate into granular and periglomerular interneurons [36]. Moreover, newly formed neurons leave the subventricular zone, migrate into the adjacent layer of granular cells in the dentate gyrus and other regions, and become integrated in the nervous network. Pathological events in adult brain injury and ischemic trauma have a stimulatory effect on neurogenesis in germinal zones.

Effect of standard therapy and human SC. A histopathological and morphometric study of samples from animals of the standard-therapy group revealed that a therapeutic effect on the morphology of neurons and capillary network is observed on day 3 after trauma. Visual examination showed that the area of focal edema and damage (hyperchromic neurons) in the cerebral cortex and subcortical regions of these rats was lower than in untreated animals. Swelling of the neuropil was less pronounced in rats of the treatment group. A histological pattern of the microcirculatory bed returned to normal in the sensorimotor cortex (Fig. 2, *c*). The intensity of cytoplasmic staining for Nissl substance was shown to increase in neurons of treated animals. VEGF expression in the epithelium of the vascular plexus increased in some rats (Fig. 3, *c*). The degree of reparative processes in the cortex and thalamus of animals from the metabolic maintenance group remained slightly elevated after 2 weeks (Table 2 and 3).

Morphofunctional criteria for activation of reparative processes in group 3 animals (treatment with adult MSC) were revealed after 2 weeks. A

