Therapeutic Benefit of Intravenous Transplantation of Mesenchymal Stem Cells After Experimental Subarachnoid Hemorrhage in Rats

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Background: Subarachnoid hemorrhage (SAH) usually occurs when an aneurysm ruptures and bleeds into the subarachnoid space. However, no information is available regarding the therapeutic potency of transplanted mesenchymal stem cells (MSCs) for SAH. Therefore, our aim was to investigate whether MSC transplantation therapy may cause stem cell activation and improve neurologic functional recovery after induction of SAH. *Methods:* Female rats were divided into 2 groups of SAH plus phosphate-buffered saline (PBS; control) and SAH plus MSCs (experimental). Both control and experimental groups received PBS or injection of 3×10^6 male rat MSCs labeled with bromodeoxyuridine (BrdU) into the tail vein 24 hours after SAH. All animals were killed 14 days after SAH. A behavioral test (Neurological Severity Score) was performed at 1, 7, and 14 days after SAH. Immunohistochemistry was used to identify MSCs and the cells derived from MSCs in brains with SAH. Terminal deoxynucleotidyltransferase mediated dUTP-biotin nick-end labeling was used to identify apoptotic cells. *Results:* Significant functional recovery ($P < .05$) was found in SAH animals infused with MSCs compared with other rats. Significantly more BrdU-positive cells were located in the parietal lobe of MSC-treated than in PBS-treated animals. MSCs were also seen to differentiate into glial cells (GFAP), neurons (Neu-N), and endothelial cells (vWF), thereby enhancing neuroplastic effects in the injured brain. Significantly fewer apoptotic cells were found in insulted cerebral tissue in SAH plus MSC rats when compared with other groups. *Conclusions:* Intravenously transplanted MSCs improve functional recovery, reduce apoptosis, and enhance neuroplastic effects after SAH in animal models. This is a promising novel procedure to repair central nervous system damage after SAH, and may provide a new way to induce plasticity in the injured brain cells. **Key Words:** Apoptosis—mesenchymal stem cell—rat—subarachnoid hemorrhage—transplantation.
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Subarachnoid hemorrhage (SAH), a major public health problem, usually occurs when an intracranial berry aneurysm ruptures and bleeds into the subarachnoid space.[1,2] It is a serious cerebrovascular disease with high morbidity and mortality rates. Despite the recent progress that has occurred in medical and surgical treatment, the incidence of SAH remains unchanged.[1] The most complicated and disastrous outcome of aneurysmal SAH is mainly related to the development of long-term cerebral vasospasm.[2] This can lead to

decreased cerebral blood flow, resulting in neurologic deterioration, cerebral ischemia or infarction, and even death.[3] Recently, Sabri et al[1] investigated the effect of SAH on neural cells in dogs. Their data showed that SAH was associated with significant increase in neuronal and astrocytic apoptosis. This was considered a primary effect of SAH. Other studies upon a variety of laboratory animal species revealed that the earliest injury following SAH occurs in the endothelial layer followed by neuronal and astrocytic cell death.[4,5] Because endothelia not only occupy a strategic anatomic location between blood and the arterial wall but also undertake several important functional roles to maintain blood circulation, any damage to them may worsen the consequences of SAH.[6]

One possible line of effective therapy for SAH may be adult stem cell transplantation. Recent studies have shown that transplantation of bone marrow mononuclear cells achieved efficacy for treatment of cerebrovascular diseases, such as stroke.[7-13] Bone marrow stromal cells or mesenchymal stem cells (MSCs) are capable of self-renewal in a number of nonhematopoietic tissues, and they have a potential for differentiation and therefore a possible application in cell therapy.[13-16] MSCs are available for autologous transplantation and have the capacity to cross the blood–brain barrier and migrate throughout the central nervous system; MSCs can also differentiate into neural cells once they reach the injury zone.[13,17] Transplantation of adult MSCs into adult rat brain was shown to reduce functional deficits associated with cerebral injuries, such as stroke and traumatic brain injury.[16,18-20] MSCs express neuronal and astrocytic cell phenotypes and also migrate when administered in damaged cerebral tissue after stroke in rats.[13,21] Several studies have shown that systemic infusion of MSCs migrated to the ischemic cortical region.[10,11] Therefore, an alternative method to direct intracerebral MSC transplantation is to infuse these cells intravenously after the in vitro expansion of bone marrow stromal cells.[8,10] Hanabusa et al[19] reported that the systemic administration of MSCs induced angiogenesis and inhibited apoptosis of neural cells after the induction of middle cerebral artery occlusion in rats.

