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Comparison of Transplantation of Bone Marrow Stromal Cells (BMSC) and Stem Cell Mobilization by Granulocyte Colony Stimulating Factor after Traumatic Brain Injury in Rat

Mehrdad Bakhtiary[1, 2], Mohsen Marzban[*2], Mehdi Mehdizadeh[1, 2], Mohammad Taghi Joghataei[1, 2], Samideh Khoei[3], Vahid Pirhajati Mahabadi[1], Bahareh Laribi[4], Mahdi Tondar[2] and Arash Moshkforoush[5]

[1]*Cellular and Molecular Research Center;* [2]*Dept. of Anatomy;* [3]*Dept. of Medical Physics; and* [4]*Dept. of Immunology, Tehran University of Medical Sciences;* [5]*Dept. of Materials Science and Engineering, Sharif University of Technology, Tehran, Iran*

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ABSTRACT

Background: Recent clinical studies of treating traumatic brain injury (TBI) with autologous adult stem cells led us to compare effect of intravenous injection of bone marrow mesenchymal stem cells (BMSC) and bone marrow hematopoietic stem cell mobilization, induced by granulocyte colony stimulating factor (G-CSF), in rats with a cortical compact device. **Methods**: Forty adult male Wistar rats were injured with controlled cortical impact device and divided randomly into four groups. The treatment groups were injected with 2×10^6 intravenous bone marrow stromal stem cell (n = 10) and also with subcutaneous G-CSF (n = 10) and sham-operation group (n = 10) received PBS and "bromodeoxyuridine (Brdu)" alone, i.p. All injections were performed 1 day after injury into the tail veins of rats. All cells were labeled with Brdu before injection into the tail veins of rats. Functional neurological evaluation of animals was performed before and after injury using modified neurological severity scores (mNSS). Animals were sacrificed 42 days after TBI and brain sections were stained by Brdu immunohistochemistry. **Results**: Statistically, significant improvement in functional outcome was observed in treatment groups compared with control group ($P<0.01$). mNSS showed no significant difference between the BMSC and G-CSF-treated groups during the study period (end of the trial). Histological analyses showed that Brdu-labeled (MSC) were present in the lesion boundary zone at 42nd day in all injected animals. **Conclusion**: In our study, we found that administration of a bone marrow-stimulating factor (G-CSF) and BMSC in a TBI model provides functional benefits. *Iran. Biomed. J. 14 (4): 142-149, 2010*

Keywords: Stem cells, Injection, Traumatic brain injury (TBI)

INTRODUCTION

Stem cell transplantation can either compensate missing population of cells or rescue cells in the injured brain or spinal cord by the production of cytokine interleukins and neurotrophic factors that ease regeneration [1]. Autologous adult stem cells e.g. bone marrow mesenchymal stem cells (BMSC), may have priorities, ethical and legislative, over fetal or embryonic stem cells. Adult bone marrow provides a source of circulating blood progenitor cells derived from hematopoietic stem cells; moreover, bone marrow is a source of non-hematopoietic cells that can differentiate into various cell types including bone [2], glia, and neurons [3, 4]. Bone marrow cells have ability to secrete substances such as cytokines (interleukins) and trophic factors [4] may assist the regeneration and rescue partially damaged cells in addition to the replacement of lost cells which results in functional recovery after traumatic brain injury (TBI). Additionally, the transplantation of acutely isolated BMSC leads to extensive remyelination [5, 6]. The transplantation of mesenchymal stem cells of BMSC

*Corresponding Author; Cell phone: (+98-936) 5764669; E-mail: mohsen136051@yahoo.com

has also been seen to ameliorate functional recovery after CNS injury [7]. The administration of granulocyte colony stimulating factor (G-CSF) is known to mobilize hematopoietic stem cells from bone marrow into the peripheral blood [8]. G-CSF has been used extensively for bone marrow reconstruction and stem cell mobilization [9]. The subcutaneous administration of G-CSF augments the availability of circulating hematopoietic stem cells to the brain and the capacity for neurogenesis and angiogenesis in rats with cerebral ischemia [10]. In addition, G-CSF itself may also have anti-apoptotic and anti-inflammatory effects in parallel with effects on neovascularization [11]. Up to now, there has been no comparison between the effect of different bone marrow cell populations on morphological and functional recovery after TBI. In the present study, we compared the effect of (1) the freshly isolated mononuclear fraction of bone marrow containing bone marrow containing MSC [12] and (2) G-CSF-mobilized endogenous stem cell not only hematopoietic stem cells, but also progenitor cells and lymphocytes.