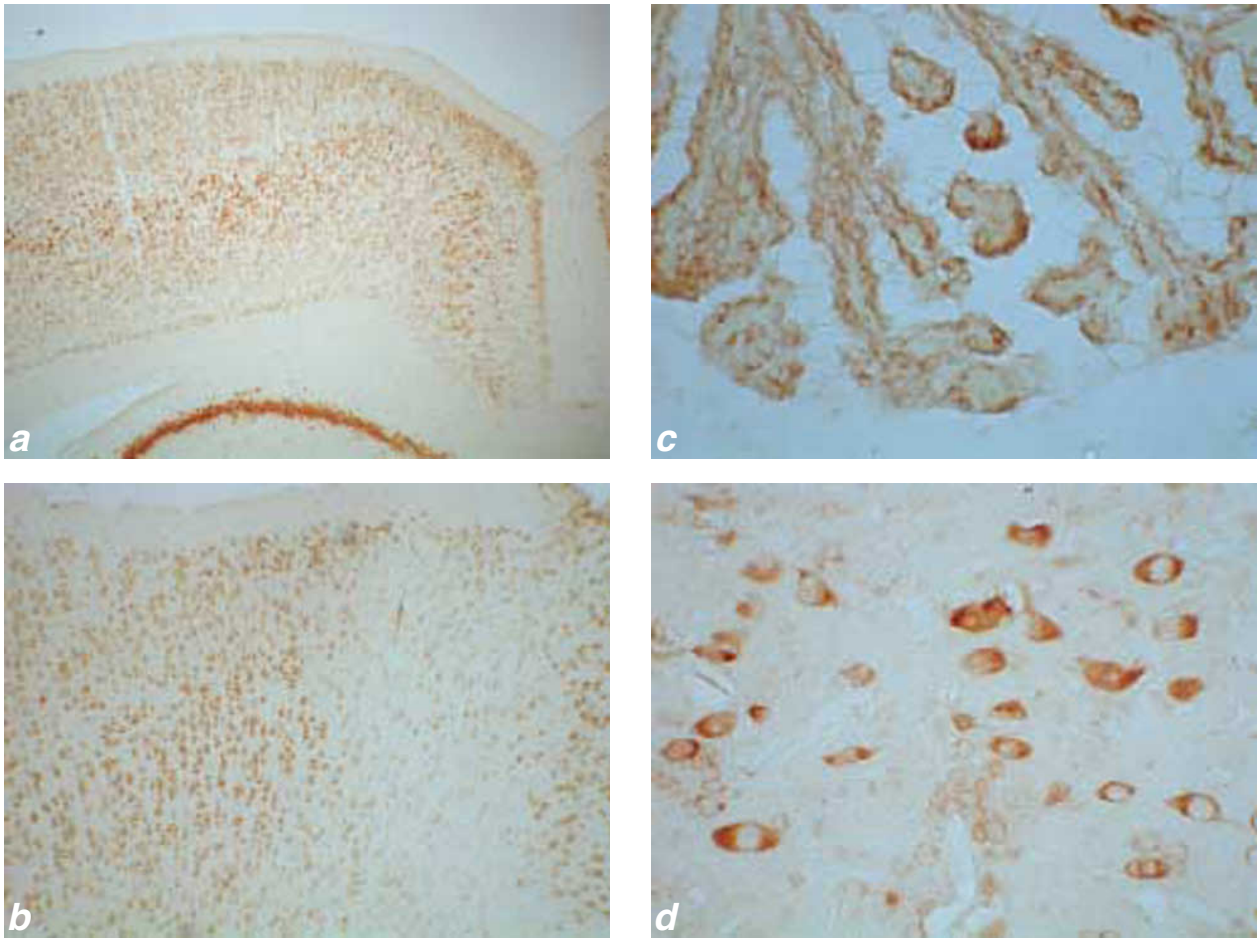

Fig. 3. VEGF expression in the sensorimotor cortex (*a*, *b*), epithelium of the vascular plexus in the third ventricle (*c*), and thalamic nucleus of rats with CCT (*d*). Control (*a*); day 3 after trauma (*b*); day 3 after standard therapy (*c*); day 3 after standard therapy and administration of fetal MSC (*d*). Method of the biotin-streptavidin peroxidase complex, diaminobenzidine. Magnification: 30 (*a*); 70 (*b*); 280 (*c*, *d*).

TABLE 3. Quantitative Characteristics of the Vascular Bed in the Sensorimotor Cortex of Rats with CCT (*M*±*m*)

Brain region, parameter	Period	Control	Trauma					
			without therapy	+ST	+MSC	+ST+MSC	+ST+fetal MSC	+ST+fetal NSC
Cortex								
optical density of immunostaining, arb. units	3 days	0.151±0.003	0.134±0.005*	0.138±0.006	0.141±0.002	0.137±0.002	0.146±0.003	0.140±0.004
	2 weeks		0.144±0.03	0.141±0.03	0.139±0.002	0.143±0.003	0.145±0.004	0.145±0.003
volume density of neurons, %	3 days	2.24±0.20	1.32±0.19*	1.80±0.34	1.31±0.13	1.96±0.19	1.63±0.30	1.55±0.25
	2 weeks		1.65±0.16	1.88±0.24	1.96±0.19	2.97±0.28+x	2.44±0.37	1.85±0.15
linear density of neurons, m/mm^2	3 days	5884±393	3765±466*	4607±741	3955±244	5174±326	4569±923	3894±597
	2 weeks		4359±306	4873±658	5030±499	6937±448+x	6145±701	4742±382
number of vascular sections per mm^2	3 days	776±11	564±50*	618±54	561±33	677±53	628±111	581±52
	2 weeks		625±15	646±43	629±32	773±20+x	697±33	623±12
Thalamus								
optical density of immunostaining, arb. units	3 days	0.152±0.003	0.155±0.004	0.143±0.010	0.151±0.004	0.156±0.004	0.161±0.004	0.151±0.005
	2 weeks		0.151±0.005	0.155±0.007	0.152±0.006	0.165±0.010	0.157±0.006	0.149±0.005
volume density of neurons, %	3 days	3.03±0.09	3.01±0.35	3.21±0.43	2.68±0.48	3.13±0.44	3.11±0.38	3.15±0.16
	2 weeks		2.92±0.46	2.90±0.37	3.81±0.54	5.37±0.83+x	4.09±0.40	3.01±0.21
linear density of neurons, m/mm^2	3 days	7465±241	7520±591	7678±590	7700±948	8013±750	7841±669	8299±588
	2 weeks		7501±939	7023±714	8221±751	9033±552	8927±419	8160±453
number of vascular sections per mm^2	3 days	761±23	814±41	785±20	763±38	808±43	728±49	742±79
	2 weeks		750±83	760±22	677±25	736±30	713±19	757±43

Note. The treatment and control groups consist of 4-5 and 7 specimens, respectively.

