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Adipose-Derived Mesenchymal Stem Cells Reduce Neuronal Death After Transient Global Cerebral Ischemia Through Prevention of Blood-Brain Barrier Disruption and Endothelial Damage

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ABSTRACT

Global cerebral ischemia (GCI) is the leading cause of a poor prognosis even after successful resuscitation from cardiac arrest. Therapeutic induction of hypothermia (TH) is the only proven therapy—and current standard care—for GCI after cardiac arrest; however, its application has been significantly limited owing to technical difficulties. Mesenchymal stem cells (MSCs) are known to suppress neuronal death after cerebral ischemia. The prevention of blood-brain barrier (BBB) disruption has not been suggested as a mechanism of MSC treatment but has for TH. We evaluated the therapeutic effect of MSC administration on BBB disruption and neutrophil infiltration after GCI. To evaluate the therapeutic effects of MSC treatment, rats were subjected to 7 minutes of transient GCI and treated with MSCs immediately after reperfusion. Hippocampal neuronal death was evaluated at 7 days after ischemia using Fluoro-Jade B (FJB). BBB disruption, endothelial damage, and neutrophil infiltration were evaluated at 7 days after ischemia by immunostaining for IgG leakage, Rat endothelial antigen-1, and myeloperoxidase (MPO). Rats treated with MSCs showed a significantly reduced FJB^+ neuron count compared with the control group. They also showed reduced IgG leakage, endothelial damage, and MPO^+ cell counts. The present study demonstrated that administration of MSCs after transient GCI provides a dramatic protective effect against hippocampal neuronal death. We hypothesized that the neuroprotective effects of MSC treatment might be associated with the prevention of BBB disruption and endothelial damage and a decrease in neutrophil infiltration. STEM CELLS TRANSLATIONAL MEDICINE *2015;4:178–185*

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INTRODUCTION

Global cerebral ischemia (GCI) is one of the most challenging clinical issues encountered during cardiac arrest and usually indicates a poor prognosis, despite great advances in cardiovascular support technology. Furthermore, severe neurological deficits will develop in 33%–50% of patients who have survived a witnessed cardiac arrest. In the rare case of survival after an unwitnessed cardiac arrest, the percentage has been 100% [1].

Various methods to overcome global cerebral ischemia have been attempted, but therapeutic induction of hypothermia (TH), which lowers and maintains the core body temperature at 32°C–34°C, is the only intervention currently demonstrated to improve neurological recovery through large, randomized controlled trials [2, 3]. Hence, it has been recommended as a mainstay of standard postcardiac arrest care in the current advanced cardiac life support guidelines [4]. However, many technical difficulties in the application of this therapy, not only with respect to induction and maintenance of the recommended core temperature, but also in maintaining the recommended rate of rewarming, have hindered the frequent and routine use of TH [5, 6]. Moreover, complications could be associated with hypothermia, such as coagulopathy and infection [7, 8]. Hence, a need exists to develop novel methods to decrease delayed neuronal injury after global cerebral ischemia as an alternative or a bridge therapeutic option.

Stem cell therapy is currently regarded as one of the most promising therapeutic options for many intractable diseases and has shown neuroprotective effects in various neuronal injury and degenerative neuronal disease models [9–14]. Ohtaki et al. [15] showed that intracerebral implantation of human mesenchymal stem cells (MSCs) 1 day after the injury decreased global ischemic brain damage in a rat bilateral carotid artery occlusion model through the modulation of the inflammation/immune response. Zheng et al. [16] showed that an intravenous administration

of MSCs 3 hours after injury also reduced cerebral injury in a rat cardiac arrest model by stimulation of brain-derived neurotrophic factor secretion. Considering the potent neuroprotective properties of MSCs shown in previous studies, a more favorable neurologic outcome might be expected via earlier administration of MSCs owing to an earlier interruption in the delayed neuronal injury process after global cerebral ischemia. Blood-brain barrier (BBB) dysfunction and endothelial damage are important mechanisms known to cause ongoing neuronal damage in the early phase of cerebral ischemia and have been suggested as possible mechanisms by which TH exerts its neuroprotective effects [17–19]. Administration of MSC can also prevent BBB disruption and the endothelial damage that is initiated in the early phase of global cerebral ischemia. However, this remains untested.

We aimed to test the effect of MSCs administered immediately after global cerebral ischemia and to suggest a possible mechanism by which this treatment would be expected to act in the more acute phases. We hypothesized that immediate administration of MSCs would decrease the delayed neuronal damage after global cerebral ischemia through the mechanism associated with the prevention of BBB permeability and endothelial damage.

Materials and Methods

Ethics Statement

The present study was performed in strict accordance with the recommendations in the NIH *Guide for the Care and Use of Laboratory Animals*. The Committee on Animal Use for Research and Education at Hallym University approved the animal studies (protocol no. Hallym 2012-28). Animal sacrifice was performed using isoflurane anesthesia, and all efforts were made to minimize the rats' suffering. The institutional review board of CHA Bundang Medical Center, CHA University, approved the whole process related to the use of human adipose-derived MSCs in the present study (institutional review board approval no. BD2012-079D).

