

Reduction of Lesion in Injured Rat Spinal Cord and Partial Functional Recovery of Motility after Bone Marrow Derived Mesenchymal Stem Cell Transplantation

Omurilik Hasarlı Sıçanlarda Kemik İliği Kaynaklı Mezenkimal Kök Hücre Transplantasyonu Sonrası Lezyonda Azalma ve Harekette Kısmi İşlevsel İyileşme

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ABSTRACT

AIM: This study aimed to analyze the effect of rat bone marrow-mesenchymal stem cells (rBM-MSCs) delivery on lesion site after spinal cord injury, and to observe the functional recovery after transplantation.

MATERIAL and METHODS: MSCs were isolated from rat femurs and tibias. The experimental rat population was divided into four groups: only laminectomy (1); laminectomy+trauma (2); laminectomy+trauma+PBS (3); laminectomy+trauma+MSCs (4). Their motility were scored regularly. After 4-weeks, rats were sacrificed, and their spinal cords were examined for GFP labeled rBM-MSCs by immunostainings.

RESULTS: In the early posttraumatic period, the ultrastructures of spinal cord tissue were preserved in Group 4. The majority of cells forming the ependymal region around the central canal were found to be MSCs. The gray-and-white-matter around the ependymal region were composed of Nestin+/GFAP+ cells, with astrocytic-like appearance. The scores showed significant motor recovery in Group 4, especially in hind limb functions. However, no obvious change was observed in other groups.

CONCLUSION: The increase Nestin+/GFAP+ cells in the gray-and-white-matter around the ependymal region could indicate the potential to self-renew and plasticity. Thus, transplantation of rBM-MSCs might be an effective strategy to improve functional recovery following spinal cord trauma. In conclusion, molecular factors in cell fate decisions could be manipulated to enhance reparative potential of cell-based therapy.

KEYWORDS: Bone marrow, Mesenchymal stem cells, Spinal cord injury, Functional recovery, Rats

ÖZ

AMAÇ: Bu çalışmada omurilik hasarı sonrası sıçan kemik iliği-mezenkimal kök hücrelerinin (sKİ-MKH) lezyon yerindeki etkisini analiz etmek ve transplantasyon sonrası işlevsel iyileşmeyi gözlemlemek amaçlandı.

YÖNTEM ve GEREÇLER: MKH'ler sıçan femur ve tibialarından izole edildi. Deney sıçanları dört gruba ayrıldı: sadece laminektomi (1), laminektomi+ travma (2); laminektomi + travma + PBS (3); laminektomi + travma + MKH'ler (4). Sıçanların hareketleri düzenli olarak skorlandı. Dört hafta sonra sıçanlar sakrifiye edildi, omurilikleri immün boyamayla GFP işaretli sKİ-MKH'lerle incelendi.

BULGULAR: Erken posttravmatik dönemde, Grup 4'teki omurilik dokusu ultrastrüktürel olarak korundu. Santral kanalın etrafındaki ependimal bölgeyi oluşturan hücrelerinin çoğu MKH'ler olarak saptandı. Ependimal bölgenin etrafındaki gri ve ak maddeler astrositik görümlü Nestin+/GFAP+ hücrelerden oluşmuştu. Grup 4'te özellikle arka bacak fonksiyonlarında motor iyileşme belirgindi. Buna karşın, diğer gruplarda belirgin değişiklik izlenmedi.

SONUÇ: Ependimal bölgenin etrafındaki gri ve ak maddede Nestin+/GFAP+ hücrelerin artışı kendini yenileme ve plastisite potansiyellerini gösterebilir ve sKİ-MKH'lerin transplantasyonu spinal kord travması sonrası fonksiyonel iyileşmede etkili bir strateji olabilir. Sonuç olarak, moleküler faktörler hücre kaderini belirleyen hücre tabanlı tedavinin onarıcı potansiyelini artırmak için uygulanabilir.

ANAHTAR SÖZCÜKLER: Kemik iliği, Mezenkimal kök hücreleri, Omurilik yaralanması, İşlevsel iyileşme, Sıçanlar

INTRODUCTION

Spinal cord injury (SCI) is a very serious and common health problem, and it is still not curable functionally. Due to the non-responsive environment of the injured spinal cord, axon regeneration does not occur (20, 22). Besides, the loss of function after SCI might be from both the primary mechanical insult and the subsequent, multifaceted secondary degenerative response. However, some experimental studies in last decades proved that the injured spinal cord could be restored (7,18). Nowadays, stem cell based therapy is promising some valuable strategies for functional recovery of the injured spinal cord. In this context, mesenchymal stem cells were used in addition to neural progenitor stem cells for functional recovery (12, 13).

We previously reported that rat bone-marrow-derived mesenchymal stem cells (rBM-MSCs) expressed myogenic (desmin, myogenin, myosinIIa, and α -actin), neurogenic (eno2, MAP2a,b, c-fos, nestin, gfap and β -tubulin), and osteogenic (osteonectin, osteocalcin, osteopontin, runx2, BMP-2, BMP-4 and type-I collagen) markers without inducing differentiation (9). The expression of these genes could be the reason of rBM-MSCs' predisposition of multi-lineage differentiation, including neurogenic lineages both *in vitro* and *in vivo*. Respectively, rBM-MSCs have crucial importance to restore some disorders including neural defects.

Our aim in this study was to analyze the healing effect of rBM-MSCs on lesion site after spinal cord injury and to observe the degree of functional recovery in motility after transplantation.

MATERIAL and METHODS

Animals

The SCI study included 18 female, nonpregnant Wistar albino rats about 2 months old with a weight of 200-300 g. In the first step of the study, six rats were sacrificed and their femurs and tibiae were excised in order to obtain MSCs. The remaining rats were divided into four groups (3 rats per group): only laminectomy (Group 1); laminectomy+trauma (Group 2); laminectomy+trauma+PBS (Group 3); laminectomy+trauma+MSCs (Group 4). Rats were sacrificed 4 weeks after transplantation. The experimental design and all procedures were approved by the Ethics Committee of Kocaeli University.