TABLE 4. Quantitative Characteristics of Cell Proliferation (PCNA) in Zones of Neurogenesis in Rats with CCT (*M*±*m*)

Brain region, parameter	Period	Control	Trauma					
			without therapy	+ST	+MSC	+ST+MSC	+ST+fetal MSC	+ST+fetal NSC
Subventricular zone of the lateral ventricles, rostral part of the brain								
area of the proliferative zone, mm^2	3 days	0.092±0.003	0.093±0.003	0.096±0.004	0.097±0.003	0.097±0.003	0.095±0.003	0.095±0.002
	2 weeks	0.093±0.004	0.102±0.004	0.104±0.009	0.108±0.011	0.106±0.008	0.106±0.008	0.103±0.010
density of PCNA-positive cell nuclei in the proliferative zone, %	3 days	17.8±1.1	20.4±0.6*	19.7±1.3	20.0±1.2	21.7±2.1	22.9±0.8^{+}	23.7±1.7
	2 weeks	15.7±1.2	17.5±1.4	17.2±1.6	22.3±1.6	22.3±1.6	24.1±2.1^{+x}	20.7±2.8
Hippocampal dentate gyrus (formation area)								
number of PCNA-positive cell nuclei per mm^2	3 days	42±5	68±9*	69±6	48±7	68±5	76±3	71±4
	2 weeks	43±4	72±8*	78±7	72±7	82±9	66±6	71±6
relative content of PCNA-positive nuclei in the subgranular layer of HDG, %	3 days	38.1	58.8*	69.6	72.9	61.8	61.8	69.0
	2 weeks	39.5	55.6*	62.8	56.9	62.1	57.6	67.6

quantitative study showed that the total number of neurons in the pyramidal layer of the cortex, numerical density of neurons, and size of thalamic neurons in group 3 rats were much higher than in group 1 animals. The neuronal cytoplasm was filled with basophilic Nissl substance (Fig. 1, *c*). A histological study of samples revealed a considerable number of neurotrophin-3-producing neurons in the cerebral cortex (Fig. 4, *c*). The expression of neurotrophin-3 was increased in diencephalon nuclei.

The content of Nissl substance in cortical neurons (Fig. 1, *d*) and thalamic nuclei of group 4-6 rats receiving combination therapy was shown to increase on day 3 after trauma. After administration of fetal NSC, the volume density and size of neurons in these rats were greater than in animals of group 1 and standard-therapy group. The recover of nerve cell morphology was accompanied by an increase in the number of VEGF-overexpressing cells in diencephalon nuclei (Fig. 3, *d*). Neurotrophin-3 expression increased in the sensorimotor cortex (Fig. 4, *d*). Combination therapy with standard drugs and adult MSC had a rapid normalizing action on the number of functioning vessels in damaged cortical regions. The degree of vascularization in the cortex and thalamus of group 4 animals reached maximum after 2 weeks (Fig. 2, *d*).

Proliferative activity of the microglia and vascular endothelium in subventricular zones and sites of reparative regeneration and restoration of the capillary network significantly increased after combination therapy (particularly in animals receiving fetal and adult MSC). The number of labeled cells increased 1-2 days after prolonged treatment with BrdU. These cells migrated from the subventricular space (Fig. 5, *g*) and were accumulated in the regenerative zone of the cortex (Fig. 5, *h*), *corpus callosum*, and thalamus. On day 3 after trauma the density of PCNA-positive cell nuclei in subventricular zones of animals from groups 4, 5, and 6 was 21.7, 22.9, and 23.7%, respectively. Two weeks after trauma these parameters were 22.3, 24.1, and 20.7%,

