Effect of adipose derived mesenchymal stem cells administration and therapeutic induction of hypothermia on delayed neuronal death after transient global cerebral ischemia

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Directed by Professor Sung Phil Chung

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This certifies that the Doctoral Dissertation of Tae Nyoung Chung is approved.

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ABSTRACT

Effect of adipose derived mesenchymal stem cells administration and therapeutic induction of hypothermia on delayed neuronal death after transient global cerebral ischemia

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Background: Global cerebral ischemia is the most important cause of poor prognosis after successful resuscitation from cardiac arrest. Therapeutic induction of hypothermia (maintenance of core body temperature between 32℃ and 34℃ for 12 to 24 hr, TH) has shown its efficacy in reduction of the neurologic damage from global cerebral ischemia through various laboratory and clinical researches, and it is recommended as one of the standard post-resuscitation managements. However, alternatives or complements for TH are necessary due to its technical difficulty in induction of recommended temperature and keeping recommended rate of rewarming, which limits the application of TH. We aimed to show the effect of stem cell on the neurologic recovery after transient global cerebral ischemia including the comparison with that of current standard therapy, TH.

Materials and methods: Rats were subjected to 7 min of transient global cerebral ischemia and randomized into 4 intervention groups: placebo control, TH, human mesenchymal stem cell (MSC), and combined TH and MSC, along with 4 sham operation groups with same intervention. Hippocampal neuronal death was evaluated at 7 days after ischemia by Fluoro Jade B staining. Activated microglia and infiltrating macrophages were evaluated at 7 days after ischemia by immunostaining for CD11b. IgG immunostaining was

performed to detect blood brain barrier (BBB) disruption, and myeloperoxidase (MPO) immunostaining was done to detect neutrophil infiltration in the hippocampus. 4HNE immunostaining was performed to detect oxidative injury. The time until the animal removed adhesive tapes on their both forepaws was measured to test the behavioral function, a week after the insult.

Results: No degenerating neuron was detected by Fluoro-Jade B staining in any of the sham operation groups. Analysis of variance (ANOVA) showed significant differences in degenerating neuron count among 4 ischemia induced groups at CA1, CA3, and hilus region ($p = <0.001$, 0.004, and 0.033, respectively). Post hoc analysis revealed significant differences: between control and TH, between control and MSC, and between control and TH/MSC in CA1; between control and MSC, and between control and TH/MSC in CA3; between control and MSC in hilus. Significant difference in microglial activation was found among 8 sham operation and ischemia groups through ANOVA ($p<0.001$). Post hoc analysis showed: no significant difference among 4 sham operation groups (control, TH, MSC, and TH/MSC with sham operation); significant differences between sham groups and control, between sham groups and TH, between sham MSC and MSC, between control and TH, between control and MSC, between control and TH/MSC, between TH and MSC, and between TH and TH/MSC. ANOVA showed a significant difference in IgG leakage among 8 sham operation and ischemia groups ($p<0.001$). Post hoc analysis revealed: no significant difference among 4 sham operation groups; significant differences between sham groups and control, between control and TH, between control and MSC, and between control and TH/MSC. ANOVA failed to show a significant difference in MPO(+) cell count among 8 sham operation and ischemia groups ($p=0.052$). Significant difference in 4HNE intensity was found among 8 sham operation and ischemia groups through ANOVA ($p<0.001$). Post hoc analysis showed: no

significant difference among 4 sham operation groups; significant differences between sham groups and control, between sham groups and TH, between control and TH, between control and MSC, between control and TH/MSC, between TH and MSC, and between TH and TH/MSC. Significant difference of the time spent to detach the adhesive tapes on forepaws among 5 normal and ischemia groups was found through ANOVA ($p<0.001$), and post hoc analysis showed the differences between placebo controlled ischemia group and other groups.

Conclusions: Administration of MSC after transient global cerebral ischemia has a prominent protective effect on delayed hippocampal neuron death comparing with TH, current standard treatment option. The present results also suggest combined treatment of MSC and hypothermia warrants a potential therapeutic strategy for intervention of global cerebral ischemia after cardiac arrest.