Isolation and Culture of rBM-MSCs

Isolation and culture of rBM-MSCs were performed as previously described (9). Under sterile conditions, both rat femur and tibiae were excised, and the bone marrow was flushed with MEM-Earle medium supplemented with 15% FBS, 1% Penicillin/Streptomycin (Gibco Invitrogen, Life Technologies, Paisley, UK). The supernatant containing thrombocytes and erythrocytes was discarded, and the cell pellet was resuspended in MEM medium. The cells were seeded on plastic tissue culture flasks and incubated for 3 days. The MSCs were isolated based on their ability to adhere on plastic. Fresh medium was added to remove the

unattached cells. After plate reached 70-80% confluency, the cells were passaged using 0.25% trypsin-EDTA solution (Gibco Invitrogen) after washing with Ca^{2+} - Mg^{2+} free phosphate-buffered saline (PBS; Gibco Invitrogen).

Flow Cytometry

Undifferentiated SCs were subjected to flow cytometry analysis to confirm that rBM-MSCs maintain their phenotypic characteristics after growth in culture. Three surface markers of rBM-MSCs at passages 3 (P_3) were assayed with antibodies against the following rat antigens: CD29 (Integrin β_1 chain; Ha2/5; FITC), CD45 (PE), and CD90 (Thy-1/Thy-1.1-FITC), and their isotype controls (IgG2a κ ; FITC). All of the antibodies were supplied by Becton Dickinson (BD Biosciences, San Diego, USA). Flow cytometry was performed using a FACSCalibur (BD Biosciences) device. The data were analyzed with Cell Quest software (BD Biosciences).

Immunostainings

The immunohistochemistry studies on the P_3 cells were performed as previously described (9), and the list of primary antibodies is given in Table I. Samples were fixed in ice-cold methanol for 10 min and treated with 0.025% Triton X-100 (Merck, Darmstadt, Germany) for permeabilization. Cells were incubated with 1.5% blocking serum solution (Santa Cruz Biotechnology, Heidelberg, Germany) for 30 min at 37 °C and incubated overnight at 4 °C with the primary antibodies after washing with PBS. Samples were incubated with appropriate secondary antibodies for 25 min and covered with mounting medium containing DAPI (Santa Cruz Biotechnology). The mounted cells were examined under fluorescence microscope (Leica DMI 4000B, Wetzlar, Germany).

In Vitro Differentiation

The in-vitro studies of adipogenic, osteogenic, and neurogenic differentiations were performed according to the methods described previously by Karaoz et al. (9).

The adipogenic differentiation was performed by incubation of MSCs in Standard culture media with supplementations (0.5 mM isobutyl-methylxanthine, 10^{-6} M dexamethasone, 10 μ g/ml insulin, 200 μ M indomethacin) for two weeks.

For osteogenic differentiation, the cells were incubated in osteogenic differentiation medium (standard culture media supplemented with 100 nM dexamethasone, 0.05 μ M ascorbate-2-phosphate, 10 mM β -glycerophosphate) for four weeks.

Neurogenic differentiation was performed by incubating the cell in culture medium supplemented with 10 ng/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor, 10 ng/ml Brain derived neurotrophic factor, 0.5 mM isobutylmethylxanthine for 24-72 h.

Labeling with GFP of Mesenchymal Stem Cells

pGFP (Clontech, Palo Alto, CA, USA), was transfected by electroporation (Neon Transfection System, Invitrogen,

Table I: Immunocytochemical Properties of rBM-MSCs

Antibody/Marker	Dilution	Source	rBM-MSCs
CD 31 (M-20)	1:100	Santa Cruz	∅
CD 34 (C-18)	1 : 150	Santa Cruz	∅
CD 45 (H-230)	1 : 150	Santa Cruz	∅
CD 71 (K-20)	1 : 150	Santa Cruz	∅
CD105 (M-20)	1:100	Santa Cruz	+
c-Fos (4)	1 : 50	Santa Cruz	+
Collagen II (2B1.5)	Prediluted	Thermo Scientific	+
Collagen Ia1 (D-13)	1 : 50	Santa Cruz	+
Beta-tubulin (KMX-1)	1 : 50	Milipore	+
Beta-tubulin	Prediluted	Thermo Scientific	+
Nestin (Rat-401)	1 : 50	Santa Cruz	+
Vimentin (C-20)	1 : 100	Santa Cruz	+
Desmin (H-76)	1 : 50	Santa Cruz	+ ¹
Desmin (D33)	Prediluted	Thermo Scientific	+ ¹
Fibronectin (EP5)	1 : 100	Santa Cruz	+
Asma	1 : 800	Thermo Scientific	+
Actin (C-2)	1 : 50	Santa Cruz	+
Osteocalcin (FL-100)	1 : 50	Santa Cruz	+
Osteonectin (SPARC)	1 : 50	Milipore	+
SPARC (H-90)	1 : 50	Santa Cruz	+
Osteopontin (AKm2A1)	1 : 50	Santa Cruz	+ ¹
MAP 2a,b (AP20)	Prediluted	Thermo Scientific	+
GFAP	Prediluted	Thermo Scientific	+
Beta3-tubulin (2Q121)	1 : 50	Santa Cruz	+
NSE/eno2	1 : 500	Milipore	+
HNK-1ST (E-20)	1 : 50	Santa Cruz	∅
Cytokeratin 18	1:50	Santa Cruz	∅
vWF (F8/86)	1 : 50	Santa Cruz	∅
CD146	1:300	Abcam	+ ¹
Ki67	1:200	Abcam	+
BrdU	1:500	Thermo Scientific	+

+ = Positive; ∅ = no expression; +/- = weak. ¹ = 10-20 % positive.

Carlsbad, CA, USA) with respect to the instructions provided by manufacturer. The transformed cells were cultured in 1 ml MEM-medium with 15% FBS. After 48 h of incubation, the cells were selected with respect to resistance against G418 (200 µg/ml).

Surgical Procedure and Cell Transplantation

For skin preparation of T10-11 spinal cord surgery, lumbar laminectomy of tracer injection, dermal surface of the related regions was cleared by hair razor, and the skin was washed by antibacterial soap followed with betadine and 70% ethanol application (2). After an overnight fast with unrestricted access to water, all 12 rats were anesthetized with intramuscular ketamine (50mg/kg) and xylazine (5mg/kg) prior to surgery.

Under dissection stereo microscope; 3 mm long laminectomy, encompassing the caudal end of T10 vertebra and the rostral end of T11 vertebra, was performed. For SCI groups (total: n=12), a severe T10-T11 contusive injury was introduced by dropping the impounder rod (1 g) from a height of 50 mm.