Therefore, in light of the utility of MSCs to treat cerebral injury and the potential intravenous route of administration, this study was designed to evaluate the therapeutic effect of MSC transplantation after experimental SAH in an animal model. To our knowledge, this is the first study to investigate the role of adult stem cells on SAH.

Materials and Methods

Animals

Sixteen female Wistar rats weighing between 275 and 300 g were used in this study. Rats were kept under standard conditions (12-hour light/dark cycle at 22-24°C, with free access to water and food). Animals were assigned to one of the 2 groups of SAH plus phosphate-buffered saline (PBS; control) and SAH plus MSCs (experimental). Six male adult rats also served as bone marrow donors. The animals were cared for in accordance with the guideline of laboratory animals at our university.

In the SAH plus PBS group, 8 rats were subjected to SAH with an injection of 0.3 mL of blood into subarachnoid space. Also, 1 mL of PBS was injected into the tail vein at 24 hours after SAH.

In the SAH plus MSC group, 8 rats were injected with 0.3 mL of blood into their subarachnoid space, followed by 3×10^6 MSCs injected intravenously 24 hours after SAH.

Induction of Subarachnoid Hemorrhage

Rats were killed with intraperitoneal injections of ketamine (80 mg/kg) and xylazine (10 mg/kg) before surgical procedures were performed. Detachment of the atlantooccipital (OA) membrane was performed by using the back of a scalpel to expose the dura and the underlying adhered arachnoid membrane as previously described.[5] A microhook was used to pierce the dura and arachnoid membrane to gain entry to the subarachnoid space. Exactly 5 mm of a 6-cm calibrated polyethylene tube (Clay Adams, Parsippany, NJ) attached distally to a 27-gauge needle was inserted in a ventrolateral direction into the opening perpendicular to the lateral side of the dorsal medulla. Therefore, the tip of the injection tube was positioned near the distal third of the basilar artery on the pons.

Donor rats were killed and their femoral arteries were isolated for blood withdrawal. Only 0.3 mL of unheparinized blood was withdrawn from the donor rat and injected through the free end of the calibrated tube into the subarachnoid space of the rats. After each injection, the tube was cleared of blood by the injection of 0.05-mL of physiologic saline. The muscle layers were used to cover the tube and the wound was closed with the skin sutured around the injection tube. Rats were given a subcutaneous injection of 10 cm^3 sterile saline and 33.3 mg/kg cefazolin antibiotic twice a day. Each rat was placed in an individual cage and allowed to recover.

Behavioral Examination

In all animals, a modified Neurological Severity Score (NSS)[22] was performed at 1, 7, and 14 days after SAH. The investigator was blinded to the examination groups. The neurologic function was graded on a scale of 0 to 18, with a normal score of 0 and a maximal deficit score of 18. NSS is a composite of sensorimotor, reflex, and balance tests. Spontaneous and motor activity of rats hung by the tail, placed on the table, and their capacity to climb a wire grid were recorded. The proprioceptive sensitivity was evaluated by reflexes from the auricle and cornea. In this examination, 1 point was awarded for the inability of rat

to perform the tasks or for the lack of a tested reflex. Scores of 1 to 6 represent mild injury; scores of 7 to 12 indicate moderate injury; and scores of 13 to 18 indicate severe injury.

Perfusion and Fixation

All animals were killed 14 days after the induction of SAH. Rat brains were fixed by transcardial perfusion with 200 mL of saline (pH, 7.2), followed by 400 mL of 4% paraformaldehyde. The entire brain was removed and stored in a laboratory jar with 4% fresh paraformaldehyde for 2 hours. The left and right parietal lobes were then dissected from each brain using a dissecting microscope (Olympus, Tokyo, Japan).