MATERIALS AND METHODS

Cell harvest and culture. Rat BMSC were harvested from the bone marrow of the femurs and tibias of 2- to 6-month male Spraw-Dawley rats by inserting a 21gauge needle into the shaft of the bone. Then, BMSC was flushed with 30 ml of complete α-modified Eagle's medium (αMEM) containing 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 ng/ml amphotericin B [2]. Cells were filtered through a 70-μm nylon filter and cells from each rat were plated into one 25 cm^2 flask. The cells were cultured in complete αMEM in 5% CO_2 at 37°C for 3 days, the medium was replaced with fresh medium, and the adherent cells were cultured to 90% confluency to obtain samples here defined as passage zero (P0) cells.

Passing rat bone marrow mesenchymal stem cells. Rat BMSC at P0 were washed with PBS and detached by incubation with 0.25% trypsin and 0.1% EDTA at 37ºC for 5 to 10 minutes. Complete medium was added to inactivate the trypsin [2]. The cells were centrifuged at 450 ×g for 10 minutes, the medium was removed, and the cells were resuspended in 1 to 10 ml of complete medium. The cells were counted in duplicate using a hemacytometer and then plated as P1 in 75 cm^2 plates at densities ranging from 5,000 cells/cm^2. Complete medium was replaced (refeeding) every 3 to 4 days over the 12- to 14-day period. All cells used for the experiments were P5 or earlier.

Stromal cell characterization. The BMSC-derived rats were cultured on chamber slides in a medium (complete αMEM containing 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 ng/ml amphotericin B) till the time when cells reached 80-90% confluency. Then, the medium was cautiously aspirated and the wells were washed two times with 1× PBS and cells were fixed by incubation in 4% paraformaldehyde for 30-40 minutes at ambient temperature. A blocking solution containing 5% normal donkey serum and 0.3% Triton X-100 in 1× PBS for at least 2 hours was applied at 4°C overnight. The cells were incubated in primary antibodies (rabbit anti-integrin β1, 1/500 dilution, Sigma,USA) overnight at 4°C. The next day, the cells were washed twice with 1X PBS (5-10 minutes for each wash) and twice with the blocking solution. At the end of the last wash, cells were left in blocking solution for at least 30 minutes and the following secondary antibodies were used: donkey anti-rabbit IgG FITC conjugated antibodies at 1:500 dilution (Sigma, USA). Cells were overlaid with the appropriate anti-rabbit secondary antibodies which were conjugated to fluorescent molecules for 2 hours at room temperature and then washed 3 to 5 times (5-10 minutes each) with 1X PBS. A glass coverslip was mounted over the chamber slides using anti-fading mounting solution. Finally, the staining cells were visualized using a fluorescent microscope.

Animal model. Adult male Wistar rats, 250 to 300 g weight, were used in this study. Animals were kept in a room at 23°C ± 2, 45% to 55% humidity and with a fixed 12-h artificial light period and allowed to eat and drink *ad libitum*. All animals were fed with standard rodent diet and received human care, as explained in the instruction for the care and use of laboratory animals. This study was approved by Ethical Committee of Tehran University of Medical Sciences (Tehran, Iran). A controlled cortical impact model in rat was used. Male rats (n = 40) were anesthetized with chloral hydrate 350 mg/kg body weight, i.p. Rectal temperature was controlled at 37° ± 0.5°C with a feedback-regulated water-heating pad. A controlled cortical impact device was used to induce the injury. Rats were placed in 6-mm

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diameter device. Craniotomies were performed adjacent to the central suture, halfway between lambda and bregma and the dura was kept intact over the cortex. Injury was induced by impacting the right cortex (ipsilateral cortex) with a piston consisting of a 5-mm diameter tip at the rate of 4 m/s and 2.5 mm of contusion [13]. Animals were divided into four groups (10 in each group) as follows:

Group 1: TBI + PBS
Group 2: TBI + bromodeoxyuridine (Brdu), i.p.
Group 3: TBI + G-CSF + Brdu (i.p.)
Group 4: TBI + BMSC