GFP labeled rBM-MSCs (3×10^5 cells/5µL) were transplanted into the injured spinal cord via Hamilton syringe (Hamilton company, Reno, NV) connected to a syringe pump (KD Scientific Inc., Holliston, MA, USA) for 5 min, respectively. PBS group received 5µL of PBS at the injured spinal cord with the same technique. The needle was removed 10 min after subcutaneous transplantation, and muscle & skin layers were closed in layers. The bladders of SCI rats will evacuate twice daily during the entire study.

BBB Scoring-Functional Tests

Functional tests were performed using the Basso, Beattie and Bresnahan (BBB) locomotor rating scale at pre-surgery, at days 1, 7, 14, 21, and 28 postinjury (p.i.). Two independent, blinded examiners observed each animal for 4 min. Hindlimb movements were recorded by video camera and locomotor functions were assessed (1). The BBB scores were presented as mean± standard error.

Tissue Harvesting and Immunohistochemical Examination

At the end of 4 weeks, rats were anesthetized with ketamine (75 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.), and transcardially perfused with saline (150 ml/per animal) and followed with 4% neutral buffered paraformaldehyde in 0.1 mol/L PBS, pH 7.4. 1.0 cm spinal cord segment encompassing the injury site was removed, the tissues were post-fixed in 4% paraformaldehyde approximately 24 h and then embedded in paraffin. Five longitudinal serial sections, each 5 µm thick, were taken from each paraffin embedded spinal cord samples.

To perform cell tracing after injection of the GFP labeled rBM-MSCs, an immunofluorescence double staining protocol was performed on sections. Slides were deparaffinized with two changes of xylene for 5 min each and rehydrated in a series of graded alcohol solutions. Sections were antigen retrieved using a steamer-citrate buffer antigen retrieval method. Endogenous peroxidases were inhibited by incubation with fresh 3% H₂O₂ in PBS buffer. Nonspecific staining was blocked with the mixture of two different serum at 1.5% in PBS for 30 min at room temperature (RT). Afterwards, the sections were incubated in a mixture of two primary antibodies in a pairwise fashion with the mouse monoclonal anti-GFP antibody (sc-9996, Santa Cruz, Heidelberg, Germany) and vimentin (sc-7557, Santa Cruz), beta3-tubulin (sc-69965, Santa Cruz) or nestin (sc-33677, Santa Cruz) or with GFAP (MS-280-R1, Thermo Scientific, NeoMarkers, Fremont, CA, USA) at appropriate dilutions in antibody dilution buffer for 1 h at RT. The sections were incubated in a mixture of two fluorescent conjugated secondary antibodies, which included the goat anti-mouse FITC (sc-2010, Santa Cruz) for the GFP antibody, donkey anti-goat TR (sc-2783, Santa Cruz) for vimentin, goat anti-mouse TR(sc-2781, Santa Cruz) for GFAP, beta3-tubulin and nestin at the dilution of 1:50 in PBS buffer for 30 min at room temperature and were mounted with mounting medium containing DAPI (Santa Cruz). The mounted cells were examined under a fluorescence microscope.

Statistical Analyses

All experiments were repeated a minimum of three times. All data presented as mean± standard error. All statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way ANOVA. Differences between groups were regarded as statistically significant when $P < 0.05$.

RESULTS

Culture of rBM-MSC

MSCs attached to the culture flasks sparsely and displayed a fibroblast-like, spindle-shaped morphology during the initial days of incubation. Following 3-4 days of incubation, proliferation started and the cells gradually grew into small colonies (Figure 1A,B). As growth continued, adjacent colonies interconnected with each other and a monolayer confluence was obtained after 12 to 15 days of incubation. In later passages, MSCs exhibited large, flattened or fibroblast-like morphology (Figure 1C,D) and did not change throughout 25 passages. Tests for bacterial and mycoplasma contamination were negative and the viability was higher than 95%. rBM-MSCs expressed CD29 and CD90, but not CD45 (Figure 1E) and maintained their phenotype in the following passages.

Immunostaining of rBM-MSCs

Immunohistochemical studies were performed to characterize the progeny of the rBM-MSCs, by using a large panel of antibodies specific to known antigens (Figure 2, 3). Immune reactivity profile for rBM-MSCs is shown in Table I. Under described standard culture conditions, α -tubulin (Figure 2A), beta-tubulin (Figure 2B, 3A), GFAP (Figure 2C), beta-actin (Figure 2E,3D), osteocalcin (Figure 2F), c-fos (Figure 2G), type-II collagen (Figure 2H), osteonectin (Figure 2I), vimentin (Figure 3B), fibronectin (Figure 3C), CD105, CD146, type-I collagen, osteopontin, nestin (data not shown) were expressed. rBM-MSCs did not express some surface markers including von Willebrand Factor (vWF), Cytokeratin (CK) 18, CD31 (endothelial marker), CD34 (Figure 2D), CD45 (hematopoietic marker) and CD71 (Table I).

Differentiation potential of rBM-MSCs

rBM-MSCs at passage 3 were used in the adipogenic differentiation experiments, in which 80-90% sub-confluence was found to be optimal for cell differentiation, which lasted for two-three weeks. At the beginning of the 3rd week, the lipid droplets enlarged and invaded the entire cytoplasm like adipocyte-differentiated rBM-MSCs (Figure 4Aa-c).

Histo- and immunohistochemical techniques for bone-specific markers were performed to cells grown in osteogenic differentiation medium and also in control medium to assess the osteogenic differentiation of rBM-MSCs. Proliferation and differentiation of both cultures were compared using phase contrast microscopy. Both cells proliferated and reached almost complete confluency after 8-10 days of incubation. Later, the cellular aggregates were observed in osteogenic differentiation cultures and gradually increased. The aggregates were characterized by the presence of amorphous material deposits (Figure 4B-a). These nodular aggregates in osteogenic cultures were stained with Alizarin red S, demonstrating that the amorphous deposits observed under microscope at day 15 were actually calcium deposits (Figure 4B-b). Cells that were incubated for 15 days also strongly stained for BMP4, osteopontin (Figure 4B-c), osteocalcin and BMP2 (data not shown).

