Effect of Cell Therapy on Recovery of Cognitive Functions in Rats during the Delayed Period after Brain Injury

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We studied the effect of systemic transplantation of human stem cells from various tissues on cognitive functions of the brain in rats during the delayed period after experimental brain injury. Stem cells were shown to increase the efficacy of medical treatment with metabolic and symptomatic drugs for recovery of cognitive functions. They accelerated the formation of the conditioned defense response. Fetal neural stem cells had a stronger effect on some parameters of cognitive function 2 months after brain injury. The efficacy of bone marrow mesenchymal stem cells from adult humans or fetuses was higher 3 months after brain injury.

Key Words: *brain injury; mesenchymal stem cells; neural stem cells; brain cognitive functions*

Severe brain injury is accompanied by inflammation and necrotic or apoptotic death of cells in the central nervous system, which results in the impairment of interneuronal communication and cognitive dysfunction. The central nervous system, as a dynamic and plastic structure, is capable of self-restoration. However, the existing neural stem cells (NSC) of the brain do not produce a sufficient number of new functional neurons in response to injury. Moreover, highly differentiated neurons cannot regenerate [16]. The development of new methods for medical treatment of brain injury (BI) and cognitive recovery is an urgent problem. Studying the efficacy of pharmacological drugs and therapeutic procedures in BI over the last 30 years shows that there are no highly-efficient methods for the therapy of brain injury [17].

Much recent attention is paid to the use of stem cells (SC) for reparative treatment. Published data show that cell therapy with MSC have a positive effect on the recovery of neurological function during local brain injury and experimental cerebral ischemia [6]. Our previous studies showed that intravenous injection of autologous MSC to rats on the next day after BI has a proliferotropic, angiogenic, and neurotrophic effect [12]. Nerve cell proliferation is initiated in the zone of neurogenesis. Fetal NSC (FNSC) [3,13,14] and bone marrow MSC exhibit therapeutic activity in relation to the nervous system [15,18]. Even heterologous MSC from adult humans can facilitate the recovery of cog-

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nitive functions in rats [11]. There are contradictory data on functional recovery of the nervous tissue after transplantation of SC from tissue cultures. The data on this problem were mainly obtained during the acute period of injury [8]. It should be emphasized that BI is a multicomponent disease. The therapeutic use of SC should be considered as a supplement to medical treatment with pharmaceutics, which affects the key stages of secondary neuronal damage and reduces the severity of hypoxia, cerebral edema, and intracranial hypertension. It is not appropriate to evaluate the efficacy of monotherapy with SC during BI. SC should be used in combination with standard drugs of symptomatic and metabolic maintenance.

Here we studied the effect of systemic transfusion of various SC in combination with pathogenetic pharmacotherapy on the recovery of cognitive functions in rats during the delayed period after BI.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats obtained from the Stolbovaya nursery (Russian Academy of Medical Sciences) and weighing 115-130 g. The animals were maintained under standard conditions and natural light/dark cycle, had free access to water, and received pelleted food.

The study was conducted with adult human MSC, fetal MSC (FMSC), and FNSC. The primary culture of adult bone marrow MSC and human FMSC and FNSC (9-10 weeks gestation, medical abortion product) were obtained as described elsewhere [1,10].

Cognitive functions of control rats and BI animals were estimated from the acquisition of the conditioned avoidance response (CAR) in the shuttle box [1,3]. Before trauma, the animals were selected by learning capacity and divided into comparable groups. All rats were tested twice for the acquisition of CAR (50 combinations at 3-day intervals). After test 1, approximately 20% animals with poor learning ability or inadequate avoidance of punishment in the shuttle box were excluded from further experiments. The results of test 2 served as the learning control to compare cognitive functions in the delayed period after BI and SC therapy. The evaluation was performed after 60 days (test 3) and 3 months (tests 4, 5, and 6 on days 90, 93, and 100, respectively).

BI was produced by a weight, which dropped through the tube (diameter 12 mm) from a height of 110 cm. The area of injury was 0.2 cm^2. The mass of a weight was different and depended on animal body weight. The stroke was applied to the frontoparietal region. Pathomorphological study of the brain in some animals was performed to evaluate the location and severity of nervous tissue injury at the site of stroke. Approximately 50% rats died over the first 5 min after trauma. Survived animals were divided into groups. These groups were comparable in terms of the following two criteria: (1) learning ability (according to the results of pre-testing); and (2) severity of brain injury (estimated from clinical symptoms and special tests over 30 min after trauma) [2].