Animal Surgery and Transient Cerebral Ischemia

Transient global cerebral ischemia was induced using the method reported by Smith et al. [20]. Male, adult, Sprague-Dawley rats weighing 250–300 g were anesthetized with 2%–3% isoflurane. A catheter was placed in the femoral artery for withdrawal of blood and monitoring of blood pressure, and another catheter was placed in the femoral vein to provide an administration route for MSCs. Bilateral burr holes were made in the temporal areas of the skull to place the electroencephalographic (EEG) probes. The body temperature was maintained at 37°C $\pm$ 1.0°C using a heating blanket and heating lamp controlled by a rectal thermistor. Both common carotid arteries were exposed and clamped, and the systemic mean arterial pressure (MAP) was decreased to 40 $\pm$ 5 mmHg by withdrawing blood (7–10 ml) from the femoral artery into a heparinized syringe maintained at 37°C. Successful induction of global brain ischemia was confirmed by the presence of isoelectricity on the EEG [21, 22]. Onset of isoelectricity was defined as the point of the last 3 cortical bursts marked within a 60-second interval. After observation for 7 minutes of the isoelectric EEG signal, perfusion was restored by unclamping the carotid arteries and reintroducing blood flow to the femoral artery. After reperfusion, which was confirmed by the restoration of the baseline EEG signal, 1×10^6 human adipose-derived MSCs suspended in 1 ml of 0.9% saline were immediately administered to the rats allocated to the MSC treatment group ($n = 5$) through the femoral venous catheter. Those allocated to the vehicle control group ($n = 5$) received 1 ml of 0.9% saline only. The sham operation group ($n = 3$) also received 1 ml of 0.9% saline. The number of MSCs for injection was determined using the protocol of Zheng et al., which had previously demonstrated the beneficial effects of systemically administered MSCs on global cerebral ischemia [16]. All the rats were closely observed for 3 hours after surgery in an incubator maintained at 36°C and then moved to the temperature-controlled recovery room.

Preparation of MSCs

Human adipose-derived MSCs were isolated as previously described [23]. Adipose tissue was obtained with written informed consent from healthy female donors undergoing elective liposuction procedures at the Department of Plastic Surgery, CHA Bundang Medical Center, CHA University, Gyeonggi-Do, Korea. The collected tissue was mixed with the same volume of phosphate-buffered saline (PBS) with 2% gentamicin and centrifuged at 1,500 rpm for 5 minutes at room temperature. Next, the centrifugate was enzymatically digested by a mixture of trypsin, DNase I, and collagenase I at 37°C for 60 minutes under shaking conditions. The digested tissue was centrifuged at 1,500 rpm for 5 minutes and resuspended in saline, for a total of 2 times. The cell pellet was filtered through a 100-μm pore-size filter and centrifuged 1 more time to separate the adipose tissue-derived stem cells from the surrounding tissue. Next, 2×10^5 isolated cells were expanded with 15 ml of the culture medium (α-minimal essential medium with 10% fetal bovine serum, 1% penicillin/streptomycin) in a T75 flask and cultured at 37°C in a 0.05% CO_2 incubator for 6–7 days until the cell count reached 3×10^6. Fluorescence-activated cell sorting analysis was used to identify the phenotype of the cells. The expression of CD44, CD73, CD90, CD105, and human leukocyte antigen (HLA)-ABC and the lack of CD45, CD34, CD31, and HLA-DR were checked to confirm the MSC identity of the cells. The cells were used at passage 2.

Tissue Preparation

The rats were euthanized 7 days after ischemia. The rats received an overdose of urethane anesthesia (1.5 g/kg i.p.) and were perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed immediately and postfixed in the same fixative for 1 hour. The brain tissues were cryoprotected by submersion in 30% sucrose overnight. Thereafter, the entire brain was frozen and sectioned using a cryosliding microtome at a 30 μm thickness and stored in cryoprotective solution.

Assessment of Neuronal Death

To identify the degenerating neurons, Fluoro-Jade B (FJB; Histo-Chem, Jefferson, AR, http://www.histo-chem.com) staining was performed as described previously [24]. In brief, the sections were immersed in a basic alcohol solution for 5 minutes and 0.06% $KMnO_4$ for 15 minutes. Next, the sections were incubated in 0.0004% FJB (Histo-Chem) for 20 minutes. The slides were washed in distilled water and dried. To quantify neuronal death, the sections were collected every third cut from 4.0 mm posterior to the bregma, and five coronal sections were analyzed from each rat. Five coronal sections were collected from each rat, spaced 80 μm apart, starting 4.0 mm posterior to the bregma. An

observer who was unaware of the treatment condition counted the number of FJB^+ neurons in the hippocampal CA1 region under a ×10 objective microscopic field. The mean count of the FJB^+ neurons were used for the statistical analyses. Three sham surgery rats were also evaluated, and these showed no detectable neuronal death.