Stem cell labeling and transplantation. To identify cells derived from bone marrow, 3 mg/mL Brdu (Sigma, USA) a thymidine analogue and marker of newly synthesized DNA, was added to the medium at 72 hours before transplantation. On harvest, cells were isolated by treatment with 0.25% trypsin and 0.5 mM EDTA at room temperature at 37 °C for 10 minutes. The digestion was stopped by adding 1 mL FBS. Cells were then washed five times with PBS. Nucleated BMSC were counted using a cytometer to guarantee sufficient cell number for transplantation. For immunostaining, BMSC were subcultured in chambered slides and more than 90% of BMSC were Brdu reactive. Approximately 2×10^6 BMSC in 200 mL PBS (n = 10) or control fluid in 200 mL PBS; n = 10) was slowly injected over a 5-minute period into each rat tail vein. Immunosuppressant was not used in any animal in this project.

Bromodeoxyuridine labeling. Brdu, a thymidine analogue that is included in the DNA of dividing cells during S-phase, was used for mitotic labeling. The labeling protocol has been previously explained [14]. Experimental rats including 10 G-CSF–treated rats and 10 control rats were injected i.p. with Brdu (50 mg/kg) every day for 10 consecutive days. Rats were then sacrificed at 42nd day after TBI.

Experimental animals and G-CSF treatment. One day after induction of TBI, rats were subcutaneously injected with recombinant human G-CSF (50 µg/kg) per day; once daily for 5 days [15]. Control animals were subjected to TBI and injected with saline.

Brdu immunohistochemical asessment. The cerebral injured tissues (5 mm) were cut into coronal paraffin blocks. Single immunohistochemical staining was used to identify cells derived from BMSC and cell mobilized by G-CSF. Series of 6-µm-thick sections at various levels (50 µm interval) were cut from this block and analyzed by a fluorescent microscope. After deparaffinization, sections were placed in boiled citrate buffer (pH 6.0) within a microwave oven (650 to 720 W). After blocking in normal serum, sections were treated with the monoclonal antibody against Brdu in mouse (Sigma, USA) diluted at 1:100 in PBS. After sequential incubation with rhodamine conjugated goat anti-mouse IgG (dilution 1:60; Chemicon, USA), the secondary antibody was bound to the first antibody against Brdu. Cells derived from bone marrow and mobilized by G-CSF were identified by morphological criteria and by immunohistochemical staining with Brdu (the tracer), present in the nuclei of donor cells. Analysis of Brdu-positive cells was based on the evaluation of an average of 10 histology slides of brain. All Brdu-reactive cells, with Brdu clearly localized to the nucleus, were counted throughout all 10 coronal sections.

Nissl staining method. This method is used for the detection of Nissl body in the cytoplasm of neurons on paraformaldehyde or formalin-fixed, paraffin embedded tissue sections. The Nissl body will be stained purple-blue. This stain is commonly used for identifying the basic neuronal structure in brain and spinal cord tissue. After fixing the brain with 4% paraformaldehyde in 0.1 M PB or 10% formalin, paraffin sections were cut at 20 µm. Then, the sections were deparaffinized in xylene 2 or 3 times, 10 minutes for each time, and then hydrated twice in 100% alcohol (5 minutes for each time) followed by 3 minutes in 95% alcohol, and finally 3 minutes in 70% alcohol. Subsequently, the sections were rinsed in tap water, then in distilled water and finally were stained in 0.1% cresyl violet solution for 3 to 10 minutes. Staining in warmed cresyl violet solution (warmed in oven in 37-50ºC) can improve penetration and even enhance staining. It is particularly beneficial for thicker sections (20-50 µm). Afterwards, the sections were rinsed quickly in distilled water and then differentiated in 95% ethyl alcohol for 2 to 30 minutes while checking microscopically for best result. Then, the sections were dehydrated twice in 100% alcohol (5 minutes for each time) then cleaned twice in xylene (5 minutes for each), and finally mounted with permanent mounting medium.

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Table 1. A set of neurologic severity scores used to grade neurologic function [16].

