Mesenchymal Cells in the Treatment of Focal Brain Injury Induced by Venous Circulation Disturbances in Rats

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Translated from *Kletochnye Tekhnologii v Biologii i Medicine*, No. 2, pp. 77-82, April, 2011
Original article submitted February 26, 2010

We studied the efficiency of bone marrow multipotent mesenchymal stem cells for correction of neurological deficit in rats with experimental sustained focal brain injury caused by venous outflow disturbances. It was found that neurological deficit in animals with transplantation of multipotent mesenchymal stromal cells decreased by 54-75% (*vs.* 14-17% in the control group) by day 21. The efficiency of mesenchymal stromal cell transplantation (intravenous or local) on day 1 of postoperation period was significantly higher than on day 7. This manifested in more pronounced decrease in the severity of neurological disorders (according to modified neurological severity score) and more rapid recovery of autonomic feeding. Moreover, transplantation of multipotent mesenchymal stromal cells on day 1 abolished the need in antiedematous therapy, while in animals receiving cell therapy on day 7, administration of glucocorticoids was necessary. Comparison of two regimens of cell administration at early terms revealed no advantages of local transplantation of multipotent mesenchymal stromal cells over intravenous injection.

Key Words: *focal brain injury; mesenchymal stromal cells; neurological deficit*

Brain damage caused by circulation disturbances is a serious medical and social problem associated with considerable economic loss, because it requires urgent hospitalization, leads to long-term disability, and occupies third place among mortality causes after tumor diseases and cardiovascular pathologies in adults [1]. The absence of effective methods of correction of brain injuries necessitates the search for new therapeutic approaches. In light of this, studies in the regenerative medicine oriented at application of different cells and factors stimulating reparation of the nervous tissue produced by them are of particular interest.

Multipotent mesenchymal stromal cells (MMSC) of the bone marrow (BM) attract special attention in this respect, because they can be easily generated in sufficient amounts in *in vitro* culture. Improvement of neurological deficit in rats with experimental local brain ischemia under the effect of MMSC transplantation was previously demonstrated [2,5,10]. Some hypotheses were put forward [8,13] that clinical improvement after MMSC transplantation can be determined by replacement effect, *i.e.* migration of these cells into damaged brain tissues and their subsequent neuronal differentiation. However, taking into account relatively rapid development of positive clinical effect and the fact that only solitary cells entering the damaged zone express neuronal markers, most authors explain the improvement of neurological deficit by production of a wide spectrum of growth and trophic factors activating regional neuronal precursors, stimulating angiogenesis

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and myelination, accelerating synaptic transmission, and suppressing inflammation and apoptosis of nerve cells in the inflammation focus [4,6,7,12].

Most studies analyzing the efficiency of MMSC in correction of neurological deficit were performed on rats or mice with experimental focal cerebral ischemia caused by occlusion of the middle cerebral artery. Under these conditions, transplantation of MMSC (intravenous, intracarotid, or intracerebral) led to considerable alleviation of neurological deficit in experimental animals [12]. However, this model reproduces moderate neurological deficit (modified neurological score <12) [5]. Moreover, neurological deficit is transient due to well-developed collateral circulation and the animals demonstrated relatively high level of spontaneous recovery.

Occlusion of the middle cerebral artery is a model simulating ischemic stroke in humans. At the same time, disturbances in venous flood flow, *e.g.* after surgical treatment of parasaggital meningiomas of the brain, can be the causes of ischemic injuries. The absence of the corresponding experimental models impedes the study of pathophysiological aspects of the development and correction of ischemic injuries caused by venous stasis. Therefore, the efficiency of cell therapy in correction of severe and stable neurological deficit caused by venous circulation disturbances is unknown.

Here we evaluated the efficiency of MMSC administered via different routes and at different terms in the treatment of focal brain injury caused by venous blood flow disturbances in rats.

MATERIALS AND METHODS

Experiments were carried out on male and female Wistar rats (mean body weight 220 g).

The animals were sacrificed under ether narcosis and suspension of BM cells was isolated from the femoral bones. The cells were washed from the femoral bones and suspended by passing through a 24 G needle. For obtaining MMSC, BM cells were incubated in Nunclon plastic flasks in DMEM (Sigma) containing 15% FCS (ICN) at 37°C and 5% CO_2. Nonadherent cells were removed after 24 h; adherent cells were washed with Hanks medium and cultured until 80-90% confluence. Mesenchymal cells were harvested with 0.25% trypsin and 0.02% EDTA. After 1-2 passages MMSC were used for transplantation to experimental animals.