Detection of Live Neurons

To identify live neurons, the brain sections were immunohistochemically stained with NeuN. Monoclonal anti-NeuN, clone A60 antibody (diluted 1:100, EMD Millipore, Billerica, MA, http://www.emdmillipore.com) was used as the primary antibody in PBS containing 0.3% Triton X-100 overnight at 4°C. The sections were washed 3 times for 10 minutes with PBS, incubated in biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and ABC complex (Vector Laboratories), diluted 1:250 in the same solution as the primary antiserum. Between the incubations, the tissues were washed with PBS 3 times for 10 minutes each. The immune reaction was visualized using 3′3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) in 0.01 M PBS and mounted on the gelatin-coated slides. The immunoreactions were observed using the Axioscope microscope (Carl Zeiss, Munchen-Hallbergmoos, Germany, http://www.zeiss.com).

Detection of BBB Disruption Using IgG Immunostaining

The rats were examined for the extravasation of presumed endogenous serum IgG after ischemia for the detection of BBB disruption [25]. Five coronal sections were collected from each rat, spaced 80 μm apart, starting 4.0 mm posterior to the bregma. Coronal sections (30 μm thick) were incubated with rabbit serum, followed by purified biotinylated rabbit anti-rat IgG (Vector Laboratories) at a dilution of 1:250. The ABC immunoperoxidase method was used to detect IgG-like immunoreactivity [26]. From the brain section images, the IgG-stained area was measured with ImageJ, version 1.47 (National Center for Biotechnology Information, Bethesda, MD, http://www.ncbi.nlm.nih.gov). Measurement of the IgG-stained area was quantified using the method modified from Tang et al. [27]. The selected part of hippocampus in the whole image was sorted, and the area of IgG leakage was expressed as the %area.

Quantification of Endothelial Vasculature Damage

At 7 days after ischemia, the integrity of the brain vasculature was evaluated by immunostaining with the Rat endothelial antigen-1 (RECA-1) antibody [28]. The RECA-1 antibody is known to identify endothelial cells and blood vessels. To quantify the RECA-1 immunoreactive area, the image was loaded into ImageJ, version 1.47, and converted to an 8-bit image through the menu options Image/Type/8-bit. Next, the image was thresholded using the menu option Image/Adjust/Threshold. The type was set to black and white and the bottom slider moved to a value sufficient to show only the RECA-1 immunoreactive area. The resulting thresholded image was binary and only reflected RECA-1 immunoreactivity. To measure the area, the menu option Analyze/Measure was selected.

Assessment of Neutrophil Infiltration

To detect neutrophil infiltration in the hippocampus after ischemia, the brain sections were immunohistochemically stained with myeloperoxidase (MPO) antibody. MPO is a heme protein synthesized during myeloid differentiation that constitutes the major component of neutrophil azurophilic granules. The ABC immunoperoxidase method was used to detect MPO^+ cells [26].

Functional Analysis

The tape removal test was performed to evaluate the functional behavior of the rats using the method of Albertsmeier et al., with slight modifications [29]. Two pieces of 10-mm × 12-mm adhesive tape were placed on both forepaws of the rats in random order. The interval until the rat had removed both pieces was measured. Observation was stopped when the duration had reached 180 seconds and was recorded as "180 seconds." One test each day was conducted for 3 consecutive days before ischemia or sham operation to familiarize the subjects with the test. The tests were performed 5 times at 7 days after transient global ischemia or the sham operation, and the median durations were used for analysis. The same investigator conducted all the measurements.

Statistical Analysis

The numerical values are expressed as the mean ± SEM. Analysis of variance with the Bonferroni post hoc test was used to compare the values of the experimental groups. Statistical significance was defined as $p < .05$. IBM SPSS Statistics software, version 21.0 (IBM Corp., Armonk, NY, http://www-01.ibm.com/software/analytics/spss/), was used for the statistical calculations. A sample size of 12 was calculated to have sufficient power to detect a significant difference in the number of FJB^+ neurons in each group (effect size f = 1.48, power = 0.95), using G*Power, version 3.1 (Heinrich-Heine Universität, Düsseldorf, Germany, http://www.gpower.hhu.de/en.html) [30].

Results

Three sham-operated, 5 vehicle-controlled, and 5 MSC-administered rats were sacrificed 7 days after ischemia/reperfusion and used for analysis. No significant differences were found in the core body temperatures and MAPs before, during, and after ischemia induction between the vehicle control and MSC treatment groups (Table 1).