Morphologic Studies

For this portion of the experiment, light microscopy was used to examine the parietal lobe of each animal. Each parietal lobe was then cut into 7 equally spaced coronal blocks and embedded in paraffin. A series of 5-μm thick sections at various levels (100-μm intervals) was cut from each block and stained with hematoxylin–eosin (H&E) for further analysis.

Isolation and Expansion of MSCs

For the isolation of MSCs, tibias and femurs were dissected from 2-month-old male Wistar rats (200-300 g) under sterile conditions. They were cleaned from adherent soft tissues and the ends were cut with a rongeur. Bone marrow plugs were elicited by flushing the bone marrow cavity with Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Gibco, Grand Island, NY). All of these procedures were carried out on ice. Mononucleated cells were isolated from the perfusate by density gradient fractionation (1.073 g/mL; Pharmacia, Miami, FL), washed and resuspended in DMEM-LG supplemented with 15% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. All cells were incubated at 37°C with 5% humidified CO_2. After 24 hours, the medium was replaced for removal of no adherent cells, and repeated every 3 or 4 days until the cultures reached 90% of confluence. They were then harvested with 0.25% trypsin and 1 mM EDTA (Gibco) for 2 minutes at 37°C, cultured into plastic flask, and expanded for 3 passages. The mesenchymal population was isolated to the culture plate as spindle-shaped adherent cells. Proliferation and growth in primary and passage culture were observed with phase contrast microscope (Olympus IX71). It was confirmed that a majority of the adherent cells were MSCs by immunohistochemical staining with Stro-1 as special marker of MSCs and differentiation ability to adipocyte/osteocyte. To detect MSCs after injection, cultured MSCs were incubated with 10 μM bromodeoxyuridine (Brdu; Sigma Aldrich, Saint Louis, MO) for 48 hours before transplantation.

Cell Transplantation of MSCs

MSC transplantation was performed at 24 hours after induction of SAH. All transplantation procedures were performed under sterile conditions. The male MSC suspension (1 mL containing 3×10^6 cells) was slowly infused into each SAH rat via the tail vein. At the same time, the rats in the control group received an equal volume of PBS (Gibco) through the tail vein.

Immunohistochemical Assessment

To detect MSC differentiation, single and double immunohistochemical staining was performed to identify cells derived from MSCs. Also, to visualize the cellular colocalization of BrdU-specific and cell-type–specific markers in the same cells, double staining was used. After deparaffinization, sections were placed in citrate buffer (pH, 6.0) at 65°C for 2 hours. After washing in the citrate buffer, sections were incubated in 2N HCl at 37°C for 30 minutes. Sections were rinsed in 0.5% Triton X-100 for 20 minutes and incubated with an anti-BrdU antibody and antibodies for each cell marker at 4°C overnight. Cerebral sections were treated with cell-type–specific antibodies as primary antibody: a neuronal nuclear antigen (NeuN) for neurons (dilution 1:200), anti–von Willebrand factor (vWF) polyclonal antibody for endothelia (dilution 1:200), and glial fibrillary acidic protein (GFAP) for astrocytes (dilution 1:200). The combination of antibodies used in each double-immunostaining experiment was (1) rat anti-BrdU antibody and mouse anti-NeuN antibody as primary antibodies and rhodamine-labeled anti-rat IgG antibody and FITC-labeled anti-mouse IgG as secondary antibodies for BrdU-NeuN; (2) mouse anti-BrdU antibody and rabbit anti-GFAP antibody as primary antibodies and FITC-labeled anti-mouse IgG antibody and rhodamine-labeled anti-rabbit IgG antibody as secondary antibodies for BrdU-GFAP; (3) mouse anti-BrdU antibody and anti-vWF polyclonal antibody as primary antibodies and FITC-labeled anti-mouse IgG antibody and rhodamine-labeled anti-rabbit IgG antibody as secondary antibodies for BrdU-vWF. Positive and negative controls were included in each assay. After staining, the sections were observed with immunofluorescence microscopy (Zeiss, Berlin, Germany). A total of 10 microscopic fields were counted to obtain the percentage of BrdU cells that were differentiated into neurons, astrocytes, and endothelia.