Ischemic brain injury caused by disturbances in venous blood outflow in the temporoparietal area was modeled. To this end, resection trepanation in the right temporoparietal area with exposure of the middle third of the superior saggital sinus was performed. The dura matter was cut at ×5 magnification. For the formation of the damage focus, the veins flowing into the superior saggital sinus were coagulated at the area of 1 cm^2 and its complete obliteration was performed (diathermocoagulation) [3]. This procedure resulted in severe and sustained focal neurological deficit in 70% animals.

Forty animals with neurological deficit were divided into 6 groups (Table 1). In group 1 rats (n=8; control for groups 3 and 4), surgical intervention without cell therapy was performed and the animals were followed up over 21 days; group 2 rats (n=8; control for groups 5 and 6) underwent the same surgery followed by introduction of a silicon catheter into the zone of ischemic injury (proximal end of the catheter was fixed under the skin of the temporal area) and did not receive cell therapy. In groups 3 and 4 animals (n=6 each), the surgery was followed by single intravenous injection of MMSC (2×10^6 cells per animal) into the caudal vein on days 1 and 7, respectively. Groups 5 and 6 (n=6 each) comprised rats receiving MMSC (2×10^6 cells per animal) on days 1 and 7 after surgery, respectively, through a catheter implanted into the zone of ischemic injury in the brain. All animals received antibacterial therapy (Sol. Enroxili 5%, 0.05 mg intramuscularly, once a day) over 1 week after surgery. Experimental and control animals receiving MMSC transplantation on day 7 after surgery (groups 4 and 6) were treated with dexamethasone (0.05 mg/day intramuscularly, antiedematous therapy) for 7

TABLE 1. Characteristics of Experimental Groups

Group	n	Surgery	Antiedematous therapy	Cell therapy	Term of transplantation, days	Administration route
1	8	+		days 1-7	-	
2	8	+	days 1-7	-		
3	6	+	once, during surgery	+	day 1	Intravenous
4	6	+	days 1-7	+	day 7	Intravenous
5	6	+	once, during surgery	+	day 1	Local
6	6	+	days 1-7	+	day 7	Local

days. In rats receiving cell therapy on day 1, dexamethasone was injected once during surgery. Until resumption of autonomic feeding the animals received baby food (Nutrikomp or Frezubim) through a pipette. If artificial feeding was impossible, the animals received intramuscular injections of physiological saline in a dose of 3-4 ml/day.

General status of animals was analyzed using Neurological severity score (NSS) [7] in our modification. NSS is an integral score in four tests assessing motor activity, sensitivity, balance, and the absence of normal reflexes and pathological movements. The maximum neurological deficit by NSS corresponds to 18. Modification consisted in two additional tests characterizing muscular tone and ability of autonomic feeding (Table 2); the maximum neurological deficit according to modified NSS was 26.

The control animals were tested on days 1, 7, and 14 and then decapitated. The severity of neurological deficit in the experimental groups (3-6) was analyzed on days 1, 7, 14, and 21. Apart from NSS, the decrease in neurological deficit severity (in %) was determined by the formula:

[1- (NSS on days 7, 14, or 21/NSS on day 1)]×100%.

The data were processed statistically by nonparametric tests for dependent (P_{ST}, sign test) and independent variables (P_U, Mann–Whitney test) using Statistica 6.0 software.