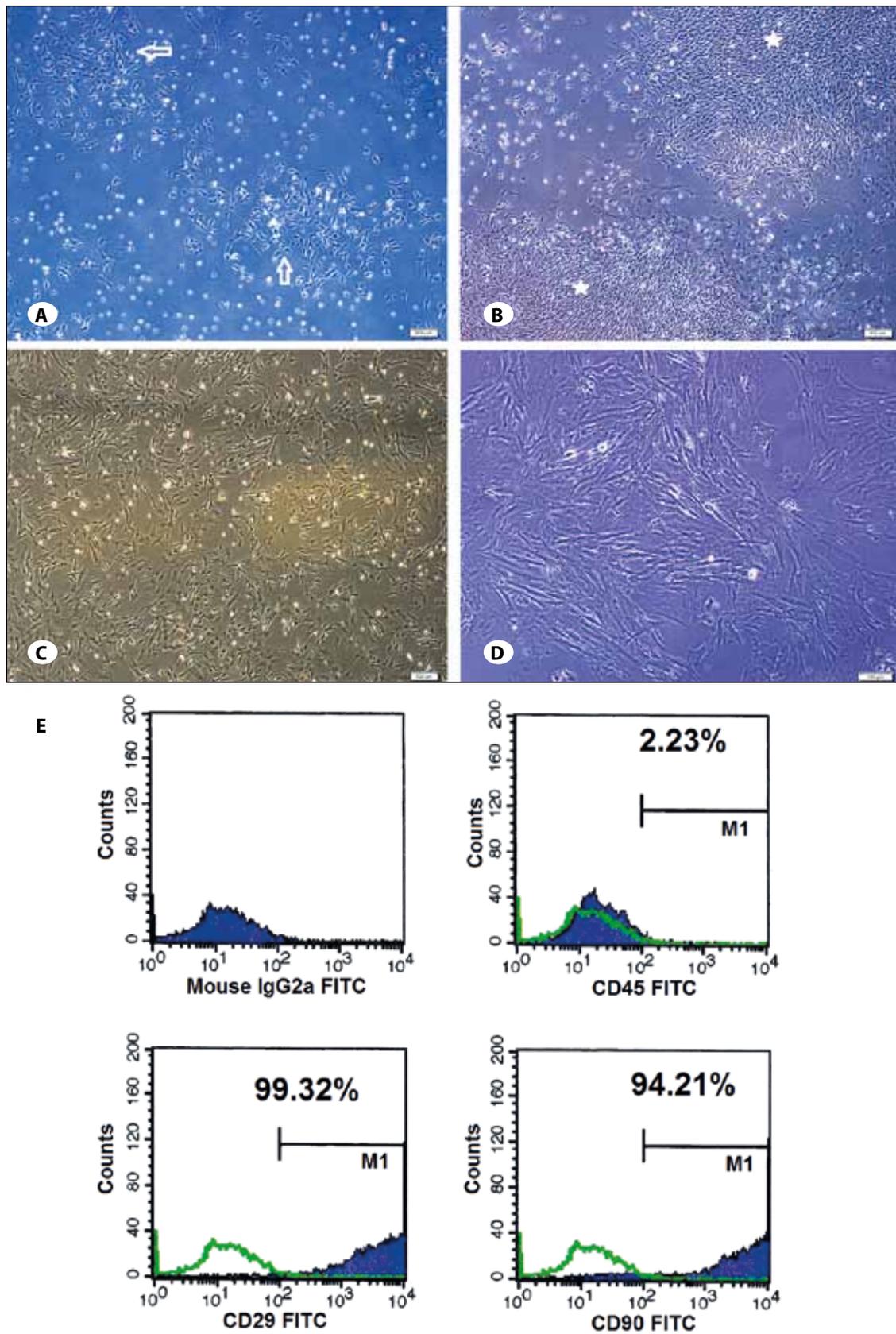


Figure 1: rBM-MSCs morphologies for primary culture (A-B) and late passages (C) P1-3rd day; (D) P3-7th day). (E) Flow cytometry analysis for P3 cells.