Detection of Apoptosis

To evaluate the rate of apoptosis of transplanted MSCs, paraffin-embedded sections were used for terminal deoxynucleotidyltransferase mediated dUTP-biotin nick-end labeling (TUNEL). TUNEL staining was performed with a commercially available kit (ApopTag plus fluorescein kit; Chemicon, Chandlers Ford, UK). TUNEL-positive cells per field were observed in slide sections by

Table 1. *Neurologic functional test as assessed with the Neurological Severity Score on days 1, 7, and 14 after induction of subarachnoid hemorrhage*

	Day no.		
Animal group	1	7	14
SAH plus PBS	16.5	8.75	7.75
SAH plus MSC	14.5	6.25	3.5*

Abbreviations: MSC, mesenchymal stem cell; PBS, phosphate-buffered saline; SAH, subarachnoid hemorrhage.
*$P < .05$.

immunofluorescence microscopy. The numbers of TUNEL-positive cells were counted (500 nuclei) using a cell apoptosis detection kit, POD (Roche Applied Science, Mannheim, Germany). Briefly, samples were fixed in 4% paraformaldehyde, stained with TUNEL reaction mixture, and exposed to the DAB (3,3-diaminobenzidine) substrate solution for color development in a dark chamber at room temperature for 15 minutes. Samples were dehydrated in ethanol, cleared in xylene, and mounted with entelan.

Statistical Analysis

All data were expressed as mean ± standard deviation. Comparisons between groups were performed using the Student *t* test or analysis of variance. A *P* value of $< .05$ was considered statistically significant.

Results

General and Gross Examination

The SAH rats developed drowsiness, but none of them showed any signs of severe paralysis. Clot accumulation was observed over the ventral surface of the brains when observed with dissecting microscope. The clot accumulation usually extended from the medulla to the superior border of the pons in contact with basilar arteries. Clots were similarly scattered in areas around the origin of middle cerebral arteries.

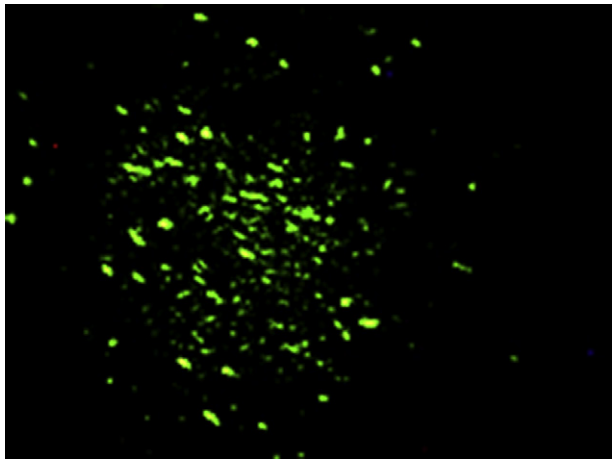

Figure 1. *Neural tissue labeling with BrdU (green for nuclei) from animals in the subarachnoid hemorrhage plus mesenchymal stem cell group (scale bar, 200 μm.).*

Neurologic Assessment

NSS values on day 1 after SAH did not differ significantly among the 2 groups (Table 1). Although both groups showed improvements in NSS assessment, scores in the MSC group on day 14 were significantly lower than those in the other group ($P < .05$). As a result, neurologic deficit decreased significantly in SAH plus MSC rats on day 14. The results showed that the highest score of 13 was recorded from rat 6 of the control group on day 1 after SAH. However, the lowest score of 2 occurred in rat 8 from the experimental group on day 14 after SAH.

Engraftment and Differentiation of Transplanted MSCs

The intravenous administration of MSCs was engrafted in the parietal lobe of brain. This was confirmed with BrdU-positive cells which were numerous in neural tissue of animals in the SAH plus MSC group (Fig 1).