After pre-testing in the shuttle box, all animals had a group of "biological control" with the same learning ability. The rats were divided into the following six groups of 17 specimens each: group 1, untreated animals; group 2, BI animals; group 3, BI animals receiving standard therapy; group 4, treatment with standard drugs and adult human MSC; group 5, BI animals receiving standard therapy and FMSC; and group 6, BI animals receiving standard therapy and FNSC.

Each rat in the group had an individual identification number and specific rank in the corresponding series (ranking by the number of conditioned reactions in test 2). This approach allows us to maintain the equivalence of experimental groups during the study of at least 3 months duration (even when some animals were excluded from observations due to death, disease, *etc*.). The groups were equalized by substitution of a withdrawn animal for a specimen with the same learning ability (reserve group) or exclusion of rats of the same rank from all groups.

BI rats were divided into groups 3, 4, 5, and 6 to receive standard therapy. They were treated with antioxidant, antihypoxic, nootropic, and anxiolytic agents on day 1 and over the next 2 days after trauma (symptomatic and metabolic maintenance, or standard therapy).

Standard therapy included Actovegin (5.0 mg/kg, twice daily, 4 injections), mexidol (5.0 mg/kg, twice daily, 4 injections), cavinton (5.0 mg/kg, once daily, 1 injection), and piracetam (200 mg/kg, once daily, 1 injection). The study was performed with solutions of cavinton and piracetam in ampoules. Actovegin and mexidol were dissolved in sterile 0.9% NaCl (8- and 10-fold, respectively). The drugs were injected intraperitoneally in a dose of 0.1 ml per 100 g body weight.

Besides symptomatic and metabolic therapy, the animals of groups 4, 5, and 6 received a single intravenous injection of MSC, FMSC, and FNSC, respectively, on day 1 after BI. Human SC (2×10^6 cells) were dissolved in 0.5 ml physiological saline. Neurospheres were obtained during culturing of neural stem/progenitor cells from the periventricular area of human fetal brain and dissociated by mechanical disintegration of colonies. Proliferative activity of SC suspension was estimated by staining of acetone-fixed smears for proliferating cell nuclear antigen (PCNA).

The rats were decapitated under nembutal anesthesia. Pathomorphological study of the brain was performed 3 days, 14 days, and 3 months after trauma.

The brain was immersed in 10% neutral formalin, 70% ethanol, or acid alcohol for 24 h. Two sagittal segments or four frontal segments were cut off from the rostral part of the lateral ventricles, hippocampus, midbrain, and cerebellum. Tissue samples were dehydrated and embedded in Paraplast Plus (Kendall). For histological study, microtome sections were stained with hematoxylin and eosin. The sections were fixed in ethanol and acid alcohol and stained with cresyl violet and thionin (method of Nissl). Histotopographic mapping of the damaged zone and accurate determination of the level of coronary sections were performed using the stereotaxic atlas of rat brain.

CAR was elicited by the intermittent delivery of a conditioned signal (light+sound) and weak (1.0 mA) electrocutaneous pain stimulation (through an electric floor of the shuttle box). The shuttle box was a dark chamber with an electric floor. This chamber was divided by a partition wall into 2 compartments. A hole was made in the wall. The rat could avoid punishment by going to a safe compartment of the chamber during conditioned stimulation. The scheme of signal delivery was set by an automatic device. The latency of running was recorded with an accuracy of up to 1 sec. Each session of the test included 50 presentations of the signals in the following sequence: light+sound, 4 sec; nociceptive electrical stimulation, 4-12 sec; between-cycle period, 20 sec. The criteria of learning in this test were proposed and described previously [5].

The results were analyzed by multivariate statistical analysis. The quantitative data (*e.g.*, 50% learning rate) were compared by Student's *t* test. Statistical treatment included the Fisher exact test, Mann—Whitney *U* test, and median test (c^2). The response latency was gradually stabilized during CAR acquisition. The absolute latency of avoidance and running was shown to approach the time limit of CAR. For quantitative analysis of the adaptive behavior, the dispersion of the true latency in various periods was evaluated by two-factor analysis of variance (Fisher's *F* test). The tendency toward variations in the latency of reactions was estimated by the median test (χ^2).