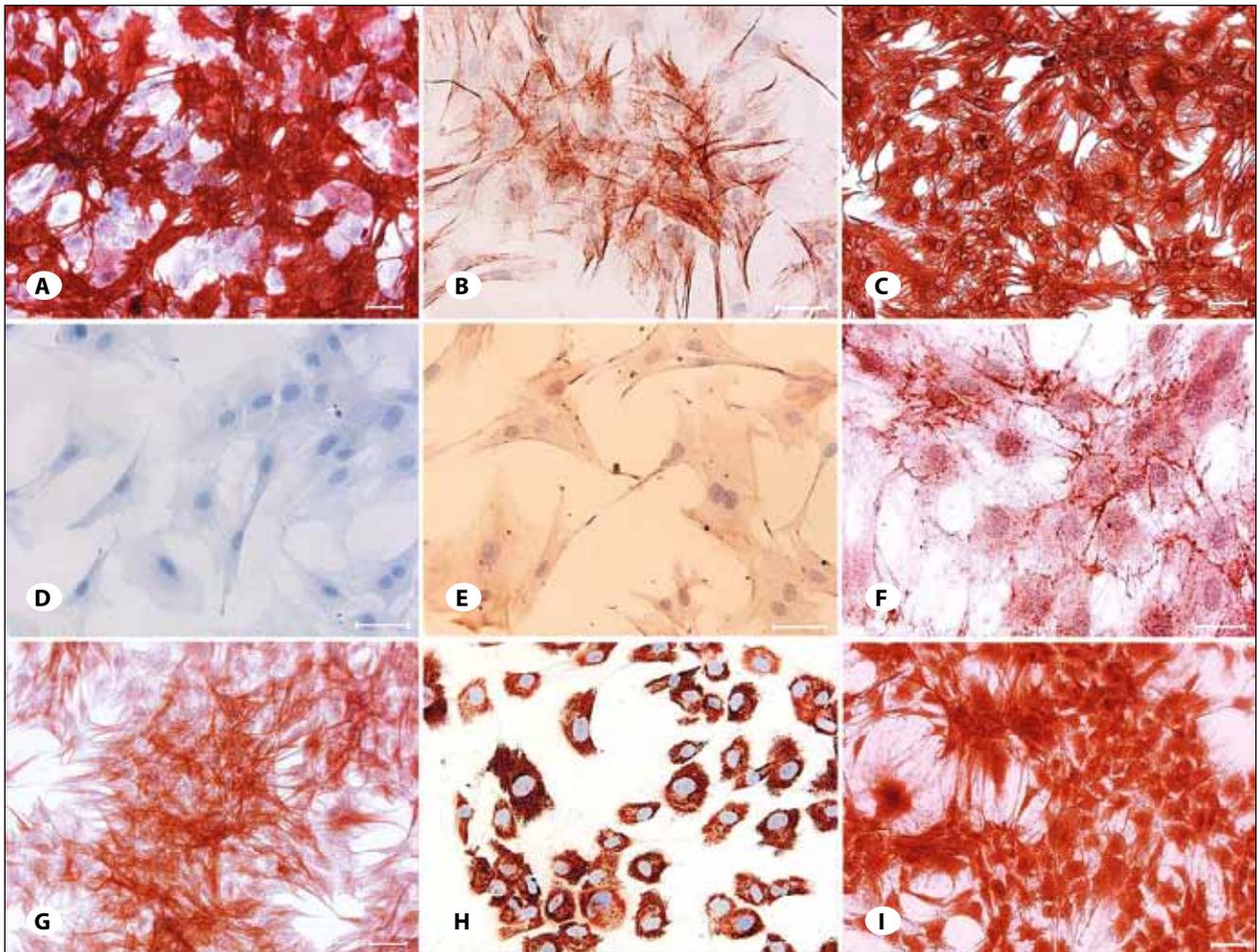


Figure 2: Immunophenotype of rBM-MSCs. Asma (A), beta-tubulin (B), GFAP (C), CD34 (D), beta-actin (E), OCN (F), c-fos (G), type-II collagen (H), and osteonectin (I). (Scale bars, 50µm).

Neurogenic differentiation

rBM-MSCs-derived neuron-like cells displayed distinct morphologies, ranging from extensively simple bipolar to large, branched multipolar cells (Figure 4C-a). To characterize neuronal identity further, differentiated rBM-MSCs were positively stained for some neuron or glial cell specific markers including MAP2a,b (Fig.4C-b), beta-tubulin (Figure 4C-c), c-fos (Figure 4C-c), GFAP, beta3-tubulin, eno2, and HNK-1ST (data not shown).

Survival and migration of rBM-MSCs

After 4 weeks, the immunofluorescence microscopic analyses of longitudinal and transversal sections of rat spinal cords from experimental groups were performed with double staining of GFP together with either GFAP, beta3-tubulin or c-fos. In all sections of Laminectomy (1) and Laminectomy&Trauma (2) groups, they showed negative staining for GFP. Noticeably, the center of the lesion was severely cavitated in the Laminectomy&Trauma group (Figure 5). However, GFP+ cells were observed in the vicinity of the damage site of Group4

at the end of 4 weeks, and most of the surviving cells were located at the periphery of the lesion site next to healthier tissue (Figure 5). GFP+ MSCs migrated into the cavitated area from the injection sites, and the majority still survived and expressed some stem cell and neural markers such as GFAP (Figure 6), vimentin (Figure 7), nestin (data not shown) and beta3-tubulin (Figure 7).

Interestingly, the sections of low scored animals of Group4 in tests showed considerably lower number of GFP+ cells migrated to the damage site than the sections of higher scored animals. Additionally, the increase of GFAP expression in this region was remarkable (Figure 6).

Functional recovery

To confirm the traumatic impact of the standardized severe weight-drop contusion injury to the T10-T11 spinal cord, the hind limb locomotion of the SCI rats were evaluated. At each assessment time point, consistent functional deficits were noted among SCI rats with the BBB locomotion scores showing profound loss initially, which then gradually improved and

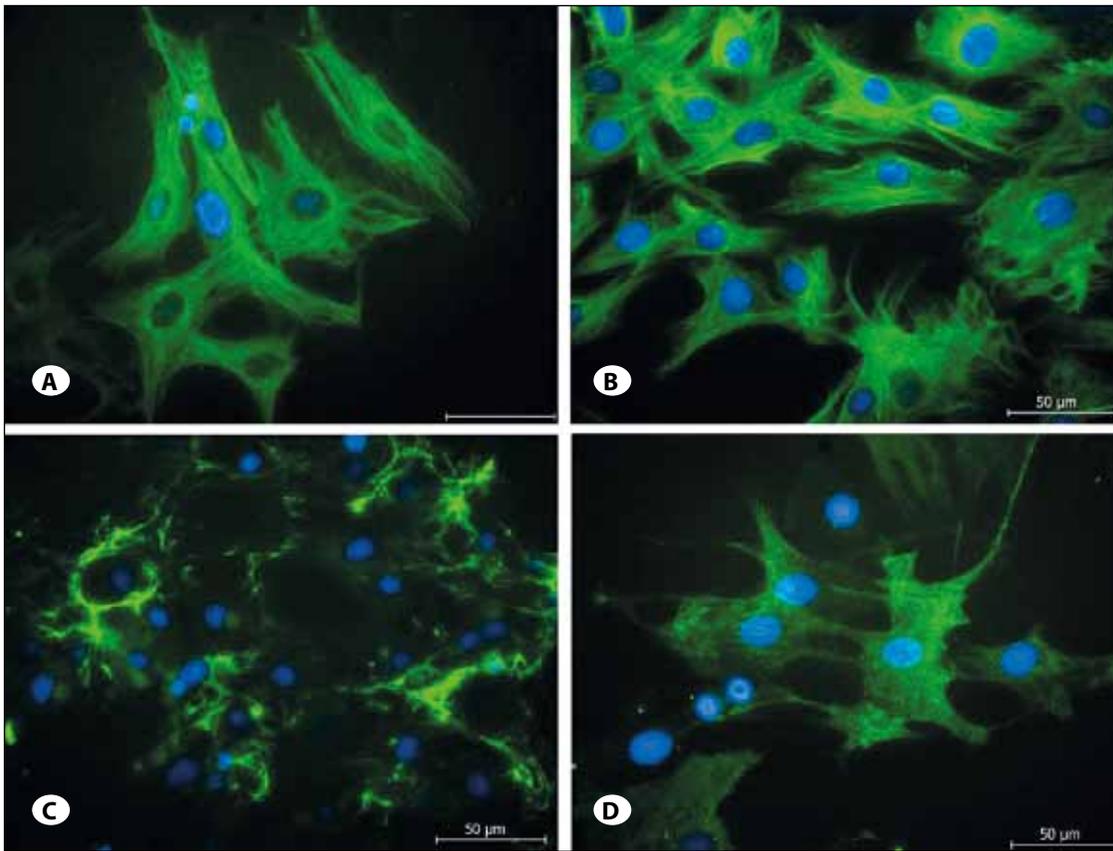


Figure 3: Expression of protein markers: Beta-tubulin (A), vim (B), fibronectin (C), and beta-actin (D). Nuclei were labeled with DAPI (blue) (Scale bars, 50µm).