MSC Treatment Reduced Hippocampal Neuronal Death After Ischemia

MSCs have been described to act in a neuroprotective manner in animal models of cerebral ischemia. To test whether MSC treatment showed neuroprotective effects after ischemia, the rats were sacrificed 7 days after ischemia with or without MSC injection. At 7 days after ischemia, we performed FJB staining to detect any degenerating neurons and NeuN staining to detect surviving neurons in the hippocampal CA1 area. No degenerating neurons were detected by FJB staining in the sham operation group. The number of FJB^+ neurons between the sham and ischemia-induced groups was highly significantly different ($p < .001$). The vehicle control and MSC groups showed significant differences in the number of degenerating neurons in the post hoc analysis (162.40 ± 17.54 vs. 17.28 ± 5.36 cells per field, $p < .001$) (Fig. 1). More $NeuN^+$ neurons were found in the MSC-treated group. Also, a significant difference was found in the count of $NeuN^+$ neurons among the sham and ischemia-induced groups ($p < .001$).

Table 1. Core body temperature and mean arterial pressure of rats before, during, and after ischemia induction and reperfusion

Variable	Vehicle control	MSCs administered	*p* value
Core BT (°C)			
Before ischemia	36.86 ± 0.22	36.74 ± 0.11	.646
During ischemia	37.10 ± 0.11	36.82 ± 0.14	.158
After ischemia	36.52 ± 0.12	36.26 ± 0.20	.304
MAP (mmHg)			
Before ischemia	112.86 ± 2.40	119.68 ± 3.72	.163
During ischemia	45.29 ± 0.55	43.02 ± 0.85	.055
After ischemia	117.46 ± 3.52	127.94 ± 9.21	.319

Data are presented as mean ± SEM. *p* values were calculated using the *t* test.
Abbreviations: BT, body temperature; MAP, mean arterial pressure; MSCs, mesenchymal stem cells.

Post hoc analysis revealed a significant difference between the vehicle control and sham groups (155.24 ± 27.93 vs. 294.83 ± 19.80 cells per field, $p = .007$) and the vehicle control and MSC-treated groups (155.24 ± 27.93 vs. 241.40 ± 14.05 cells per field, $p = .047$) (Fig. 2).

MSC Treatment Reduced Ischemia-Induced BBB Disruption

To evaluate the putative breakdown of the BBB, we searched for a leakage of serum IgGs using immunohistochemistry [25]. In normal animals, the IgGs will be restricted to the vessels. In contrast, in the ischemic rats, we observed an increase in diffuse IgG immunoreactivity throughout the hippocampus compared with the sham-operated rats (Fig. 3). Immunoglobulin leakage by BBB breakdown was apparent in the vehicle-treated group. However, administration of MSCs clearly diminished ischemia-induced immunoglobulin leakage through BBB disruption in the hippocampus parenchyma (Fig. 3). A significant difference was found in IgG leakage among the sham and ischemia-induced groups ($p < .001$). Post hoc analysis revealed significant differences between the vehicle control and sham groups (3.08 ± 0.24 vs. 1.19 ± 0.06 %area, $p < .001$) and between the vehicle control and MSC-treated groups (3.08 ± 0.24 vs. 1.51 ± 0.09 %area, $p < .001$) (Fig. 3).

MSC Treatment Reduced Ischemia-Induced Damage of Endothelial Vasculature

In normal rats, RECA-1 immunoreactivity was relativity weak, and the vascular structure seemed to be well preserved. In contrast, in the ischemic rats, we observed increased RECA-1 immunoreactivity and engorged vasculature throughout the hippocampus compared with the sham-operated rats (Fig. 4). RECA-1 immunoreactivity due to endothelial damage was apparent in the vehicle-treated group. However, administration of MSCs diminished ischemia-induced RECA-1 immunoreactivity in the hippocampus parenchyma (Fig. 4). A significant difference was found in RECA-1 immunoreactivity among the sham and ischemia-induced groups ($p < .001$). Post hoc analysis revealed a significant difference between the vehicle control and sham groups (23.46 ± 1.14 vs. 12.70 ± 0.42 %area, $p < .001$) and the vehicle control and MSC-treated groups (23.46 ± 1.14 vs. 14.94 ± 0.48 %area, $p < .001$) (Fig. 4).

MSC Treatment Reduced Neutrophil Infiltration Induced by Ischemia

It has been reported that infiltrating neutrophils contribute to neurological brain damage [31, 32]. To test whether infiltrating neutrophils were observed with ischemic brain injury and whether treatment with MSCs afterward could reduce the presence of infiltrating neutrophils, we performed immunohistochemistry with anti-MPO to observe the degree of neutrophil infiltration in the postischemic hippocampus. In the saline- or MSC-treated rats, the MPO^+ cells were sparse in the hippocampus. Transient cerebral ischemia increased the number of infiltrating neutrophils in the CA1 pyramidal layer, an area vulnerable to ischemia. However, MSC administration reduced neutrophil infiltration in the hippocampal formation, mainly in the CA1 layer (Fig. 5). A significant difference was found in the MPO^+ cell count among the sham and ischemia-induced groups ($p < .001$). Post hoc analysis revealed a significant difference between the vehicle control and sham groups (20.00 ± 3.13 vs. 0.27 ± 0.03 cells per field, $p < .001$) and the vehicle control and MSC-treated groups (20.00 ± 3.13 vs. 3.12 ± 0.62 cells per field, $p < .001$) (Fig. 5).