RESULTS

Pathomorphological changes in the brain of rats during the early period after BI were similar to those observed previously on the same model of trauma [7,12]. BI was followed by diffuse injury to brain tissues in the parietal cortex, thalamus, hippocampus, and brainstem. This conclusion was derived from the presence of diffuse and local edema, swelling of the neuropil, bundles of ischemic and contracted neurons with pericellular edema in the parietal cortex and thalamus, local death of cells (necrosis), and petechial hemorrhages between the cortex and *corpus callosum*. Nerve cells of modified shape and color were found in some fields of the hippocampal pyramidal layers. The degree of staining with basophilic Nissl substance became less pronounced after trauma. This parameter reflects the total content of ribonucleoproteins in the neuronal cytoplasm. Empty vessels and paretically widened or collapsed profiles of the vascular section were seen in the sensorimotor cortex. The signs for delayed injury and degeneration of hippocampal neurons were revealed after 3 months. These changes suggest the development of a chronic traumatic state in animals.

The method of CAR acquisition allows us to evaluate cognitive function from the number of CAR (over one session of training), some integral parameters, and speed indexes. The ability for skill reproduction, *i.e.*, short-term memory (series of CAR) and long-term memory (repeated sessions for consolidation of experience, CE), could be estimated by visual examination of the behavioral reactions, overall excitability, and psychic state of the animals. Preliminary testing of the experimental animals was conducted to compare the group indexes at a certain stage after BI. This approach allowed us to compare the dynamics of cognitive recovery in animals of various groups (relative to the baseline). The distribution of animals in the group (ranking by learning ability before BI) allowed us to perform the differential analysis for an increase in learning capacity compared to the baseline. This analysis was performed for each rat and the whole group of animals. The use of differential analysis for evaluation of intergroup differences at a certain stage (relative to the baseline in control and treated specimens) excludes error due to intragroup variability [4].

The psychophysiological state was evaluated from CAR acquisition (Tables 1 and 2). Two and three months after BI, cognitive functions of rats not receiving therapy and SC were significantly reduced compared to the biological (sham-operated) control. For example, BI animals were characterized by a longer lag phase (many combinations before the first CAR); greater number of animals exhibited no running reaction; most animals of this group demonstrated shorter duration of several consecutive CAR. Otherwise, the series of such reactions were absent. These changes reflect impairment of attention and short-term memory. Two months after trauma, the number of rats with series of 5 CAR decreased by 2 times. However, the number of these animals increased slightly in the control group (Table 1). Impaired learning in treated specimens was confirmed by some changes in speed indexes, including the decrease in the B-regression coefficient for "success-failure" at various stages of study (Table 2) and increase in the number of attempts

to reach a 50% learning rate (Table 1). Significant intergroup differences were found in the final performance (*i.e.*, number of CAR over one session of 50 attempts). Learning capacity in BI animals decreased by 17.4 and 6.6% after 2 (test 3) and 3 months (test 4), respectively. By contrast, this parameter in control rats increased by 8.4% after 3 months (test 4). The most significant differences were revealed between groups 2 and 1 with poor learning capacity (due to a long interval between test sessions). These differences became less pronounced on day 100 after BI (consolidation of experience).

No differences in the integral criteria for learning were found between animals of groups 3 and 2. The regression coefficient calculated for estimated speed indexes showed that standard therapy slightly increased the rate of learning (by 22%) in the delayed period after BI (2nd and 3rd months). Combined treat-

TABLE 1. Integral Criteria for CAR Acquisition in Rats 2 and 3 Months after BI (Various Therapeutic Regimens)