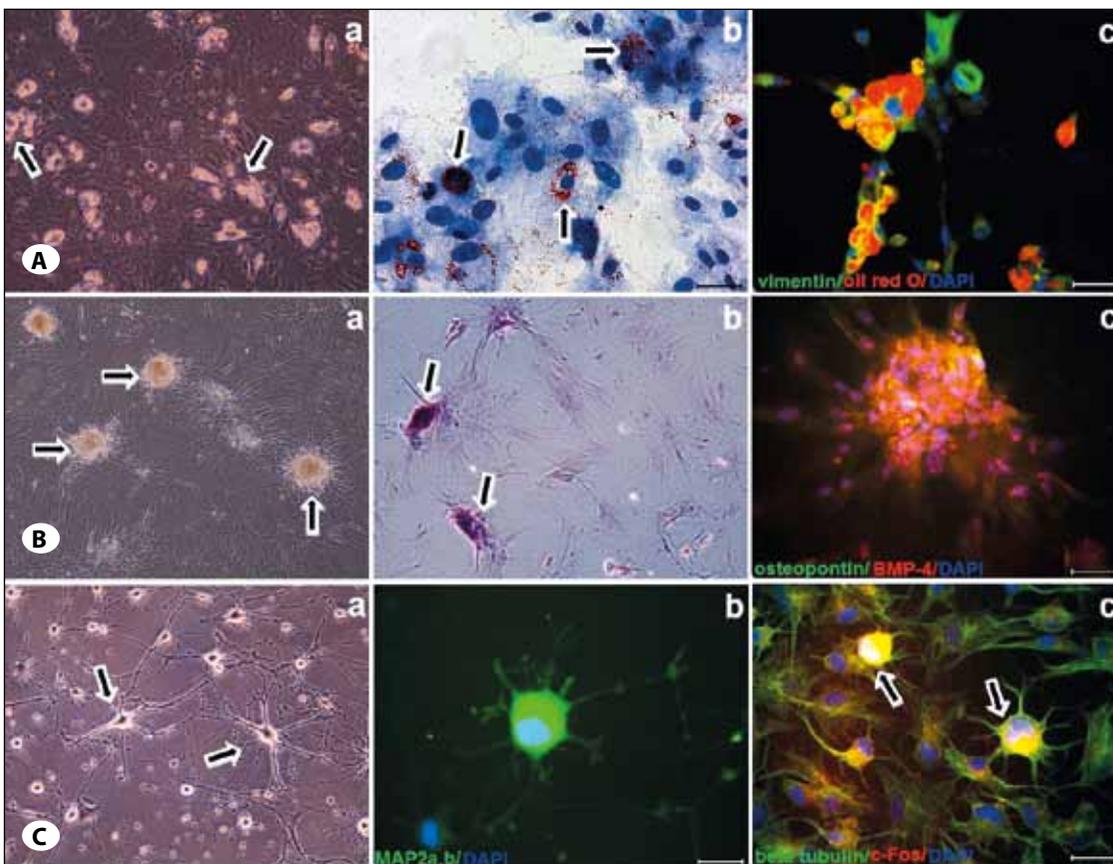


Figure 4: Differentiation of rBM-MSCs into adipogenic (A), osteogenic (B) and neural (C) lineages. Red-Oil (A-b), Alizarin-Red-S stainings (B-b). (Scale bar, 50 µm).

approached a plateau level of spontaneous recovery typical for this type of injury by 4 weeks p.i. (Table II, III).

According to the BBB locomotor activity test, the performances of Group 4 were statistically different than Group 2 and 3 ($p < 0.05$, Table II). The injured rats (Group 2 and 3) showed

markedly lower activity score than the mesenchymal stem cell injected group (Group 4) in the BBB locomotor rating score. All experimental groups (Group 2-4) showed BBB locomotion score increase to some extent but the highest scores were observed in the mesenchymal stem cell injected group (Group 4) (Figure 8).

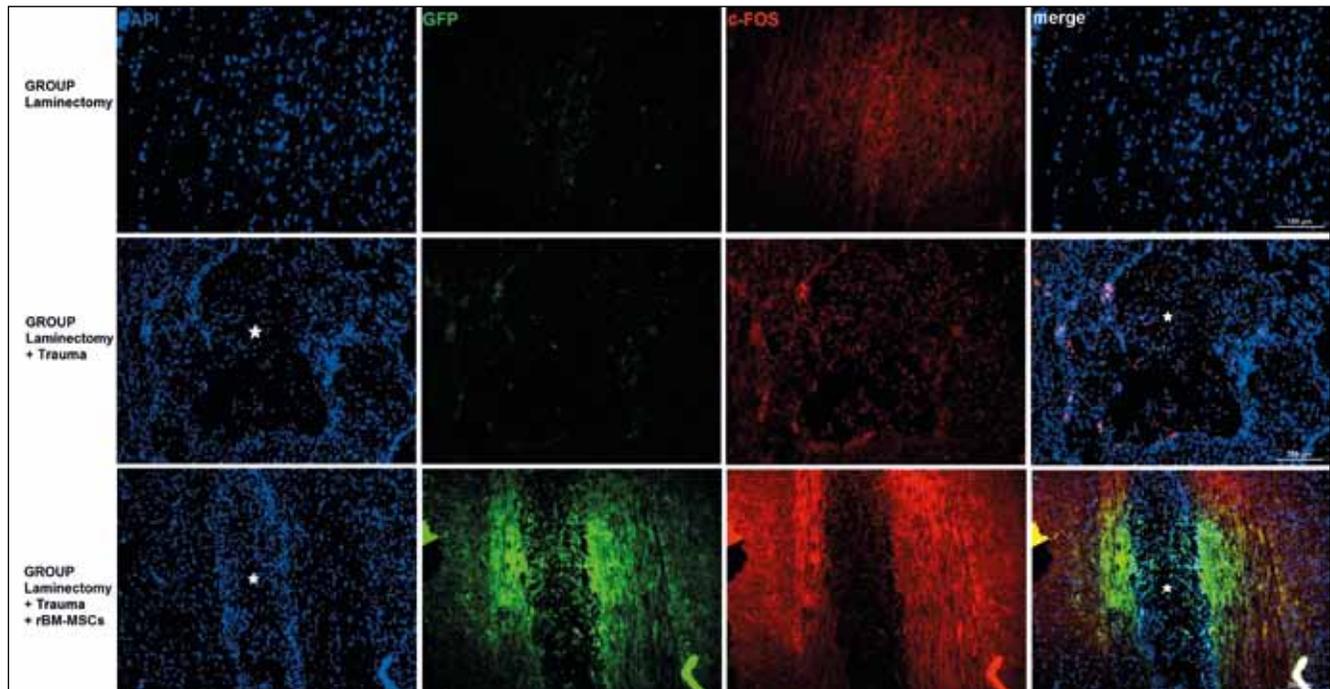


Figure 5: Longitudinal sections of rat spinal cords. After 4 weeks, GFP+ cells were migrated to the damaged site (*) and survived in Laminectomy&Trauma&MSC (Group 4) animals' sections.