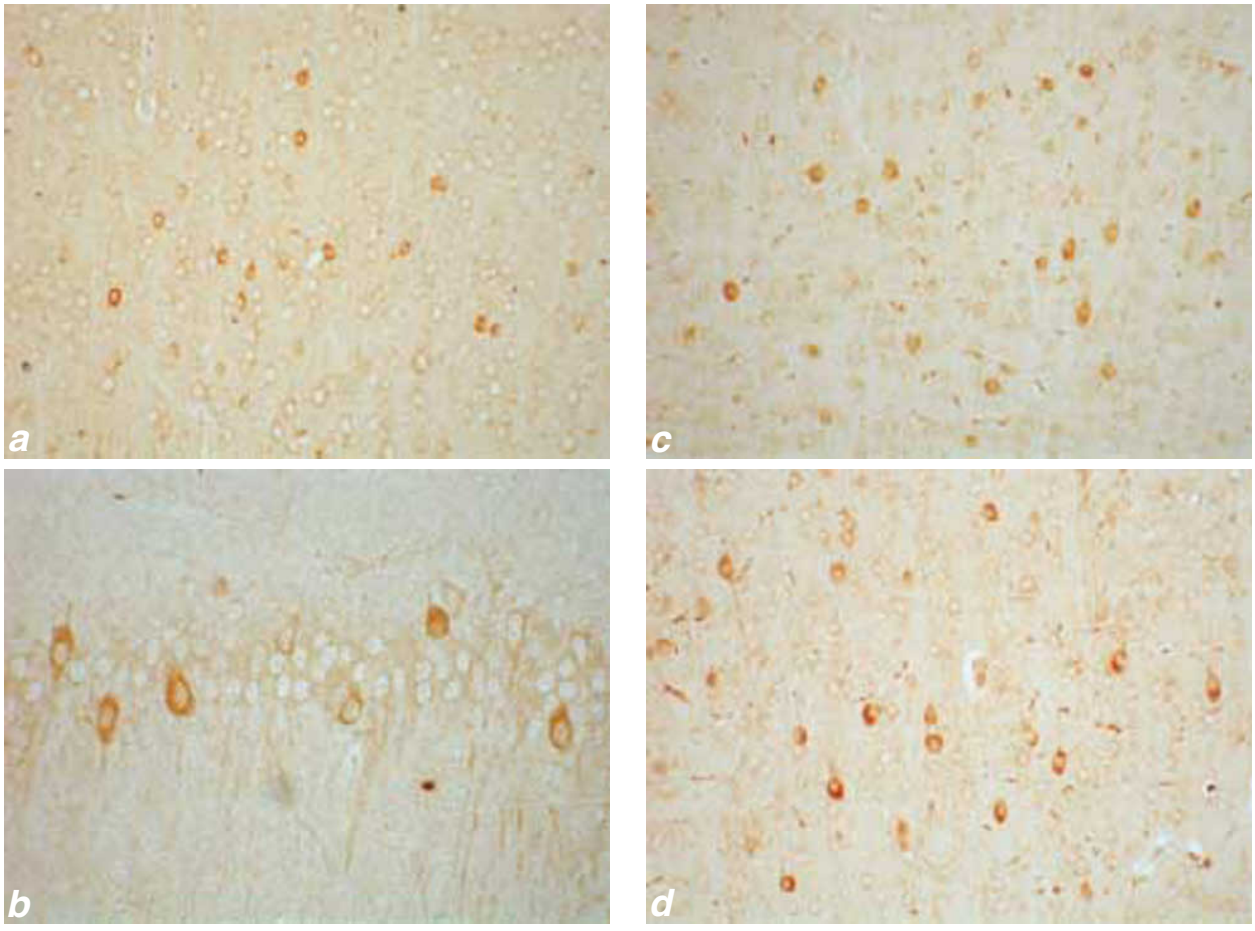

Fig. 4. Neurotrophin-3-producing cells in the polymorphic layer of the sensorimotor cortex (*a*, *c*, *d*) and pyramidal layer of the hippocampus (*b*) during CCT. Control (*a*); day 3 after trauma (*b*); day 14 after trauma and administration of adult MSC (*c*); day 3 after standard therapy and administration of fetal MSC (*d*). Method of the biotin-streptavidin peroxidase complex, diaminobenzidine. Magnification: 140 (*a*, *c*, *d*); 280 (*b*).