Period	Group	Lag phase	Total number of CAR	Refusals	Number of rats with a series of 5 CAR
Baseline (test 2)	BC	5.9±2.1	27.3±3.3	2.7±1.0	9
	BI	5.7±1.4	27.9±3.2	2.7±1.2	12
	ST	5.1±1.5	27.6±3.2	1.5±0.6	11
	MSC	5.9±2.2	27.9±2.9	2.1±0.7	13
	FMSC	6.8±1.8	27.5±3.1	1.7±1.1	11
	FNSC	4.8±1.3	27.9±3.2	3.3±0.9	9
60 days (test 3)	BC	6.5±3.3	25.3±2.9	2.3±0.8	10
	BI	8.2±2.7^{x}	19.2±3.5*	2.0±0.8	6*
	ST	8.1±3.1	21.8±2.8	2.5±0.9	7
	MSC	9.4±2.6	18.7±3.1	3.9±1.1	7
	FMSC	2.4±1.0*^{o}	25.0±2.7	1.7±0.5	11
	FNSC	6.9±3.0	27.7±3.3^{o}	3.6±2.1	11
90 days (test 4)	BC	3.9±2.0	31.5±2.9	0.7±0.2	13
	BI	5.1±3.3^{x}	24.6±3.9*	3.1±1.4^{x}	11
	ST	4.2±2.0	31.9±3.4	3.0±1.9	12
	MSC	4.2±1.6	31.4±3.5	2.0±1.1^{+}	14
	FMSC	2.1±1.5	29.3±2.9	1.5±0.7^{+}	13
	FNSC	4.9±2.4	28.3±3.5	2.3±1.5	11
93 days (test 5)	BC	3.1±0.7	33.0±2.0	0.7±0.4	14
	BI	2.3±0.6	28.3±3.5	4.3±2.1^{x}	12
	ST	6.0±1.4	27.9±2.8	3.4±1.9	11
	MSC	2.7±1.1*	33.8±2.2*	0.9±0.4^{+}	14
	FMSK	2.1±0.6*	35.2±1.8*	2.3±1.1^{+}	15*
	FNSC	3.8±1.9	29.2±3.6	3.3±1.5^{+}	10
100 days (test 6)	BC	1.9±0.5	35.3±2.7	0.5±0.3	14
	BI	4.4±1.7^{x}	33.6±3.2	2.5±1.2^{x}	13
	ST	5.1±1.5	28.9±2.6	3.7±1.1	12
	NSC	1.3±0.5*	35.1±2.5	1.7±0.8*	14
	FMSC	2.2±1.0	39.2±1.6*	1.1±0.7^{+}	15
	FNSC	3.9±1.2	30.3±3.7	3.4±1.2^{+}	12

Note. $p \leq 0.05$: *compared to the baseline (Student's *t* test, differencial comparison); ^{o}compared to standard therapy (ST, Student's *t* test); ^{x}compared to the biological control (BC, *U* test); $^{+}$compared to ST (*U* test).

ment with SC and standard drugs was more effective in recovering the cognitive function after BI. The reproduction of CAR by FMSC-treated rats was accelerated by the 2nd month after BI. This conclusion was derived from a decrease in the lag phase. The relative rate of learning was estimated from the B-regression coefficient for the ratio of successful and unsuccessful attempts and increase in the number of CAR. These parameters in FNSC-treated animals were higher than in group 3 rats (BI+standard therapy; by 18 and 45%, respectively) and untreated specimens with brain injury (by 40 and 67%, respectively). After pre-testing, the degree of CE in FNSC-treated animals was higher than in rats of the standard therapy group. CE was evaluated as the ratio of the initial to optimal level of learning in tests 3 and 2, respectively. The degree of CE was estimated in animals of groups 5 and 6 (6 and 11%, respectively), group 3 (standard therapy, 5%), and group 2 (BI without treatment, 4%). We performed the regression analysis for the increase in the percentage of CAR during testing. This parameter at the beginning of testing was higher in group 6 rats. The α-coefficient in group 6 rats was 2-fold higher than in group 3 animals (29.3±3.7 and 12.4±3.5, respectively). No intergroup differences in the α-coefficient were found before trauma. The final effectiveness of learning in group 5 and 6 animals was improved at this stage of study. The number of CAR increased significantly in group 6 rats ($p \leq 0.05$) and tended to increase in group 5 animals. The number of rats with a series of CAR was shown to increase in groups 5 and 6. The duration of CAR was also increased in these animals ($p \leq 0.05$, χ^2 test). These changes reflect the improvement of short-term memory and reproducibility of successful reactions. At this stage of study, learning ability of group 4 animals (treatment with human bone marrow MSC) was slightly improved in comparison with group 2 rats. It was manifested in a slight increase in estimated speed indexes. Final performance did not change under these conditions (except for a decrease in the latency of CAR, $p \leq 0.05$). These data indicate that therapy with standard drugs and SC has