Modified neurological severity score (mNSS)	Points
Motor tests	
Raising the rat by the tail 1 Flexion of forelimb 1 Flexion of hind limb 1 Head moved more than 10° to the vertical axis within 30 s	3
Walking on the floor (normal = 0; maximum = 3) O Normal walk 1 Inability to walk straight 2 Circling toward the paretic side 3 Falling down to the paretic side	3
Sensory tests 1 Placing test (visual and tactile test) 2 Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles)	2
Beam balance tests (normal = 0; maximum = 6) 0 Balances with steady posture 1 Grasps side of beam 2 Hugs the beam and one limb falls down from the beam 3 Hugs the beam and two limbs fall down from the beam, or spins on beam (>60 s) 4 Attempts to balance on the beam but falls off (>40 s) 5 Attempts to balance on the beam but falls off (>20 s) 6 Falls off: no attempt to balance or hang on to the beam (>20 s)	6
Reflexes absent and abnormal movements 1 Pinna reflex (a head shake when the auditory meatus is touched) 1 Corneal reflex (an eye blink when the cornea is lightly touched with cotton) 1 Startle reflex (a motor response to a brief noise from snapping a clipboard and paper) 1 Seizures, mycolonus, myodystony	4
Maximum points	18

One point is awarded for the inability to perform the task or for the lack of a tested reflex: 13-18 = severe injury; 7-12 = moderate injury and 1-6 = mild injury.

Neurological functional evaluation. As shown in Table 1, neurological function in the rats was assessed using the modified neurological severity scores (mNSS).The mNSS is composed of motor (muscle status and abnormal movement), sensory (visual, tactile and proprioceptive), reflex, and beam walking tests. In the severity scores of injury, one point is awarded for the inability to correctly perform the tasks or for the lack of a tested reflex. The higher the mNSS score is, the more severe the injury will be. The evaluation of all rats was started before TBI and performed after TBI in one week, and weekly thereafter. All measurements were performed by observers who were blinded to individual treatment [16].

Statistical analysis. Statistical analysis was performed using One-way analysis of variance (ANOVA) followed by Duncan post-hoc test with SPSS 15.0 for Windows. The data are presented as the Mean ± SD. P value less than 0.05 was considered statistically significant difference, and $P<0.01$ was defined as a very significant difference. All P values >0.05 were interpreted as representing no significant difference.

RESULTS

Isolation, expansion and characterization of bone marrow mesenchymal stem cells. Colonies of fibroblastic cells began to appear in the culture flasks 5 to 7 days after plating of BM nucleated cells. The non-adherent hematopoietic cells in the culture were removed during changes of medium. Initially, fibroblastic cells in a single colony were often separated from each other (Fig. 1A); however, after continuous culturing for 1 week, the number and density of cells were greater in the colonies.

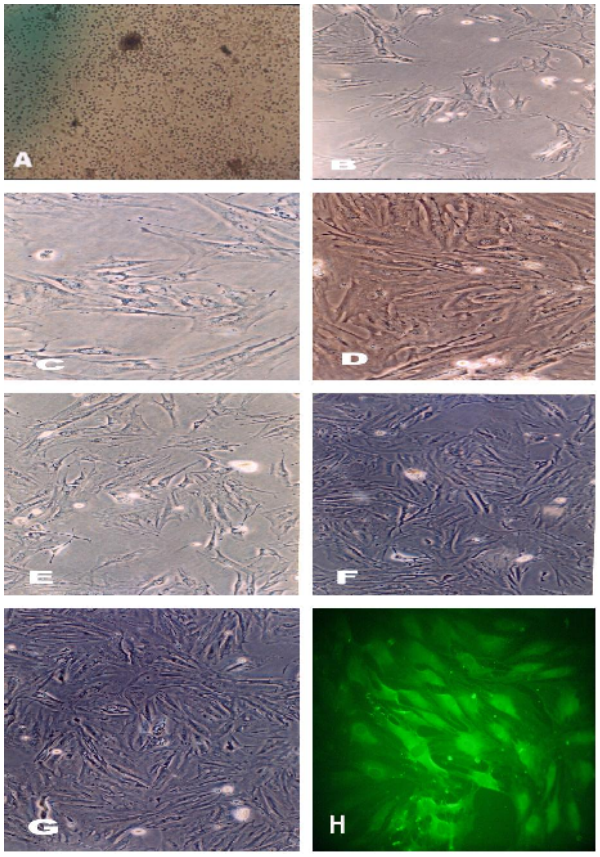

Fig. 1. Stem cells from the bone marrow. (A-H)Appearance and growth of fibroblastoid cells or bone marrow stromal stem cell at primary culture **(A)** passage 1 on days 3, 7 (**B** and **C**), passage 2 (**D** and **E**) and passage 3 (**F** and **G**), respectively. Briefly, when BMSC initially grow outward from explants, two morphologically distinct populations of cells are present: spherical or flat mesenchymal cells. Bone marrow stromal stem cells express mesenchymal stem cell markers, integrin β1 (**H**).