respectively. The area of proliferation tended to increase under these conditions. During this period, the fraction of proliferating cells in group 5 rats was much greater than in untreated animals. After 2 weeks the number of proliferating cells in these rats was higher than in untreated specimens and group 3 animals (metabolic maintenance). However, a comparative quantitative analysis showed that the

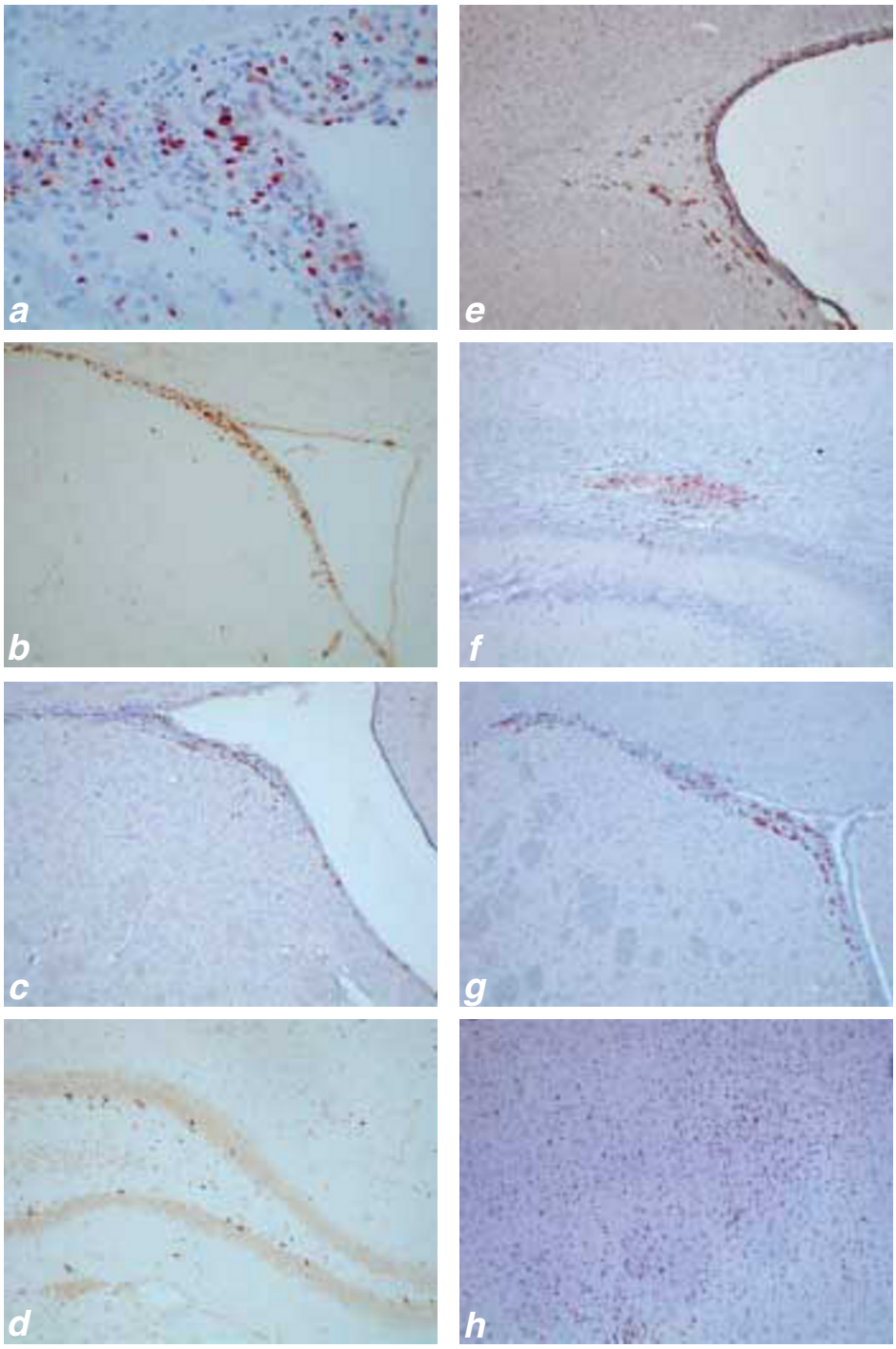

Fig. 5. Proliferative activity of rat cells: subventricular zone of the rostral part of the lateral ventricles (*a-c*, *e*, *g*) under control conditions (*a-c*), on day 1 after trauma (*e*), and 2 weeks after standard therapy and administration of fetal MSC (*g*); hippocampal dentate gyrus, 3 days after trauma (*d*); site of injury in the *corpus callosum* (day 3, *f*) and sensorimotor cortex (after 2 weeks, *h*), standard therapy and administration of fetal MSC. Sagittal sections (*a*, *e*); frontal sections (*b-d*, *f-h*). Immunohistochemical reaction of cell nuclei with anti-PCNA antibodies (*a*, *b*, *d*) and anti-BrdU antibodies (*c*, *e-h*): 1 h (*c*, *e*), 1 day (*f*, *g*), and 2 days after administration of the label (*h*). Method of the biotin-streptavidin peroxidase complex: diaminobenzidine (*b-e*); aminoethylcarbazole (*a*, *f-h*). Magnification: 280 (*a*); 70 (*b-h*).

intensity of cell proliferation in the dentate gyrus of group 4-6 rats is similar to that in untreated animals. Stimulation of neurogenesis in the dentate gyrus during diffuse brain injury is probably determined by the severity of hippocampal damage, but not by therapeutic treatment (*e.g.*, metabolic maintenance and administration of human SC).

Our results indicate that standard drugs contribute to the recovery of the capillary bed and stimulate reparative regeneration. A therapeutic effect of adult MSC on the morphology of neurons was evaluated 2 weeks after transplantation in the absence of metabolic maintenance. Combination therapy with human SC of different tissue origin was followed not only by the increase in reparative processes, but also by the activation of neurogenesis. During the early period after