TABLE 2. Increase in the Level of Learning in Treated Rats 2 and 3 Months after BI (Decrease in the Ratio of Successful and Unsuccessful Attempts)

Group	Time after BI, days	Level of learning, success/failure ratio in 10 attempts		B-coefficient	CE, %	Relative rate of learning, %
		initial	optimal			
Biological control	60	0.97±0.58	6.83±1.70	0.133±0.008	16	100
	90	0.83±0.19	8.48±2.10	0.204±0.009	12	100
	93	0.93±0.27	8.70±2.20	0.270±0.020	12	100
BI	60	0.33±0.10	4.18±1.00	0.055±0.003*	4	41
	90	0.80±0.30	5.86±1.40	0.092±0.004*	19	45
	93	0.96±0.58	6.68±1.60	0.125±0.009*	15	46
Standard therapy	60	0.34±0.12	4.91±1.20	0.084±0.005^{+}	5	63
	90	1.66±0.66	7.9±1.9	0.138±0.009	34	68
	93	0.32±0.13	7.18±1.80	0.184±0.008^{+}	4	68
MSC	60	0.36±0.16	4.51±1.10	0.075±0.006^{+}	5	56
	90	0.79±0.28	8.49±2.10	0.192±0.020^{+x}	17	94
	93	0.84±0.28	8.69±2.20	0.439±0.060^{+x}	10	162
FMSC	60	0.40±0.10	5.29±1.30	0.108±0.004^{+x}	6	81
	90	0.84±0.26	7.67±1.90	0.177±0.016^{+x}	16	87
	93	0.87±0.29	9.53±2.40	0.581±0.040^{+x}	11	216
FNSC	60	0.68±0.22	7.38±1.80	0.143±0.002^{+x}	11	108
	90	1.95±0.79	6.22±1.50	0.073±0.008	26	36
	93	0.74±0.21	6.86±1.70	0.116±0.010	12	43

Note. $p \leq 0.05$: *compared to the biological control; $^{+}$compared to the BI group. $^{x}p \leq 0.001$ compared to animals receiving standard therapy (the same period of study).

a positive effect 2 months after trauma (particularly in group 6 rats).

Threefold testing (tests 4, 5, and 6) was performed 3 months after BI. A strong positive effect was observed in animals receiving adult human MSC and FMSC (Tables 1 and 2). Despite the absence of reinforcement over a long period of time (1 month) after 2-month testing, parameters of learning in test 4 reflects progressive formation and retention of conditioned reflex relations in all groups. At the same time, speed indexes for the increase in CAR were better in SC-treated animals (particularly of the MSC group). The relative rate of learning in rats receiving standard drugs was elevated by 21%. This parameter in animals of the MSC and FMSC groups was increased by 49 and 42%, respectively, and approached the control level. Some differences in the final performance were found between treated and untreated animals with BI. We revealed an increase in the number of CAR and improvement of a 50% learning rate. At this stage of study, the degree of CE was higher in animals receiving standard drugs and FNSC. A progressive increase in the level of learning was found in SC-treated animals during further testing. These rats differed from BI animals that received or did not receive standard drugs. A further increase in learning ability was not revealed in rats of the standard therapy group. However, SC were potent in preventing the consequences of BI. This conclusion was derived from studying the parameters for acquisition and retention of CAR. After test 5, the index of group learning (*i.e.*, number of CAR) in SC-treated animals of groups 4 (MSC), 5 (FMSC), and 6 (FNSC) increased to 67.6, 70.4, and 58.4%, respectively (*vs*. 55.8% in rats of the standard therapy group; Fig. 1). The animals of all groups were characterized by a decrease in the lag phase of learning, latency of conditioned avoidance reactions ($p \leq 0.001$), and module dispersion for this parameter (estimated by the median test). The rapid/slow CAR ratio was modified toward an increase in the number of rapid reactions. A 50% learning rate was increased by 2 times. These changes reflect a decrease in the number of attempts to reach the expected level of 50% learning (Fig. 2) and series of 5 consecutive CAR (learning criterion) in MSC-treated animals. The behavior of these rats was more stable. They exhibited lower rate of run refusal. Despite a rapid reaction in the test, these animals did not demonstrate the stressogenic responses. The rate of intersignal running in animals of this group was lower than in rats receiving standard therapy. These results illustrate a greater psychophysiological stability of MSC-treated animals. Similar results were obtained in comparing the groups at various stages of study. More-