MSC Treatment Reduced Behavioral Impairment

To evaluate the behavioral function of the rats, we measured the interval required for the rats to remove the adhesive tapes from both forepaws. A significant difference was found in the tape removal time among the sham and ischemia-induced groups ($p < .001$). Post hoc analysis revealed significant differences between the vehicle control and sham groups (168.6 ± 11.4 vs. 20.0 ± 7.9 seconds, $p < .001$) and the vehicle control and MSC-treated groups (168.6 ± 11.4 vs. 46.6 ± 9.8 seconds, $p < .001$).

Discussion

The results of the present study suggest, proof-of-principle, that administration of MSCs immediately after transient global cerebral ischemia/reperfusion can prevent neuronal death. This neuroprotection is associated with a suppression of BBB disruption, endothelial damage, and neutrophil infiltration during the ischemic neuronal damage process.

The hippocampus, especially the CA1 zone, is known to be one of the most vulnerable areas for delayed neuronal death after global cerebral ischemia and is considered to provide a possible target for pharmacological intervention [33–35]. This was why most studies of global cerebral ischemia, including ours, have focused on providing neuroprotection of the hippocampal CA1 region. The result of FJB staining showed a marked decrease in the dysfunctional neuron count at the hippocampal CA1 region in the MSC-administered group compared with the vehicle control group. This trend was concordant with the results of NeuN staining, which showed a greater number of live neurons in the MSC group. However, the trend shown by NeuN staining was relatively less prominent compared with the result of FJB staining. It might that this discrepancy resulted from differences in the two staining techniques and how they reflect neuronal viability [36]. However, the results of FJB and NeuN staining both suggested a possible neuroprotective effect of MSC treatment and correlated well with the results of previous studies [15, 16]. This conclusion was also supported by the results of the functional analysis. In addition to the previous evidence, our results showed that immediate

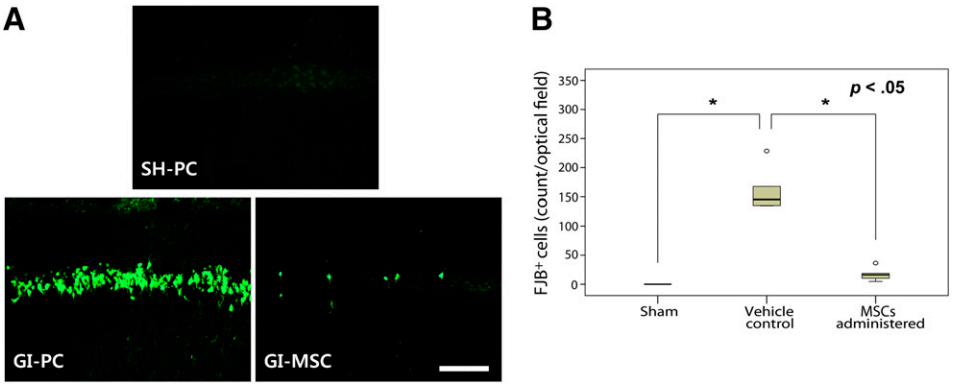

Figure 1. Ischemia-induced degeneration of hippocampal neurons is decreased by MSC treatment. **(A):** Transient cerebral ischemia caused neuronal death in the hippocampal CA1 region 1 week after insult. Fluorescence images show several FJB^+ neurons in the CA1 area after ischemia. Intravenous injection of MSCs after reperfusion provided protective effects on hippocampal neuronal death after ischemia compared with the vehicle-treated group. Scale bar = 100 μm. **(B):** Box whisker plot shows the quantification of neuronal degeneration in the hippocampus. The number of FJB^+ neurons was significantly different among the groups on analysis of variance ($p < .001$). The post hoc analysis revealed significant differences between the vehicle control and MSC-treated groups ($p < .001$) and the vehicle control and sham operation groups ($p < .001$) in the hippocampus. ∗, statistically significant result from post hoc analysis; ○, outlier case. Abbreviations: FJB, Fluoro-Jade B; GI-MSC, human MSC-treated group after ischemia; GI-PC, vehicle control group after ischemia; MSC, mesenchymal stem cell; SH-PC, sham operation group with vehicle control.