Keywords: Global cerebral ischemia, mesenchymal stem cell, therapeutic induction of hypothermia

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I. Introduction

Although recent advances in resuscitation techniques have allowed for increases in the rate of return of spontaneous circulation (ROSC) to 25-50%, only 2-10% of cardiac arrest victims achieve significant neurologic recovery.[1-3] Furthermore, severe neurological deficits develop in 33-50% of patients who have survived from a witnessed cardiac arrest. In the rare case of survival from an unwitnessed cardiac arrest, the figure is 100%.[4] Post-cardiac arrest syndrome, which includes symptoms such as decreased myocardial function, systemic ischemia/reperfusion reaction, and delayed apoptotic neuronal cell death has been suggested as a cause of the poor prognosis of cardiac arrest survivors.[5] Vigorous resuscitative efforts using hemodynamic monitoring or the percutaneous cardiopulmonary bypass have markedly increased the survival of the heart but have not been shown to affect neurologic outcome. Therefore, the latent and persistent neurological injuries that accompany global cerebral ischemia due to cessation of systemic blood flow may be the

greatest obstacle to a full recovery from cardiac arrest.[4]

Therapeutic induction of hypothermia (TH), which lowers and maintains the core body temperature between 32℃ and 34℃, has been shown to reduce neurologic damage following global cerebral ischemia through various laboratory and clinical research studies. Thus, it is recommended as one of the standard post-resuscitation management strategies in the current guidelines for advanced cardiac life support.[6-9] TH can be achieved by various methods including cold saline infusion, ice bag application, and use of commercial devices such as cooling blankets and cold water circulating gel patches. However, there are many technical difficulties in application of this therapy, not only with respect to induction and maintenance of the recommended core temperature but also in keeping the recommended speed of rewarming, and for these reasons the frequency of TH remains low.[10, 11] Moreover, considering that the definite clinical evidence demonstrating the efficacy of TH is limited to cases of cardiac arrest due to ventricular fibrillation and ventricular tachycardia, the results of one recent study that failed to show an improved neurological prognosis - despite TH being applied after an in-hospital cardiac arrest - suggests that TH may not be a singular treatment option and that alternative or complementary strategies need to be identified.[12, 13]

Other potential neuroprotective materials such as PEP-1-SOD1, which has been shown to provide bioavailable superoxide dismutase, and sodium sulfide (Na2S), which has been shown to lower core body temperature by metabolic slowing, have been suggested as agents for protecting the brain from global ischemic injury during cardiac arrest.[14, 15] However, no neuroprotective agents have thus far been shown to match the clinical value of TH.

Stem cell therapy is currently one of the most promising strategies for the treatment of many incurable diseases, and has shown neuroprotective effects

in various neuronal injury and degenerative neuronal disease models.[16-21] Ohtaki et al.[22] showed that direct implantation of mesenchymal stem cells (MSC), which were derived from human bone marrow, into dentate gyrus of the brain of rat decreased global ischemic brain damage from bilateral carotid artery occlusion by modulation of inflammation/immune response. Zheng et al.[23] showed that an intravenous administration of human bone marrow-derived MSC lessened the brain damage following cardiac arrest by secretion of brain-derived neurotrophic factor (BDNF) in rat model. These results may strongly encourage the use of stem cell for neuroprotection in global cerebral ischemia. However, there are no studies that directly compare the effect of MSC with that of TH. In order to be suggested as an alternative option or complement of TH, MSC must be able to demonstrate a superior or equivalent level of neuroprotective compared to TH, and should not show any antagonistic effect in case of combined use with TH. Furthermore, a more complete explanation of possible mechanisms of action of MSC on global cerebral ischemia is necessary, including the effect on more delayed neuronal damage.

We aimed to evaluate the effect of MSC on transient global cerebral ischemia and directly compare it to that of TH. Also, we aimed to assess possible interaction between MSC and TH in affecting the prognosis of delayed neuronal death. Furthermore, we attempt to put forth a comprehensive mechanism of the effect of MSC on transient global cerebral ischemia.

II. Materials and Methods

The surgical and animal care procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Hallym University in Chuncheon, Korea (Protocol # Hallym 2012-28). This manuscript was written up in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines. [24]

1. Experimental preparations

(A) Animals

Adult male Sprague-Dawley rats were used in this study (250-300 g, DBL Co, Korea). The animals were housed in a temperature- and humidity-controlled environment (22 ± 2 °C, 55 ± 5 % and a 12 hr light: 12 hr dark cycle), and supplied with Purina diet (Purina, Gyeonggi-Do, Korea) and water *ad libitum*.

(B) Ischemia – reperfusion model and experimental groups

Transient global cerebral ischemia was induced by the method of Smith et al.[25] Male adult Sprague-Dawley rats weighing 250-300 g were anesthetized with 2-3 % isoflurane in 70 % NO_2 / balanced O_2 via nose cone. Once anesthetized, the animals were maintained on a small animal ventilator / anesthesia machine and the isoflurane was reduced to 1-2 %. A femoral artery catheter was placed for withdrawal of blood and monitoring of blood pressure, and a femoral vein catheter to provide an administration route for MSC.