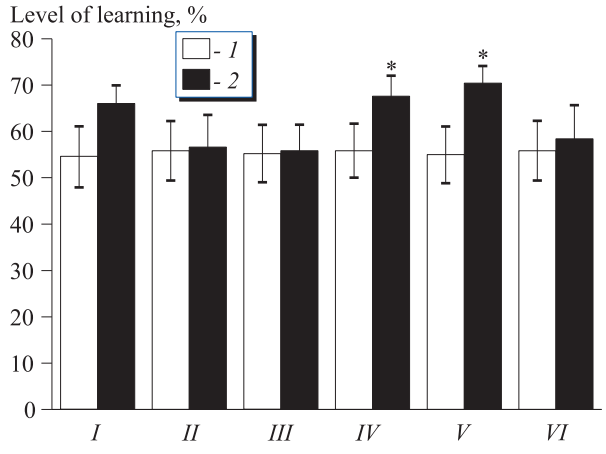

Fig. 1. Dynamics of rat learning 3 months (test 5) after BI and combination therapy with SC and standard therapy (ST). Biological control (BC, *I*); BI (*II*); BI+ST (*III*); BI+ST+MSC (*IV*); BI+ST+FMSC (*V*); BI+ST+FNSC (*VI*). Baseline (*1*); treatment (*2*). *p<0.5.

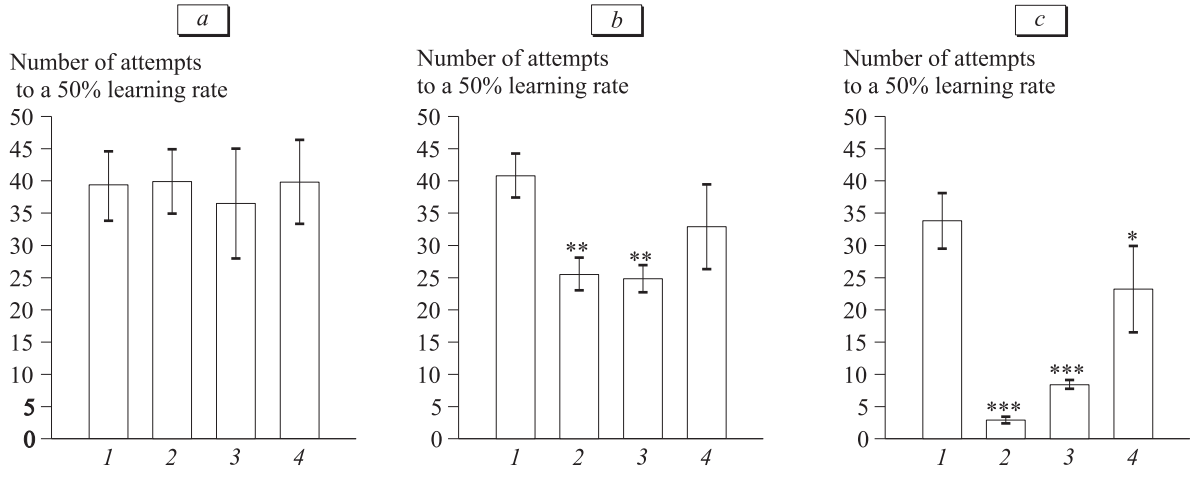

Fig. 2. Dynamics of a 50% learning rate in rats 3 months after BI. BI+ST (*1*); BI+ST+MSC (*2*); BI+ST+FMSC (*3*); BI+ST+FNSC (*4*). *p<0.5, **p<0.01, and ***p<0.001 compared to the baseline.