RESULTS

Simulation of venous stasis in the temporoparietal area was accompanied by pronounced brain edema and development of severe neurological disturbances during the first 24 h after surgery. Fifteen of 55 operated animals died within the first 3 days, which attested to the severity of induced injuries. NSS scores in survivors of groups 1 and 2 were 22.20±0.13 and 22.50±0.18, which confirmed severe brain injuries. Antiedematous therapy and artificial feeding were required during 1 week after surgery. In group 1 animals (control), the severity of neurological deficit by days 7 and 14 significantly decreased by 9±1 (P_{ST}=0.013) and 17±1% (P_{ST}=0.013), respectively, but still remained high even after 2 weeks (mean NSS was 18.4±0.43). Group 2 animals (subcutaneous catheter in projection of the ischemic focus) also demonstrated partial spontaneous recovery. Neurological disturbances in this group by days 7 and 14 decreased by 9±1 (P_{ST}=0.013) and 14.0±1.5% (P_{ST}=0.013), respectively; neurological status by the end of week 2 recovered less markedly. The difference in NSS between groups 1 and 2 on day 14 was insignificant (P_U=0.066), but manifested as a clear-cut tendency. This suggests that insertion of a catheter can impede partial spontaneous recovery and confirms the need in the formation of a special control group for the analysis of the efficiency of cell transplantation into the ischemic focus.

In animals of groups 3 and 4 (intravenous injection of MMSC), the severity of neurological disturbances on day 1 after surgery was 21.50±0.38 and 22.30±0.56, respectively, which was comparable with the mean NSS in group 1 (Fig. 1, *a*). Recovery of the neurological status in these groups was more pronounced. For instance, in group 3 (MMSC transplantation on day 1 after surgery), neurological deficit by days 7 and 14 decreased by 15±2 (*vs.* 9±1% in the control, P_U=0.028) and 44.0±3.2% (*vs.* 17±1% in the control, P_U=0.002), respectively. By the end of the 3rd week, the severity of neurological disorders decreased by 75±5% (P_U=0.028) and animal status corresponded to mild neurological disturbances (NSS=5.5±1.1).

In group 4 (MMSC injection on day 7) NSS also significantly decreased by day 14 and was comparable to that in group 3. The positive dynamics was observed up to day 21, but NSS in group 4 almost 2-fold surpassed that in group 3 (9.1±0.8 *vs.* 5.5±1.1; P_U=0.033). The decrease in neurological deficit by the end of the 3rd week was also less pronounced than in group 3 (by 60.0±4.3% compared to 75±5%; P_U<0.05). More successful recovery in group 3 compared to group 4 manifested in more rapid resumption of autonomic feeding over the first 7 days of observation in animals receiving MMSC on day 1.

Analysis of neurological status in groups with local transplantation of MMSC into the damaged area showed that the mean NSS values in experimental groups 5 and 6 and in control group 2 were similar on day 1 after surgery (Fig. 1, *b*). In group 5 animals, neurological disturbances by the end of the first and second weeks decreased by 15±3 and 34±5%, respectively (*vs*. 9±1 and 14.0±1.5% in the control), while by day 21 this decrease attained 68%. In group 6 animals, NSS on days 7 and 14 were similar to those in group 5, while on day 21 this decrease was less pronounced (54.0±3.9% *vs*. 68±6% in group 5; P_U=0.04). Similarly to groups with intravenous injection of MMSC, autonomic feeding in group 5 animals recovered earlier than in group 6.

Comparison of the two routes of cell administration on days 1 and 7 revealed no significant differences in the dynamics of neurological status improvement, though a tendency to more pronounced correction of neurological disturbances was noted after intravenous injection of MMSC.

Our experiments showed that MMSC can correct neurological disorders in rats with severe neurological deficit on the model of focal brain damage caused by

TABLE 2. Modified Neurological Severity Score

Tests	Score	Maximum score
Motor tests		
raising rat by the tail		3
forelimb flexion	1	
hindlimb flexion	1	
head turn >10° over 30 sec	1	
placing rat on the floor		3
normal walk	0	
inability to walk straight	1	
circling towards the paretic side	2	
falling down to the paretic side	3	
Sensory tests		2
placing test (visual and tactile tests)	1	
proprioceptive test (deep sensation, pushing the paw towards the table edge to stimulate limb muscles)	2	
Beam balance test		6
balances with steady posture	0	
grasps sides of the beam	1	
hugs the beam and one limb falls down from the beam	2	
hugs the beam and two limb falls down from the beam or spins on the beam (>60 sec)	3	
attempts to balance on the beam, but falls off (>40 sec)	4	
attempts to balance on the beam, but falls off (>20 sec)	5	
falls off: no attempt to balance or hang the beam	6	
Reflexes absent and abnormal movements		4
pinna reflex (head shake when touching the auditory meatus)	1	
corneal reflex (eye blink when lightly touching the cornea)	1	
startle reflex (motor response to a brief noise)	1	
seizures, myoclonus, myodystony	1	
Tone of limbs and tail		2
normal	0	
high	1	
low (flaccid)	2	
Feeding		6
autonomic (all kinds of food)	0	
autonomic (liquid food)	2	
forced (through a pipette)	3	
food refusal	6	
Maximum score		26

Note. 20-26: severe disturbances; 10-19 moderate disturbances; 1-9 minor disturbances.