trauma, administration of fetal NSC accelerated the recovery of morphological characteristics in ischemic neurons. Two weeks after trauma the effect of NSC was smaller than that of MSC. A quantitative analysis revealed that fetal MSC have a prolonged effect on reparative processes and activation of endogenous precursor cells in the subventricular zone of neurogenesis. By contrast, adult MSC were more potent in improving the vascular bed in ischemic regions. Small differences were observed in the effect of various SC. These cells had a similar effect, which was accompanied by the increased expression of VEGF and neurotrophin-3.

Published data show that neurotrophic and growth factors play a particular role and constitute the niche-forming environment for SC. They have an important role in the induction of differentiation and growth of neurons, maintenance of survival and stability of nerve cells, and activation of reparative processes in nervous tissue during postnatal development and disorders of the central nervous system [2]. The activity of neurotrophin-3 is similar to that of other compounds from the family of neurotrophins. For example, neurotrophin-3 promotes the development and viability of neurons. Previous experiments revealed that neurotrophin-3 is involved in the differentiation of hippocampal progenitor cells and formation of spatial memory [30]. VEGF is a secreted mitogen, which accelerates the proliferation of endothelium and growth of vessels. Moreover, VEGF increases vascular permeability. Recent studies showed that VEGF has neurotrophic activity and stimulates neurogenesis. A correlation exists between VEGF function, growth of vessels, and neurogenesis. These processes protect the brain after injury [14]. Our studies demonstrated that systemic treatment with human SC during regeneration of nervous tissue not only activates the proliferation of progenitor cells in the lateral ventricles, but also stimulates the migration of BrdU-labeled cells into the site of reparative regeneration. The mechanisms of these effects remain unclear. It may be suggested that this process involves neurotrophic and growth factors.