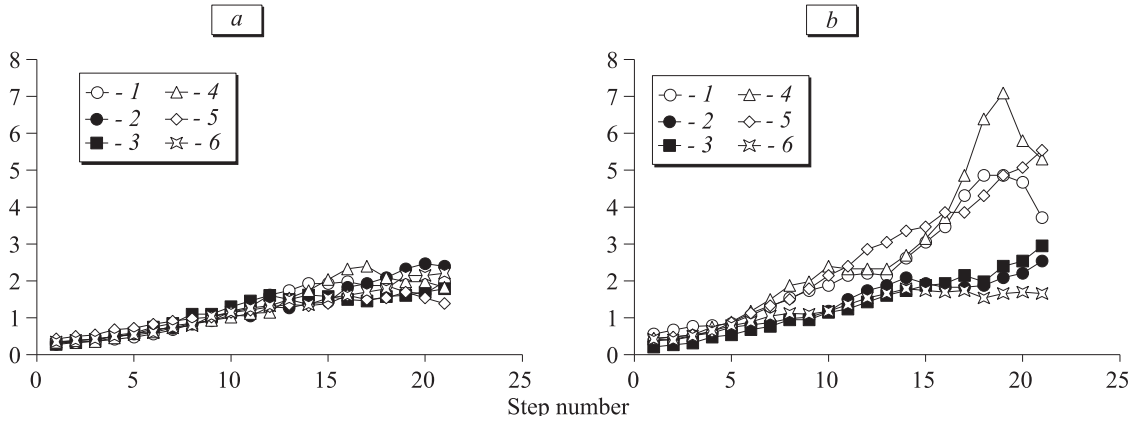

Fig. 3. Dynamics of the learning level. Ratio of successful and unsuccessful attempts in rats before (baseline-test 2, *a*) and 93 days after BI and therapy (test 5, *b*). BC (*1*); BI (*2*); BI+ST (*3*); BI+ST+MSC (*4*); BI+ST+FMSC (*5*); BI+ST+FNSC (*6*).

over, comparison of individual and group parameters (final stage of training, test 6) with the baseline level yielded the same results. Despite one-week interval after test 5, the differences in CAR number for animals of the standard therapy group and MSC group (relative to the baseline level) became more significant in this period. The degree of learning was highest in group 4 and 5 rats. The rate of conditioned avoidance reactions in these animals was 78.4 and 70.2%, respectively (*vs.* 57.8% in the standard therapy group). This parameter did not differ from the biological control.

The regression analysis was performed to evaluate the increase in the degree of learning (ratio of successful and unsuccessful attempts; Table 2). The calculation of the B-coefficient revealed a significant increase in the rate of learning in MSC-treated rats. The relative rate of learning in group 3 rats was 22% higher than in untreated animals with BI. The relative rate of learning in animals of the MSC and FMSC groups was elevated by 116 and 170%, respectively (100% in the control).

Changes in the ratio of successful and unsuccessful attempts in tests 2 and 5 (10 attempts with 2 attempt step) reflect accelerated learning in these groups (increase in CAR; Fig. 3). FNSC-treated rats exhibited no rapid increase in learning ability during testing. At this stage of the study, the final performance did not differ between FNSC-treated rats and control animals. However, the retention of memory of the previous test (CE) in these rats was better than in animals of other groups. Similar differences were found after 2 months. It was probably related to higher functional level of brain structures responsible for long-term memory.

We conclude that systemic transfusion of SC in combination with metabolic maintenance is more effective in recovering the cognitive function of the brain in rats during the delayed period after BI (compared to standard therapy). Adult and fetal MSC produced a stronger positive effect. FMSC-treated animals differed from other rats in the greater speed indexes. Studying the dynamics of conditioned response acquisition showed that these rats are characterized by a well-balanced and stable behavior in performing the test. The wave-like increase or decrease in the dynamics of conditioned response acquisition was typical of MSC-treated animals (Fig. 3). It was probably associated with the emotional component of behavior. Administration of FNSC did not improve cognitive function of the brain in rats during the delayed period after BI. However, FNSC had a positive effect on memory retention (CE). These data are consistent with the results of our previous experiments. We showed that therapeutic treatment with MSC, FMSC, and FNSC improves angiogenesis, increases the concentration of vascular endothelial growth factor, and prevents hypoxia in rats with BI [9]. Early recovery of cerebral vascularization in traumatized rats after systemic transplantation of SC probably improves cognitive function of the brain. SC of various origins have a modulatory effect on the recovery of brain cognitive function in rats with BI. These data indicate that SC hold much promise for the use in combination with reparative therapy.

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