Bilateral burr holes were made in the temporal areas of the skull for the placement of electroencephalogram (EEG) probes. Body temperature was maintained at 37 ± 1.0 °C by means of a heating blanket and heating lamp controlled by a rectal thermistor. Both common carotid arteries were exposed with a neck incision. The exposed carotid arteries were clamped and systemic mean arterial pressure was lowered to 40 ± 5 mmHg by withdrawing blood (7 to 10 ml) from the femoral artery into a heparinized syringe maintained at 37 ºC. Successful induction of global brain ischemia was confirmed by the isoelectricity on EEG monitor. Perfusion was restored by unclamping the carotid arteries and reintroducing the blood to femoral artery, after observing 7 min of isoelectric EEG signal. The incisions were closed, the femoral artery and vein catheters were removed, and anesthesia was discontinued. Rats assigned to the sham ischemia group underwent the same surgical procedure but with no blood withdrawal and no clamping of the carotid arteries.

After reperfusion, which was confirmed by the restoration of baseline EEG signal, animals were randomized into 4 groups of 7: placebo control, therapeutic hypothermia (TH), MSC treatment, and combined TH and MSC treatment (TH/MSC) for comparing short term neuronal death (7 days after injury). Rats treated with MSC received an initial dose of 1 X 10^6 cells by intravenous injection in a volume of 1 ml of 0.9 % saline immediately after ischemia. Placebo control and MSC treatment groups received injections of 1 ml saline vehicle alone. Rats allocated to TH group were wrapped by wet towel and cooled using an electric fan immediately after ischemia. Their core temperatures were lowered and maintained at the range between 32 ºC and 34 ºC. Identical procedures were performed on sham operated 4 groups of 3. Cyclosporine A (10 mg/Kg/day, i.p.) was administered to all animals from the day before surgery for 7 days.

(C) Tissue preparation

Rats were euthanized 7 days post-ischemia. Animals received an overdose of urethane anesthesic (1.5 g/kg, i.p.) and were perfused transcardially with saline followed by 4 % paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH 7.4). The brains were removed immediately and post-fixed in the same fixative for 1 hr. The brain tissues were cryoprotected by submersion in 30 % sucrose overnight. Thereafter, the entire brain was frozen and sectioned with a cryo-sliding microtome at 30 μm thicknesses and stored in cryoprotecting solution.

(D) Preparation of MSC

Adipose tissue was obtained with written informed consent from healthy female donors undergoing elective liposuction procedures in the Department of Plastic Surgery, CHA Bundang Medical Center, CHA University, Gyeonggi-Do, Korea. Collected tissue was mixed with same volume of phosphate buffered saline (PBS) including 2 % gentamicin and centrifuged at 1500 rpm for 5 min in room temperature. Then the centrifugate was put under enzymatic digestion by use of a combination of trypsin, DNase I, and collagenase I at 37 ℃ for 60 min under shaking condition. The digested tissue was centrifuged at 1500 rpm for 5 min and resuspended in saline, a total of two times. The cell pellet was filtered through a 100μm pore-size filter and centrifuged one more time to separate the adipose tissue derived stem cell form surrounding tissue. $2x10^5$ of isolated cells were expanded with 15 ml of the culture medium (alpha MEM with 10 % fetal bovine serum, 1 % penicillin/streptomycin) in T75 plask and cultured at 37 ℃, 0.05 % CO_2 incubator for 6~7 days until the cell count reached $3x10^6$. Flourescence activated cell sorting (FACS) analysis was used to identify the phenotype of the cells. The cells were used at the passage between 4 and 6.

2. Outcome measures

(A) Detection of neuronal death

Fluoro-Jade B (FJB) staining was used to identify degenerating neurons in brain sections obtained from sham and 7 days post-ischemia. The sections were rinsed in distilled water, and mounted onto gelatin-coated slides and then dried on a slide warmer. The slides were immersed in 100 % ethanol for three min, followed by 70 % ethanol for one min and distilled water for one min. The slides were then transferred to 0.06 % potassium permanganate for 15 min and gently agitated. After rinsing in distilled water for one min, the slides were incubated for 30 min in 0.001 % FJB (Histo-Chem Inc. Jefferson, AR), freshly prepared by adding 20 ml of a 0.01 % stock FJB solution to 180 ml of 0.1% acetic acid, with gentle shaking in the dark. After rinsing for one min in each of three changes of distilled water, the slides were dried, dehydrated in xylene and coverslipped with *p*-xylene-*bis*-pyridinium bromide (DPX) permount (Sigma-Aldrich Co., St. Louis, MO). To quantify neuronal death, sections were collected every third cut from 4.0 mm posterior to bregma and five coronal sections were analyzed from each animal. An observer blinded to the treatment condition counted the number of FJB-positive neurons in the hippocampal CA1, CA3 and hilus from both hemispheres under 10X objective microscopic field. The mean numbers of FJB (+) neurons from each region were used for statistical analyses. Three sham surgery rats were also evaluated, and these showed no detectable neuronal death.