No localization of BrdU-positive cells was noticed in SAH plus PBS rats. Some MSCs were positive for NeuNs and GFAPs (Figs 2 and 3). Then mean numbers of NeuNs and GFAPs distributed in parietal lobes of experimental rats were 40.37 ± 4.5 and 39.85 ± 5.6, respectively

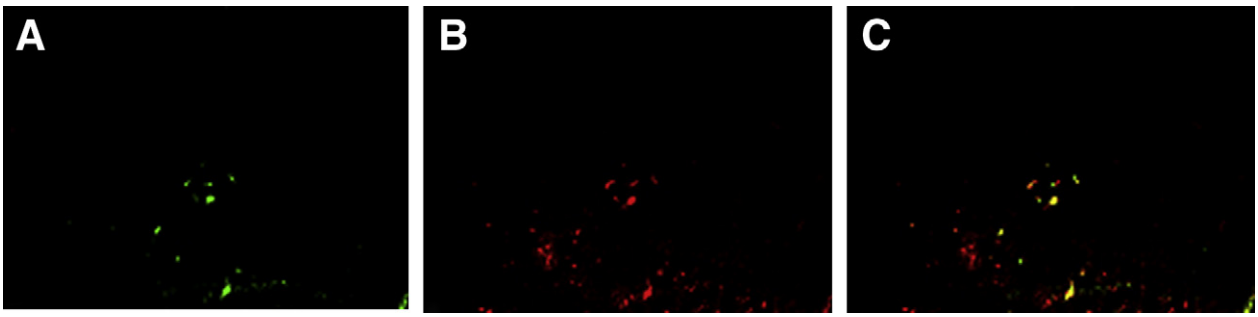

Figure 2. *Differentiation of transplanted mesenchymal stem cells (MSCs) into neurons (NeuN marker).* (A) *Labeled with BrdU (green).* (B) *Some BrdU-positive MSCs (red) express neuronal marker.* (C) *BrdU-NeuN. (Scale bar, 200 μm.).*

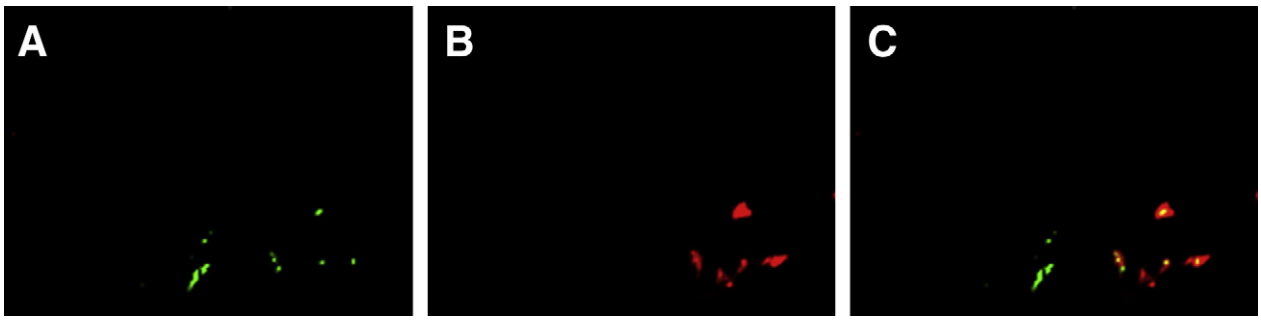

Figure 3. *Differentiation of transplanted mesenchymal stem cells (MSCs) into astrocytes (GFAP marker).* (A) *Labeled with BrdU (green).* (B) *Some BrdU-positive MSCs (red) express astrocyte marker.* (C) *BrdU-GFAP. (Scale bar, 200 μm.).*

(Table 2). This indicates that some MSCs in the injured territory of the parietal lobe exhibited signs of differentiation towards neurons and astrocytes. Also, MSCs were positive for endothelial marker vWF (Fig 4; Table 2).

Antiapoptotic Effects of MSCs on Neural Cells

Apoptotic cells (TUNEL-positive) were observed in the cerebral tissues of both SAH and SAH plus PBS rats (Fig 5). Quantitative analysis revealed that the number of TUNEL-positive cells in the SAH plus MSC (9.30 ± 1.7%) rats was significantly lower than that in the SAH plus PBS (17.42 ± 3.3%) group ($P < .05$). The data showed that infusion of MSCs decreased numbers of apoptotic cells in SAH rat model.