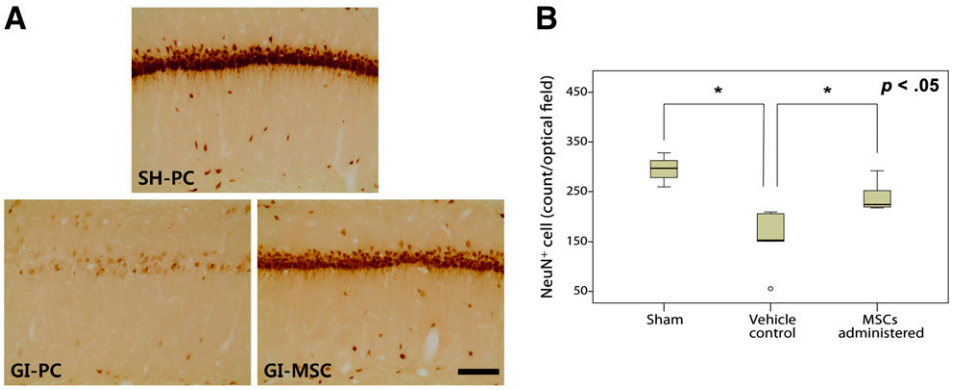

Figure 2. Survival of hippocampal neurons after transient global cerebral ischemia was increased by MSC treatment. **(A):** More $NeuN^+$ neurons in the CA1 area were observed in the sham and MSC-administered group than in the vehicle control group. Scale bar = 250 μm. **(B):** Box whisker plot shows the quantification of surviving neurons in the hippocampus. The number of $NeuN^+$ neurons was significantly different among the groups on analysis of variance ($p < .001$), and post hoc analysis revealed significant differences between the vehicle control and MSC-treated groups ($p = .047$) and the vehicle control and sham operation groups ($p = .007$) in the hippocampus. ∗, statistically significant result from post hoc analysis; ○, outlier case. Abbreviations: GI-MSC, human MSC-treated group after ischemia; GI-PC, vehicle control group after ischemia; MSC, mesenchymal stem cell; SH-PC, sham operation group with vehicle control.

administration of MSCs after global cerebral ischemia has a significant neuroprotective effect. However, the exact timing with respect to the administration of MSCs might not be a critical factor in explaining the fundamental action of MSCs on global cerebral ischemia. Considering the known multimodal actions of MSCs, they might be helpful, not only for neurologic recovery, but also for supporting unstable systemic conditions, including cardiovascular instability. We can expect a more favorable prognosis by the comprehensive action of MSCs in the postcardiac arrest state, the most important and common clinical condition causing global cerebral ischemia, if administered earlier. However, we did not compare the difference in MSC efficacy as a function of the timing of administration but only showed the effectiveness of immediately administered MSCs. Additional evaluations might be necessary to uncover the possible merit of administration of stem cells in postcardiac arrest models.

The results of IgG staining revealed a marked decrease in the IgG-stained area in the MSC-administered group, similar to that of the sham operation group and compared with the vehicle treated control group. This suggests that neuroprotection via MSC administration might be associated with either restoration or protection from BBB disruption, one of the most important mechanisms observed to be related to ischemic neuronal injury [19]. This result correlates with that of RECA-1 and MPO staining, which showed marked decreases in endothelial damage and neutrophil infiltration in the MSC-administered group. We can assume that administered MSCs might decrease the damage to the endothelial vasculature and stabilize BBB disruption and the subsequent influx of immune cells, which can prevent or slow ongoing neuronal injury after cerebral ischemia/reperfusion. Prevention of BBB permeability has been suggested as a mechanism of action of TH on global cerebral ischemia [18] but not previously for stem cell administration. The prominent IgG leakage observed in the vehicle control group might also be able to explain how systemically administered MSCs can reach the brain. Increased BBB permeability due to ischemia might play a role in importing MSCs,