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Table 2. Statistical analysis performed using One-way analysis of variance (ANOVA). It was followed by Duncan's post-hoc test with SPSS 15.0 for Windows. The data are presented as the mean ± SD.

Group	1 Week	2 Weeks	3 Weeks	4 Weeks	5 Weeks	6 Weeks
Sham PBS	10.3 ± 0.483	9.4 ± 0.699	8.6 ± 0.516	7.1 ± 0.567	6.1 ± 0.567	5.5 ± 0.527
Brdu alone	10.3 ± 0.483	9.4 ± 0.699	8.6 ± 0.516	7.1 ± 0.567	6.1 ± 0.567	5.5 ± 0.527
G-CSF	9.3 ± 0.483*	8.3 ± 0.483*	6.8 ± 788*	5.9 ± 0.567*	4.9 ± 0.567*	4.2 ± 0.421*
BMSC	9.0 ± 0.471*	8.1 ± 0.737*	6.5 ± 0.527*	5.7 ± 0.483*	4.6 ± 0.516*	3.8 ± 0.421*

*$P<0.01$

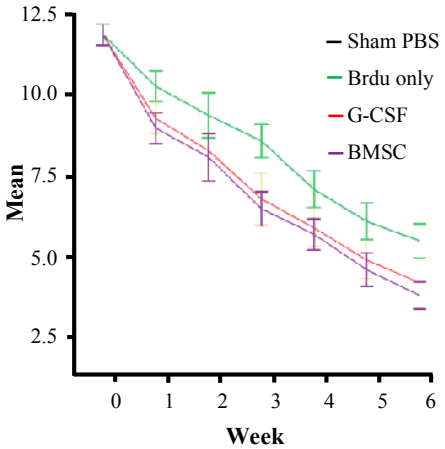

Fig. 2. Results of behavioral functional tests (mNSS test) before and after TBI. Rats (10 in each group) were injured to TBI and were injected with PBS (Control), Brdu (control), G-CSF and BMSC one day after TBI. Significant functional recovery was detected in rats treated with BMSC and G-CSF treated group compared with control and sham. The data are presented as the mean ± SD. *$P<0.01$.

Typically, more than 20 colonies were obtained from each marrow preparation in the primary culture. Freshly isolated cells (P1) principally displayed a fibroblast-like appearance over the first days of culture (Fig. 1B and 1C). BMSC were fed and changed with culture medium twice weekly and passaged as necessary (passage 2; D-E and passage 3, F-G).

Neurological and motor function evaluation. Injury in the right hemispheric cortex of rats caused neurological functional deficits, as measured by the mNSS, which is a combination of motor, sensory, reflex and beam walking tests. These rats presented with high scores on motor, sensory, reflex, and beam balance tests in the early phase after injury induction. All rats achieved the minimum score of 0 before TBI. One day after traumatic brain contusion, all rats had a mNSS score of 12. Recovery began at the end of the First week and persisted at all subsequent evaluation times in all groups. Motor function was tested by the mNSS recovered faster than sensory and beam balance functions. Modified NSS scores for all BMSC and G-CSF-treated groups were significantly lower than those of PBS-treated and control groups, indicating an improvement in the functional outcome. In all treatment groups, the mNSS scores were significantly lower at weeks 1 to 6 than those seen in control animals. mNSS values showed no significant differences between the BMSC and G-CSF treated groups during the study period (Table and Fig. 2).

Injury site Nissl histology and presence of Brdu positive cells. Brdu-reactive cells were detected from an average of 10 histology slides per treatment animal from multiple areas of the ipsilateral hemisphere, including cortexes, striatum of the