SC hold much promise for the therapy of neurodegenerative diseases, stroke, and CCT. Previous experiments showed that various types of SC, including bone marrow MSC [3,6,21-23], umbilical blood SC [27,32,35], and NSC [8,9,11,15] (*e.g.*, immortalized [10] and genetically modified lines of human NSC [18]), can induce the expression of growth and trophic factors, increase the proliferative activity, and contribute to functional recovery of the brain.

However, little is known about the mechanisms for action of cell therapy. Other problems are associated with a selection of the optimal cell source, adequacy of the treatment procedure, and possibility for functional integration of transplanted cells in the recipient nervous tissue. Ischemic brain injury involves not only cells of various neural phenotypes, but also the vascular endothelium. For replacement therapy, SC should be characterized by high proliferative activity and phenotypic plasticity to differentiate into nervous, glial, and endothelial cells (depending on the corresponding ectopic site) [29]. Several types of cells are of limited use as a potential source for implantation. The recovery of complex relationships between neurons, glia, and endothelium requires a wide range of precursors, but not only phenotypically committed cells. Much attention was paid to differentiation activity of NSC and MSC. It was shown that cultured NSC can spontaneously differentiate into neurons, oligodendrocytes, and astrocytes. Moreover, native and implanted MSC can express neural markers [20-23,34,37]. However, little is known about the neural pathway of MSC differentiation. A comparative study was performed to evaluate the differentiation of human NSC and MSC in the tissue culture and after intracerebral transplantation to rats [1]. NSC differentiate into all types of neural cells, migrate, and have reciprocal relationships with the recipient brain. However, MSC do not differentiate into neurons under conditions of cultivation or transplantation.

A large body of evidence indicates that intravenous (systemic) or intracerebral injection of MSC and NSC is mainly followed by cell migration into the site of experimental brain injury [10,11,16-18, 20-23]. The microenvironment probably expresses chemotactic signals/factors that direct and contribute to the migration of circulating SC into the ischemic region [31]. After intravenous injection, only a small number of transplanted cells can enter

the site of injury in target tissues (including the recipient brain). These cells should survive, proliferate, differentiate, and become integrated into nervous tissue [16]. There is no evidence that donor cells can be integrated into the existing neuronal chains or form new structures. The therapeutic activity of SC from various origins is probably related to the secretion of physiologically active substances that have the angiogenic, antiapoptotic, antioxidant, and mitogenic properties. Much attention is paid to the role of these compounds in stimulation of regenerative processes [2,3,6,9,16,18,21,22,25,27,31, 32]. Published data [29] suggest that the recovery is not assisted with cell differentiation into neurons or integration of the implant in damaged zones of nervous tissue. This process is related to the release of trophic factors, which activates the endogenous mechanism of reparation, prevents cell death, and stimulates angiogenesis and neurogenesis. The efficacy of cell therapy in reparation of nervous tissue and improvement of neurological symptoms is determined by a variety of factors, including the time of transplantation, state of the vascular network in damaged regions, and expression of signal molecules for SC homing. For example, during the acute period of traumatic injury the release of excitotoxic transmitters, free radicals, and antiinflammatory compounds may be hazardous for survival of transplanted cells in the site of damage [19]. Inflammation is followed by activation of the microglia, which can inhibit endogenous neurogenesis and suppresses the growth and survival of transplanted cells [12]. However, local reparation and release of neurotrophic factors during the early period contributes to survival, differentiation, and integration of the implant.

Our findings indicate that systemic treatment with xenogeneic human SC of different age groups and tissue origin has a positive therapeutic effect during diffuse brain injury under conditions of metabolic maintenance. Clinical trials should be performed to evaluate the efficacy of autologous/allogeneic SC in combination therapy for patients with CCT. This treatment will improve the trophic state of nervous tissue and activate endogenous neurogenesis and reparation. These processes are of considerable importance for the recovery of functional morphology in damaged neurons.

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