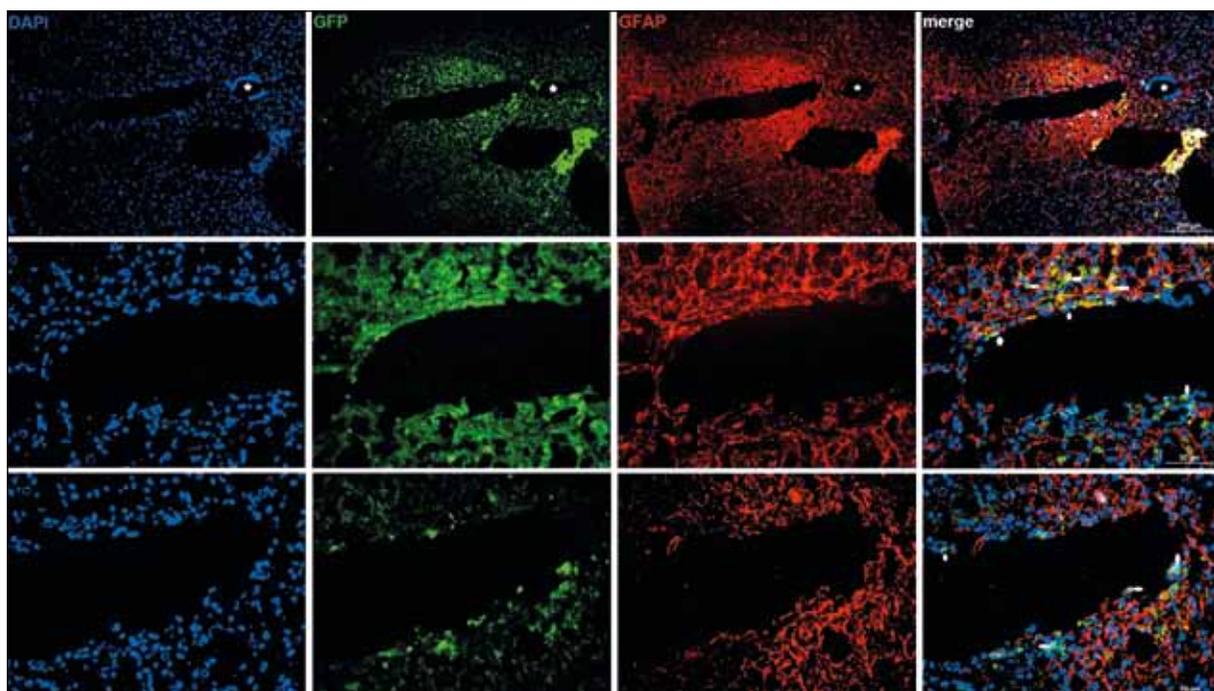


Figure 6: Low-scored animals of Group 4 has lower number of GFP+ cells migrated to the damage site than those of higher scored (arrow). Canalis centralis (*).

DISCUSSION

Different types of stem/progenitor cells have been transplanted into experimental models of spinal cord injury (SCI) with promising results but minimal functional benefit, and phase 1 and 2 trials of stem/progenitor cells have already been performed in humans with SCI (15). In the present study, we focused on bone marrow derived MSCs because they have the ability to differentiate into neuronal or glial cells both *in vitro* and *in vivo* studies (6,11). In addition, BM-MSCs were used because of their easy accessibility, potential for

autologous transplantation and promising pre-clinical studies (3,10, 14, 22). Additionally, BM-MSCs might have advantages over embryonic or fetal stem cells as MSCs have less tumor formation potential and ethical problems.

The general strategy for identifying *in vitro* cultivated BM-MSCs is to analyze the expressions of cell surface markers such as CD29, CD44, CD90 and CD106 (9, 18, 22). FACS experiments indicated that rBM-MSCs of this study were negative for CD45, a cell surface marker associated with lymphohematopoietic cells. Therefore, there was no evidence of hematopoietic

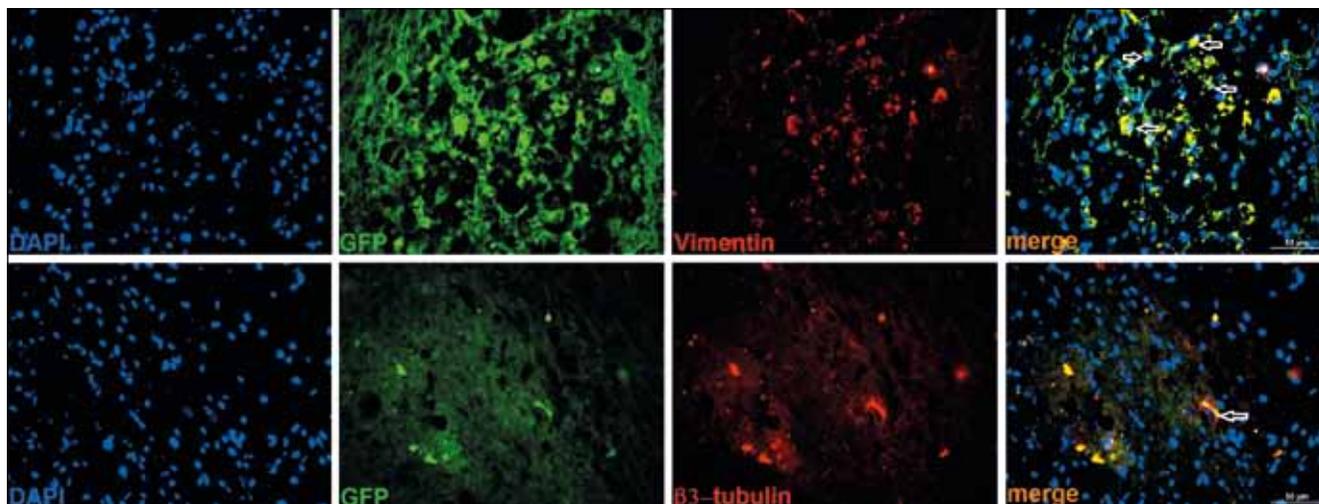


Figure 7: GFP (green), vimentin and beta3-tubulin (red) staining of longitudinal sections of Group 4. GFP+ and vim+ staining could be observed in damage regions and GFP+ and beta3-tub+ in adjacent zone (arrows).