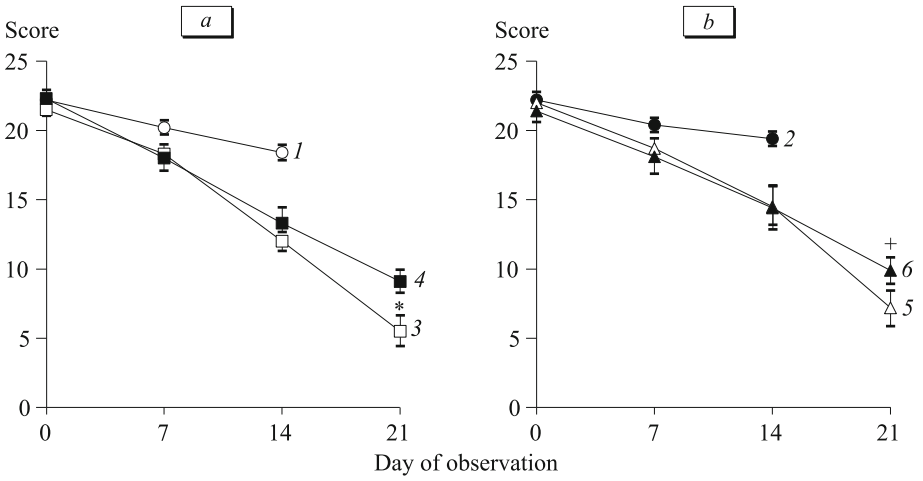

Fig. 1. Effect of intravenous (*a*) and local (*b*) administration of MMSC on the severity of neurological disturbances in rats with experimental focal brain damage caused by venous stasis. P_U<0.05 compared to: *group 4, +group 5.

venous stasis characterized by low level of spontaneous recovery. Thus, neurological deficit in animals receiving cell therapy decreased by 54-75%, while in the control groups, spontaneous recovery was 14-17%. By day 21, the neurological status of experimental animals corresponded to mild neurological disorders. Analysis of the efficiency of MMSC transplantation at different terms after damage showed that the effect of cell therapy injected on day 1 of postoperation period (intravenously or locally) was significantly higher than after cell injection on day 7. This manifested in more pronounced decrease in the severity of neurological disorders (according to NSS values) and more rapid recovery of autonomic feeding. Moreover, the animals receiving MMSC transplantation on day 1 survived without antiedematous therapy, while in animals receiving cell therapy on day 7, administration of glucocorticoids was necessary. It is known that MMSC exhibit pronounced immunosuppressor activity related to production of suppressive cytokines (transforming growth factor-β, hepatocyte growth factor, IL-10, *etc.*) and expression of 2,3-dioxygenase and HLA-G molecules [9]. Since these substances inhibit activation of T cells, macrophages, and dendritic cells and production of anti-inflammatory cytokines, MMSC can effectively suppress the inflammatory process [11] reproducing the effect of glucocorticoids.

Comparison of the two transplantation routes revealed no advantages of local MMSC administration into the ischemic focus. On the contrary, the animals receiving local cell transplantation demonstrated a tendency to less effective recovery compared to rats receiving intravenous injections of MMSC. It can be hypothesized that local administration of MMSC via the implanted catheter is associated with additional damage due to premedication with ketamine and repeated surgery (separation of the wound edges, isolation of the proximal end of the catheter followed by its removal and repeated suturing after cell transplantation). Moreover, the damaging effect can also be associated with additional hydrodynamic pressure during injection of the cell suspension.

On the other hand, higher clinical efficiency of intravenous MMSC transplantation suggests that MMSC effectively migrate to the damaged zone at the specified terms and hence no local intracerebral transplantation of cells is required. Thus, technically simpler route, intravenous injection of MMSC, is more preferable during cell therapy at earlier terms after damage.

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