Discussion

For the first time, we treated SAH in female rats with the intravenous administration of male MSCs. Our data indicate that at 14 days after transplantation, intravenously injected MSCs increased the expression of neural and vascular endothelial cells, reduced neural cell apoptosis, and improved functional recovery. This was not, however, observed in the other rat group, which was treated with PBS only after induction of single SAH. MSCs were shown to survive and some express cell-type–specific markers (NeuN, GFAP, and vWF). In addition, a significant improvement in functional outcome was found in animals receiving MSCs. Functional recovery was not obtained at the early stage, but took place toward the end of the second week after injection with MSCs. It is unlikely that these cells integrate into the cerebral tissue to make appropriate connections. It is more likely that these cells act as sources of tropic factor production at the site of injury following subarachnoid bleeding. Chen et al[8] found a relation between stem cell dose and the effect in rats with stroke. They noticed a better recovery of somatosensory and NSS in animals infused intravenously with 3 million MSCs at 24 hours after stroke than did animals infused with only 1 million cells. Therefore, we also decided to use the same dose of MSCs (3 million) and the same route of infusion 1 day after SAH induction in female rats.

Table 2. *Quantitative analysis using numbers of NeuN for neurons, GFAP for astrocytic cells, and vWF for endothelial cells as measured in animals with subarachnoid hemorrhage plus mesencymal stem cells*

Parameter	Mean ± SD	Least	Most
NeuN	40.37 ± 4.5	33	45
GFAP	39.85 ± 5.6	32	41
vWF	34.44 ± 5.8	29	36

Abbreviations: BrdU, bromodeoxyuridine; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclear antigen; vWF, von Willebrand factor.

The results also indicate that MSCs were settled in the damaged area of SAH brain. This may indicate that impaired cerebral tissue may have induced migration of MSCs into the area. It should be noted that other mechanisms may have also promoted the migration of MSCs. Therefore, our results suggest that early treatment with mesenchymal cells after SAH may facilitate the migration of MSCs into the damaged cerebral region. This may therefore lower the rate of cell apoptosis and, on the contrary, improve functional recovery, as was shown in our animal model. In their study, Li and Chopp[13] reported that the migration of MSCs towards damaged brain tissue depends on the specific signals expressed in astrocytes, neurons, and endothelial cells. Other evidence suggests that the injured cerebral environment promotes the secretion of neurotrophic and bioactive factors by MSCs after neurologic insults.[9] Another study revealed that transplanted MSCs secrete neurotrophins, growth factors, and other supporting molecules after brain injury for the purpose of therapeutic benefits.[23] It has been reported that the subventricular zone in the adult brain is a good source of neuronal precursors that is capable of recruiting into nearby lesion zones.[24] Lu et al[25] believe that neuronal precursors synthesize and secrete trophic factors that may promote neural repair after insult. It has been known that various brain injuries affect the subventricular zone

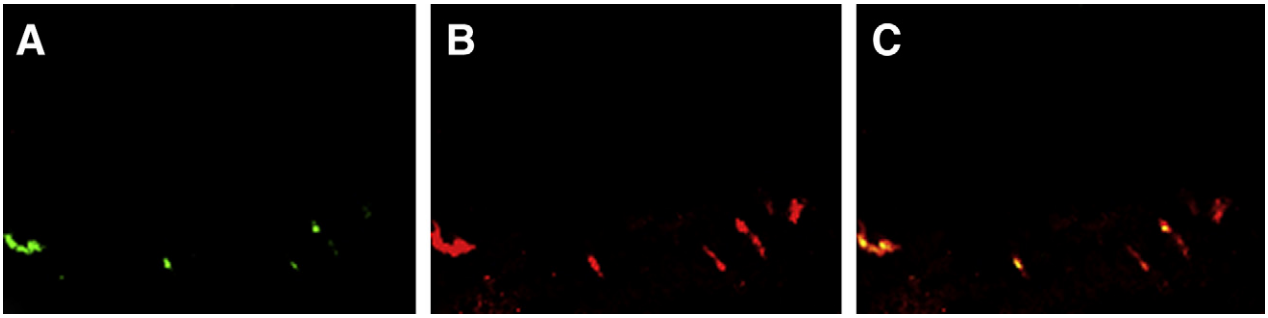

Figure 4. *Differentiation of transplanted mesenchymal stem cells (MSCs) into endothelial cells (von Willebrand factor [vWF] marker). vWF-positive cells were observed around the prevascular and endothelial regions of rats with subarachnoid hemorrhage that were treated with MSCs.* (A) *Labeled with BrdU (green).* (B) *Some BrdU-positive MSCs (red) express endothelial marker.* (C) *BrdU-vWF. (Scale bar, 200 μm.).*