(B) Evaluation of microglial activation

Rats were euthanatized at 1 wk after ischemia-reperfusion. Serial 30 μm cryostat sections were collected, and immunostaining was performed with a mouse antibody to rat CD11b (AbD serotec, UK) at a 1:200 dilution as

described previously.[26] After washing, the sections were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Invitrogen, Grand Island, NY) at a dilution of 1:250 for 2 hr at room temperature. Sections were then subsequently washed, mounted and imaged with a Zeiss confocal laser-scanning microscope. Secondary antibody alone without prior incubation with primary antibody showed no staining. Microglial activation was evaluated by a blinded observer. Five sections from each animal were evaluated for scoring. Microglial activation criteria were based on the number of CD11b immunoreactive cells and their morphology.[26, 27]

(C) Detection of blood brain barrier (BBB) disruption by IgG immunostaining

Rats were examined for the extravasation of presumed endogenous serum IgG after ischemias.[28] Animals were sacrificed 7 days after the transient global cerebral ischemia. The ABC immunoperoxidase method was employed to detect IgG-like immunoreactivity.[29] Brains were fixed by perfusion (4.0 % paraformaldehyde). Coronal sections (30 μm thick) were incubated with rabbit serum, followed with purified biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:250. From the brain section images, the IgG stained area was measured by ImageJ (NCBI, MD, USA). Measurement of the IgG stained area was quantified using the method modified from Tang et al.[30] Briefly, to quantify the area of IgG leakage, the image was loaded into ImageJ and converted into an 8-bit image. Then, the image was thresholded using the menu option. The type was set to Black & White and the bottom slider moved to a value of 106. The resulting thresholded image is binary and will only show the region of IgG leakage. To measure this area, the menu option Analyze → Measure was selected. The selected part of hippocampus in the whole image was sorted, and then the area of IgG leakage was expressed as % area.

(D) Detection of neutrophil infiltration in the hippocampus after ischemia

To detect neutrophil infiltration in the hippocampus after ischemia, 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 employed to detect MPO (+) cells. Brains were fixed by perfusion (4.0 % paraformaldehyde). Coronal sections (30 μm thick) were incubated with rabbit serum, followed with purified biotinylated rabbit anti-rat MPO (Vector Laboratories, Burlingame, CA) at a dilution of 1:250.

(E) Detection of oxidative injury

Oxidative injury was estimated by evaluating levels of the lipid peroxidation product, 4HNE (4-hydroxy-2-nonenal). Immunostaining with 4HNE (Alpha Diagnostic Intl. Inc., San Antonio, TX) antibodies was performed as described previously.[31] Tissues were incubated in mixture of polyclonal rabbit anti-HNE antiserum (diluted 1:500, Alpha Diagnostic Intl. Inc., San Antonio, TX) in PBS containing 0.3 % Triton X-100 overnight at 4 ℃. After washing three times for 10 min with PBS, sections were also incubated in a mixture of Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (Grand Island, NY) at a dilution of 1:250 for 2 hr at room temperature. The sections were washed three times for 10 min with PBS, and mounted on gelatin-coated slides. 4HNE fluorescence intensity was measured using ImageJ.

(F) Behavioral analysis

Tape removal test was done to evaluate the behavioral function of the animals following the method of Albertsmeier et al.[32] Two pieces of 10 mm X 12 mm sized adhesive tapes were placed on the both forepaws of the animals

in random order. The time until the animal removed both adhesive tapes was measured. Observation was stopped when the time reached 180 s and was recorded as “180 s”. Animals were tested 1, 3 and 7 days after transient global ischemia or sham operation. One test per day was repeated for 3 consecutive days before ischemia or sham operation for the animals to be familiarized with the test. All measurements were conducted by same investigator. The results of global cerebral ischemia induced animals were compared with those of normal animals which did not receive any operation or intervention (n=4).