Table II: BBB Scoring for All Groups (mean± standard error)

	Laminectomy (Group1)	Laminectomy+Trauma (Group 2)	Laminectomy+Trauma+PBS (Group 3)	Laminectomy+Trauma+MSC (Group 4)
post-op	21.0 ±0.0	1.0 ±0.0	1.0 ±0.0	2.3 ±0.6
Week 1	21.0 ±0.0	2.3 ±1.2	3.3 ±0.6	8.0 ±1.0
Week 2	21.0 ±0.0	3.4 ±1.5	4.3 ±0.6	14 ±3.6
Week 3	21.0 ±0.0	5.6 ±2.3	5.3 ±1.5	15.3 ±2.1
Week 4	21.0 ±0.0	9.0 ±2.0	8.0 ±2.0	16.0 ±2.7

Table III: BBB Scores

	Mean (±SE)	Min.	Max.
Laminectomy (Group1)	21.00 (± 0.00)	21.00	21.00
Laminectomy &Trauma (Group2)	4.26 (± 3.14)	1.00	9.00
Laminectomy&Trauma&PBS (Group3)	4.38 (± 2.57)	1.00	8.00
Laminectomy&Trauma&MSC (Group4)	11.12 (± 5.85)	2.30	16.00

One way ANOVA (for all groups) (p<0,05)

Laminectomy vs Laminectomy&Trauma	p=0.000	p<0.05
LaminectomyTrauma vs Laminectomy&Trauma&PBS	p=1.000	p>0.05
Laminectomy vs Laminectomy&Trauma&PBS	p=0.000	p<0.05
Laminectomy vs Laminectomy&Trauma&MSC	p=0.002	p<0.05
Laminectomy&Trauma vs Laminectomy&Trauma&MSC	p=0.035	p<0.05
Laminectomy&Trauma&PBS vs Laminectomy&Trauma&MSC	p=0.037	p<0.05

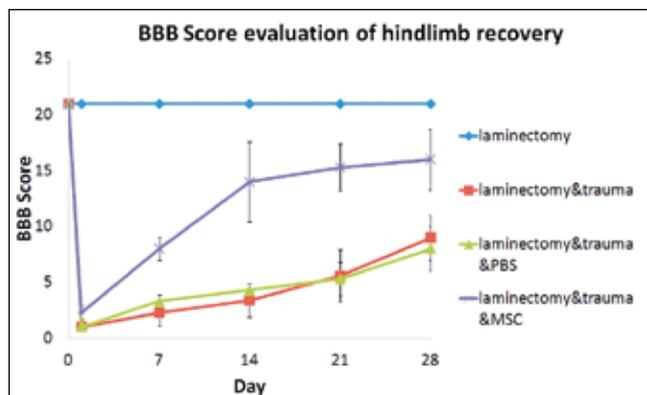


Figure 8: Effect of T10-11 SCI on general hind limb function over time after SCI. Deficits are expressed as a BBB locomotion score.

precursors in the cultures. In contrast, the expression of surface antigens was the same in accordance with the previous reports regarding murine MSCs, indicating that the cells used in this study had the characteristics of MSCs (1, 4).

The present study demonstrated that the GFP labeled donor cells survived in the spinal cord injury area. rBM-MSCs were observed around the transplant and on the lesion sites, suggesting migration toward and into the injury, but not rostral or caudal. Besides, there was significant reduction in the cavitation area in the rats receiving BM-MSCs in comparison with Group 2 and 3 only after 4 weeks. SCI induced high continuous expression of nestin and GFAP in cells from gray and white matter located around the ependymal region of the central canal.

SCI is associated with the loss of both neurons and glia. Improved functional outcomes after SCI may be elicited by neuroprotective approaches that limit secondary tissue loss and thus the loss of function. It has been postulated that this neuroprotection may occur as a result of growth factor production by transplanted cells, and this theory is supported by transfection studies with growth factors such as glial cell line derived neurotrophic factor that have been shown to improve functional recovery after stroke in a rodent model (12). The other options may be inhibition of the host immune response and inflammation (20, 23), improvement of vascularization (5) and suppression of glial scar formation (23). Alternatively, functional recovery could be elicited by axon growth and neural plasticity-promoting approaches that result in restoration of damaged and formation of new neural circuits that could become involved in functional recovery (5, 8, 16).

Regarding locomotor activity, most animals were not able to move their hindlimbs or showed only slight movement of their hip and/or knee joints immediately after contusive injury at the T10-11 segment. The locomotor deficits rapidly recovered during the first two weeks regardless of treatment. Animals with rBM-MSCs continuously improved locomotor score even after 3 weeks of injury to the extent where they could regain coordination between the fore- and hindlimbs with almost

consistent weight-supported plantar steps (average BBB scale:11). One-Way ANOVA revealed a significant treatment effect over time ($p < 0.05$).

Interestingly, we determined that there was considerably lower number of GFP+ cells migrated to the damage site in the sections of low scored animals of the Laminectomy&Trauma&MSC group (Group 4) in tests than those of higher scored ones. The results of function test scoring show parallelism with the number of the labeled cells in the damaged site after 4 weeks. This result show that there is a correlation between cell viability and migration of MSCs towards the damaged area and functional tests. These results suggest that placing rBM-MSCs into the contused spinal cord enhances recovery of open field locomotion and improves quality of the hindlimbs movement during locomotion.

CONCLUSION

The present study showed that transplantation of rBM-MSCs into the contused spinal cord enhances the extent of myelination in the spared white matter and improved locomotor recovery. Transplantation of BM-MSCs might be an effective strategy to improve functional outcomes following traumatic injuries to the spinal cord. Our study further suggests that manipulation of molecular factors governing cell fate decisions during development could influence the fate of BM-MSCs and positively affect the reparative potential of the transplantation therapy. Clinical trials with BM-MSCs in traumatic spinal cord injury might be started since the encouraging results of applications of rBM-MSCs in experimental models bode well for its therapeutic use in injuries of the human spinal cord.

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