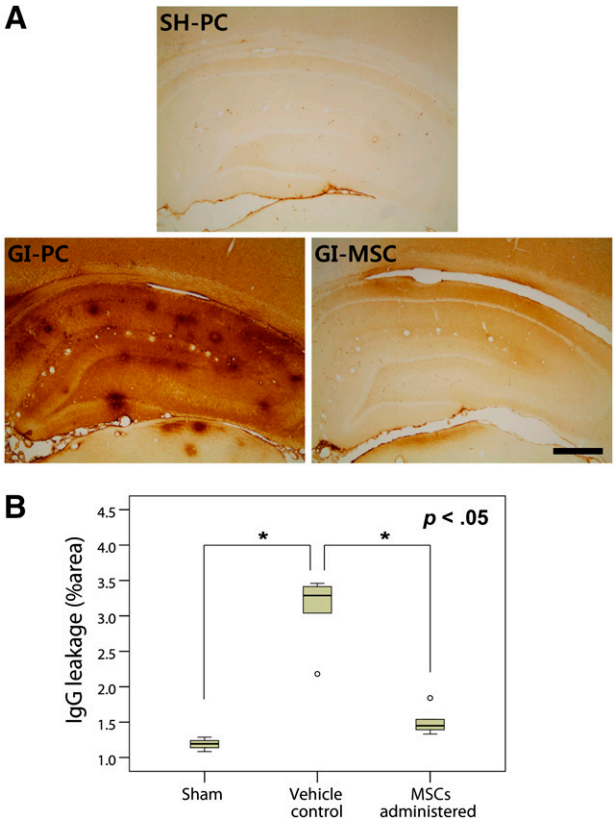

Figure 3. Ischemia-induced blood-brain barrier (BBB) damage was reduced by MSC treatment. BBB damage in the hippocampus after ischemia shown. **(A):** Low-magnification photomicrographs showing IgG-stained coronal hippocampal sections. Sham-operated rats showed sparse IgG staining in the hippocampus. At 1 week after ischemia, the entire hippocampus was intensely stained with IgG immunoreactivity, indicating that substantial BBB damage had occurred in the vehicle-treated rats. Injection of MSCs after ischemia reduced the intensity of IgG staining in the hippocampus compared with that in the vehicle-treated group. Scale bar = 500 μm. **(B):** Box whisker plot shows the quantification of IgG intensity in the hippocampus. The intensity was significantly different among the groups on analysis of variance ($p < .001$), and post hoc analysis revealed significant differences between the vehicle control and MSC-treated groups ($p < .001$) and vehicle control and sham operation groups ($p < .001$) in the hippocampus. *, statistically significant result from post hoc analysis; ○, outlier case. Abbreviations: GI-MSC, human MSC-treated group after ischemia; GI-PC, vehicle control group after ischemia; MSC, mesenchymal stem cell; SH-PC, sham operation group with vehicle control.

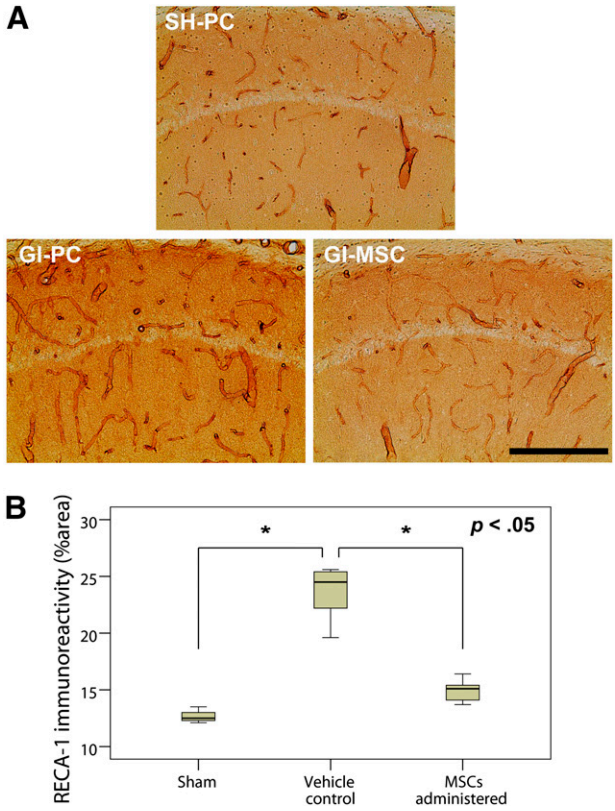

Figure 4. Ischemia-induced damage of endothelial vasculature was reduced by MSC treatment. The damage to the endothelial vasculature in the hippocampus after ischemia is shown. **(A):** High magnification photomicrographs show RECA-1-stained hippocampal sections. Sham-operated rats showed sparse RECA-1 immunoreactivity in the hippocampus. At 1 week after ischemia, the entire hippocampus was intensely stained with RECA-1 immunoreactivity, indicating that substantial endothelial damage and engorgement and distortion of vessels had occurred in the vehicle-treated rats. Injection of MSCs after ischemia reduced the intensity of RECA-1 immunoreactivity in the hippocampus compared with that in the vehicle-treated group. Scale bar = 200 μm. **(B):** Box whisker plot shows the quantification of RECA-1 immunoreactivity in the hippocampus. The intensity was significantly different among the groups on analysis of variance ($p < .001$), and post hoc analysis revealed significant differences between the vehicle control and MSC-treated groups ($p < .001$) and vehicle control and sham operation groups ($p < .001$) in the hippocampus. *, statistically significant result from post hoc analysis; ○, outlier case. Abbreviations: GI-MSC, human MSC-treated group after ischemia; GI-PC, vehicle control group after ischemia; MSC, mesenchymal stem cell; RECA-1, Rat endothelial antigen-1; SH-PC, sham operation group with vehicle control.

which cannot directly pass through the BBB under normal conditions, to the brain.

We would not expect the replacement of damaged neurons by newly differentiated cells arising from the grafted MSCs in our experiment, considering that the cells were administered systemically to an immunocompetent xenograft host, which might represent a poor setting for survival. Furthermore, systemically injected cells will not be expected to pass the BBB in general. However, some previous studies have reported the beneficial effect of systemically injected human MSCs on rat cerebral ischemia and the survival of the grafted cells in the host brain [16, 37]. Recently, the beneficial effects of MSCs on brain ischemia have been explained by the effect of various cytokines and chemokines induced by MSC treatment, rather than by the survival and differentiation of grafted cells [38]. Its mechanisms of actions were suggested as the neuroprotective effect of neurotrophic factors secreted from MSCs such as brain-derived neurotrophic factor, stromal cell-derived factor-1, insulin-like growth factor, vascular endothelial growth factor, and glial cell line-derived neurotrophic factor [16, 37, 39, 40], increased endogenous neurogenesis [40, 41], and modulation of the inflammatory and immune responses [15, 40]. In our study, those neuroprotective effects were thought to be related to the prevention of BBB disruption and endothelial damage. Additional investigation is necessary to elucidate the specific mechanisms underlying the action of grafted MSCs and their effects on the stability of the BBB and endothelium of the host brain.