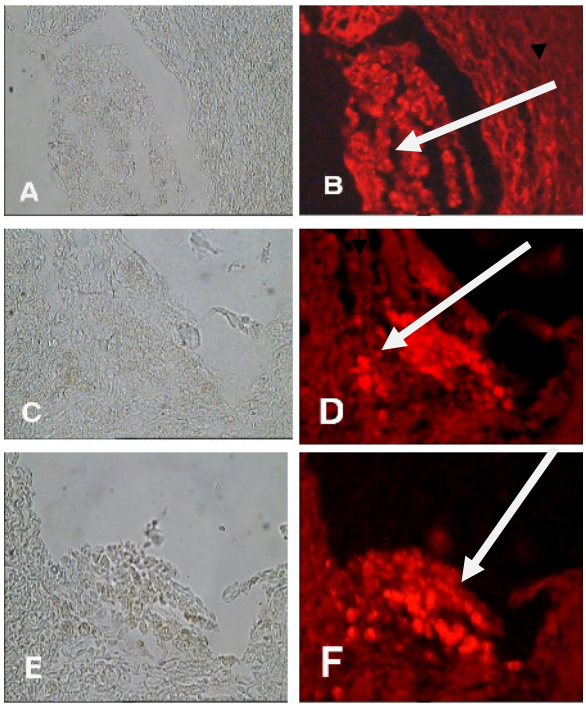

Fig. 3. Brdu Immunohistochemistry. Forty two days after traumatic brain injury, 41 days after intravenous transplantation of BMSC and subcutaneous injection of G-CSF, cells derived from BMSC were identified by rhodamin conjugated secondary antibody (**E** and **F**) and G-CSF mobilized stem cell (**C** and **D**) and endogenous stem cell (Brdu alone) (**A** and **B**) distribute in the territory of the TBI (40×). Arrows show red spots.

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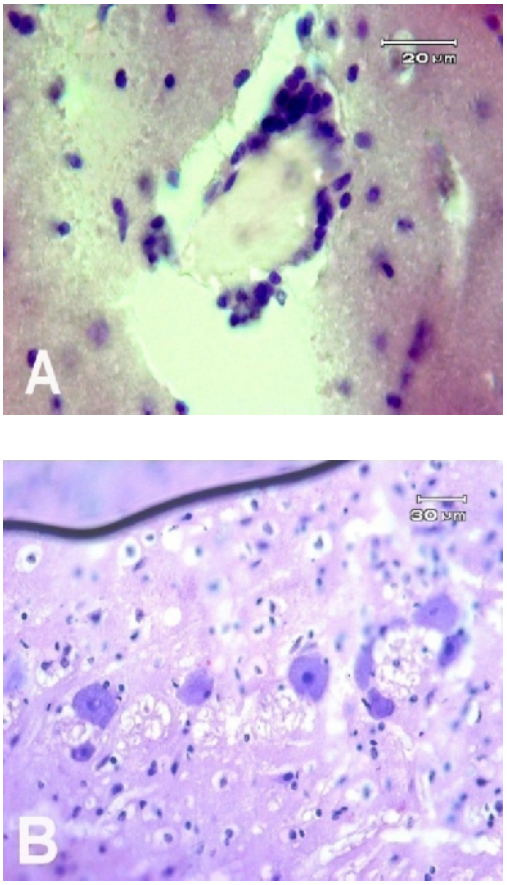

Fig. 4. Degeneration of ipsilateral white matter of traumatic zone in rat brain revealed by cresyl violet staining at 7 days after TBI. **(A)** Nissle staining of traumatic brain site inflammatory cells (glial and microglial) is around traumatic zone. **(B)** Most of neurons are shrunk with condensed nuclei and sparse Nissl bodies.

ipsilateral hemisphere. The vast majority of Brdu-labeled cells were located in the traumatic core and its boundary zone. Few cells were observed in the contralateral hemisphere. In summary, BMSC and G-CSF–treated traumatic rats exhibited significantly increased numbers of Brdu immunoreactive cells in their traumatic core compared with saline- and Brdu alone injected traumatic rats. (Fig. 3-5).

DISCUSSION

In our study, we showed that the intravenous injection of BMSC as well as the mobilization of stem cell with G-CSF remarkably ameliorated the recovery of motor and sensory function in rats with a traumatic brain contusion lesion. We found ameliorated behavioral factors after TBI was same in Brdu control and saline-treated group. The recovery of motor and sensory score was faster and more tangible after injection of BMSC and G-CSF and similar results were seen histologically.