differently.[26] However, the exact mechanisms of lesion-induced activation of the ventricular zone during MSC migration is not established in brain-injured mammals.

In the central nervous system, astrocytes provide many supportive activities that are essential for normal neuronal functioning.[27] These cells contact all parts of neurons and cerebral capillaries. They increase the expression of various nuurotrophic and growth factors following MSC administration in laboratory animals.[28] In addition, MSCs reduce intrinsic factors that inhibit axonal outgrowth, which may form after glial scar formation.[29] After SAH, many cerebral capillaries with their functional endothelial cells may become severely injured. It has been reported that MSCs have the capability to stimulate angiogenesis and create new blood vessels or repair them after insult. It has been revealed that MSC treatment promotes vascular endothelial growth factor (VEGF) secretion by reactive astrocytes. VEGF is considered an angiogenetic factor that can increase the survival and proliferation of endothelial cells. MSCs also mediate angiogenesis and vascular integrity through asrocytic cells.[30]

Our findings also show that MSC transplantation induced angiogenesis and lowered apoptosis of neural cells after the induction of SAH in rats. Also, differentiation of transplanted MSCs into neuronal cells took place, all of which resulted in an improvement of neurologic functions. In other investigations, MSC transplantation has been shown to improve neurologic architectures and functions in animal stroke models.[8,19,29] The beneficial effects are primarily mediated by the elevation of endogenous angiogenic and antiapoptotic factors, as well as differentiation of MSCs into neuronal and astrocytic cells.[8,19,31] MSCs in vivo have neuroprotective effects through their differentiation and secretion of angiogenic and antiapototic factors. Furthermore, MSC transplantation markedly improved neurologic functions in rats with SAH. Our results are similar to those of Chen et al,[10] who noticed a significant reduction in apoptotic cells in the ischemic boundary after MSC treatment in rats.

In conclusion, intravenously administered MSCs survive, migrate, and improve functional recovery after SAH. Because stem cells are widely available, they may be an excellent source of cells for early treatment of SAH. Therefore, these findings may be considered a novel and promising line of therapeutic strategy for the treatment of SAH. Additional studies are necessary to examine the safety and efficacy of this MSC-based therapeutic approach before its application in clinical use can be introduced.

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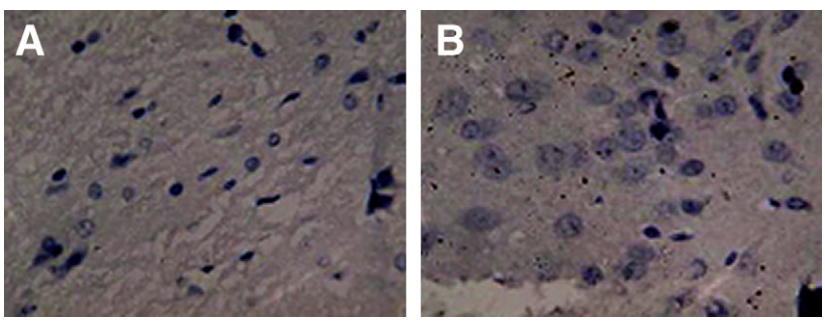

Figure 5. *Photomicrographs of neural cell apoptosis (TUNEL). The number of TUNEL-positive cells (dark nuclei) in the subarachnoid hemorrhage (SAH) plus mesenchymal stem cells (MSCs) group were markedly lower than that in the other group.* (A) *SAH plus phosphate-buffered saline group.* (B) *SAH plus MSC group. (Scale bar, 100 μm.).*

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