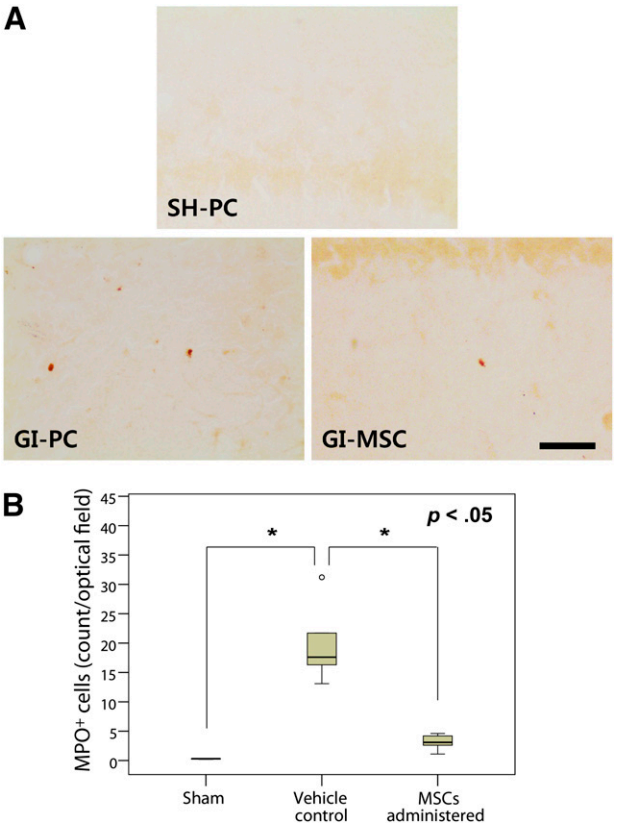

Figure 5. Ischemia-induced neutrophil infiltration in the hippocampus was reduced by MSC treatment. **(A):** Light photomicrographs from coronal sections of the rat hippocampus demonstrating that neutrophil infiltration by MPO staining 1 week after ischemia. MPO is a heme protein synthesized during myeloid differentiation that constitutes the major component of neutrophil azurophilic granules. At 1 week after ischemia, MPO^+ cells were detected in the hippocampal CA1. Injection of MSCs immediately after ischemia reduced the number of MPO^+ cells in the hippocampus compared with the vehicle-treated group. Scale bar = 20 μm. **(B):** Box whisker plot shows the quantification of MPO^+ cells in the hippocampus. The number of MPO^+ cells was significantly different among the groups on analysis of variance ($p < .001$), and post hoc analysis revealed significant differences between the vehicle control and MSC-treated groups ($p < .001$) and vehicle control and sham operation groups ($p < .001$) in the hippocampus. *, statistically significant result from post hoc analysis; ○, outlier case. Abbreviations: GI-MSC, human MSC-treated group after ischemia; GI-PC, vehicle control group after ischemia; MPO, myeloperoxidase; MSCs, mesenchymal stem cells; SH-PC, sham operation group with vehicle control.

Although it lacked statistical significance, the relatively low p value (slightly greater than .05) calculated from the comparison of MAPs from the vehicle control and MSC-administered groups during the ischemia induction procedure might indicate a possible difference between both groups in the present study. However, this would not seem likely to lead to a significant difference in the ischemic insult, considering that the difference was only approximately 2 mmHg, and an approximately 70-mmHg reduction in MAP at baseline was applied to both groups to achieve an isoelectric EEG. Furthermore, the MAP was lower in the MSC-administered group, which would be thought on its own to cause a more severe ischemic insult in the MSC group. Hence, the superior results shown in the MSC group could be regarded as the effect of MSC administration and not the differential effectiveness of the ischemic insult.

Our study did contain a notable limitation. Just as in all previous studies that have assessed the neuroprotective effect of MSCs, we did not compare the efficacy of MSC-based neuroprotection after global cerebral ischemia with that of TH, the current standard therapeutic option. However, the effect of MSC administration on global cerebral ischemia appeared to be superior—or at least not inferior—to that of the current standard.

Conclusion

Administration of MSCs decreased the delayed neuronal damage in a transient global cerebral ischemia model by prevention of BBB disruption, endothelial damage, and neutrophil infiltration.

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Author Contributions

T.N.C.: conception and design, financial support, manuscript writing; J.H.K. and B.Y.C.: collection and/or assembly of data, data analysis and interpretation; S.P.C.: administrative support, data analysis and interpretation; S.W.K. and S.W.S.: conception and design, financial support, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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Adipose-Derived Mesenchymal Stem Cells Reduce Neuronal Death After Transient Global Cerebral Ischemia Through Prevention of Blood-Brain Barrier Disruption and Endothelial Damage

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