The transplantation of autologous stem cells from adult bone marrow might therefore be a promising strategy for the treatment of CNS traumatic injuries and neurodegenerative diseases. The use of cells originating from bone marrow has several advantages over other cell sources, like the immediate availability of healthy donor tissue, and no reported tumorigenicity. Bone marrow as a source of cells has already been approved in human medicine for the treatment of hematopoietic diseases; however, the clear molecular and cellular mechanisms that promote recovery after TBI are still unknown. A preponderance of data indicates that BMSC-induced functional recovery from stroke and brain injury is not due to BMSC replacing the damaged neurons, but rather by BMSC inducing growth factor production and promoting intrinsic neurorestorative functions of the brain [17].

The vital role of neurotrophic growth factors in neural repair and regeneration has been well established [17-20]. It has previously been shown that BMSC express as well as induce intrinsic parenchymal cells to express multiple growth factors such as brain-derived neurotrophic factor, nerve growth factor, fibroblast growth factor and vascular endothelial growth factors both *in vitro* as well as *in vivo* [4, 21, 22]. In addition to growth factors, MSC have recently been shown to produce cerebral perfusion, lowering intracranial pressure and decreasing cerebral edema [23]. BNP production by BMSC is augmented by adding growth factors to brain natriuretic peptide (BNP), an important

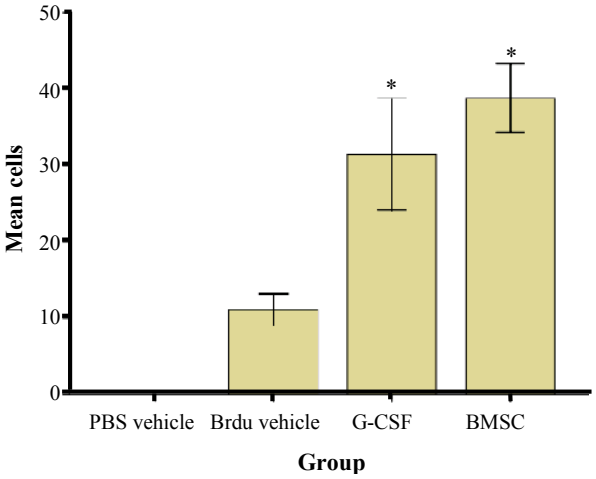

Fig. 5. Comparition of Brdu positive cells. Data presented as mean ± SD, Brdu-labeled cells were increased in the BMSC and G-CSF injected group than alone Brdu group 41 days after injection (*P<0.01).

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vasoactive factor that exercises powerful natriuretic, diuretic and vasodilatory effects [23, 28]. BNP may ease recovery from neural injury by improving their culture media [23] and since BMSC intrinsically produce growth factors, this may serve as an autoinductive stimulant to raise the expression of BNP.

G-CSF in humans mobilizes bone marrow cells that are a mixture of hematopoietic progenitor cells (mainly $CD34^+$) similar in characteristics to marrow-derived stem cells [24]. In addition to the mobilization of hematopoietic progenitor cells, G-CSF itself might have several mechanisms by which it can have an influence on recovery from neuronal injuries. G-CSF receptors are found on neurons and glia; therefore, G-CSF may exert a specific neuroprotective effect on these cells [25]. Thus, G-CSF may confer neuroprotection after TBI by inhibiting excitotoxicity and inducing the transcription of neuroprotective genes [25].

G-CSF (filgrastim) has been used after neuronal injury to reduce the risk of sepsis in patients with TBI [26]. In our study, we found that the effect of G-CSF treatment was less profound than that of BMSC implantation. The improvement of motor and sensory after G-CSF treatment had a slower time course than that of other cell treatment groups. It was shown that the number of white blood cells in the peripheral blood increases after G-CSF treatment [24]. Therefore, G-CSF may not only exert a direct beneficial effect on traumatic brain tissue, but also can lead to an increase in the number of systemic neutrophils. This neutrophilia can then have an adverse effect on the development of a traumatic brain lesion as part of the inflammatory processes in the lesion. This hypothesis is in agreement with Fukumoto's findings [29], in which he and his colleagues reported that treatment with G-CSF can lead to the aggregation of mobilized inflammatory cells that induce cerebral and myocardial infarctions. In contrast, Taguchi *et al.* [27] found that $CD34^+$ cells play a positive role in neuroregeneration by inducing neovascularization in the ischemic zone of the mouse brain. $CD34^+$ cells produce growth factors or cytokines and provide a favorable environment for neurogenesis in the spinal cord. In summary, our results show that treatment with different cell populations, obtained from bone marrow, and the endogenous mobilization of bone marrow cells has advantageous effects on behavioral and histological end results after TBI.

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