3. Statistical analysis

Numerical values were expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) with Bonferroni post hoc test was used to compare the values of each experimental group. Statistical significance was defined as $p < 0.05$. If the analysis did not show any significant result and there was an extreme outlier (defined as a case containing the value 3 times or more interquartile range farther from quartiles), then the analysis was repeated without the extreme outlier case. IBM SPSS Statistics 21.0 (IBM Corp., Armonk, NY, USA) software was used for statistical calculation.

III. Results

One in control group and one in TH/MSC group died before 7 days following transient global cerebral ischemia, while all in sham operation groups survived: 2 groups of 7 (TH, MSC groups), 2 groups of 6 (control, TH/MSC groups), and 4 groups of 3 (sham operation groups) were enrolled in analysis. There was no difference in core body temperature and mean arterial pressure of the animals before, during, and after the induction of global cerebral ischemia among ischemia induced groups (Table 1).

Table 1. Core body temperature and mean arterial pressure of the animals before, during, and after the induction of global cerebral ischemia. P-values were calculated from analysis of variance (ANOVA), and numerical values were expressed as 'mean ± SEM'. TH: therapeutic induction of hypothermia, MSC: mesenchymal stem cells, TH/MSC: combined application of TH and MSC, BT: body temperature, MAP: mean arterial pressure.

	Control	TH	MSC	TH/MSC	*p*-value
Core BT (°C)					
Pre-ischemia	36.85 ± 0.18	36.64 ± 0.24	36.93 ± 0.15	36.67 ± 0.24	0.703
During-ischemia	37.08 ± 0.09	36.71 ± 0.21	37.00 ± 0.15	36.82 ± 0.27	0.512
Post-ischemia	36.53 ± 0.09	36.04 ± 0.18	36.29 ± 0.15	36.07 ± 0.20	0.160
MAP (mmHg)					
Pre-ischemia	115.00 ± 2.91	112.73 ± 3.07	116.60 ± 3.85	121.77 ± 2.25	0.259
During-ischemia	45.33 ± 0.45	44.90 ± 0.37	43.26 ± 0.65	44.17 ± 0.65	0.060
Post-ischemia	116.50 ± 3.03	119.30 ± 3.54	122.64 ± 7.29	118.45 ± 3.53	0.840

1. *Transient global cerebral ischemia*-induced neuronal death

No degenerating neurons were detected by Fluoro-Jade B staining in the

sham operation groups. ANOVA showed significant differences in degenerating neuron counts among 4 ischemia induced groups at CA1, CA3, and hilus region ($p = <0.001$, 0.004, and 0.033, respectively). Post hoc analysis revealed: the differences between control and TH (146.83 ± 21.15 vs. 66.50 ± 20.42, $p=0.008$), between control and MSC (146.83 ± 21.15 vs. 19.85 ± 4.16, $p<0.001$), and between control and TH/MSC (146.83 ± 21.15 vs. 12.60 ± 8.79, $p<0.001$) in CA1; the differences between control and MSC (26.38 ± 9.25 vs. 1.40 ± 0.38, $p=0.006$), and between control and TH/MSC (26.38 ± 9.25 vs. 2.12 ± 1.78, $p=0.011$) in CA3; the difference between control and MSC (6.78 ± 2.83 vs. 0.43 ± 0.14, $p=0.040$) in hilus (Figure 1).

2. *Transient global cerebral ischemia* -induced hippocampal microglial activation

Significant difference in microglial activation was found among 8 sham operation and ischemia groups through ANOVA ($p<0.001$). Post hoc analysis showed: no significant difference among 4 sham operation groups (control, TH, MSC, and TH/MSC with sham operation); the differences between sham groups and control (0.34 ± 0.06; 0.43 ± 0.06; 0.24 ± 0.06; 0.34 ± 0.06 vs. 2.85 ± 0.09, $p<0.001$ for all comparisons), between sham groups and TH (0.34 ± 0.06; 0.43 ± 0.06; 0.24 ± 0.06; 0.34 ± 0.06 vs. 1.61 ± 0.16, $p<0.001$ for all comparisons), between sham MSC and MSC (0.24 ± 0.06 vs. 0.99 ± 0.16, $p=0.030$), between control and TH (2.85 ± 0.09 vs. 1.61 ± 0.16, $p<0.001$), between control and MSC (2.85 ± 0.09 vs. 0.99 ± 0.16, $p<0.001$), between control and TH/MSC (2.85 ± 0.09 vs. 0.58 ± 0.07, $p<0.001$), between TH and MSC (1.61 ± 0.16 vs. 0.99 ± 0.16, $p=0.013$), and between TH and TH/MSC (1.61 ± 0.16 vs. 0.58 ± 0.07, $p<0.001$) (Figure 2).

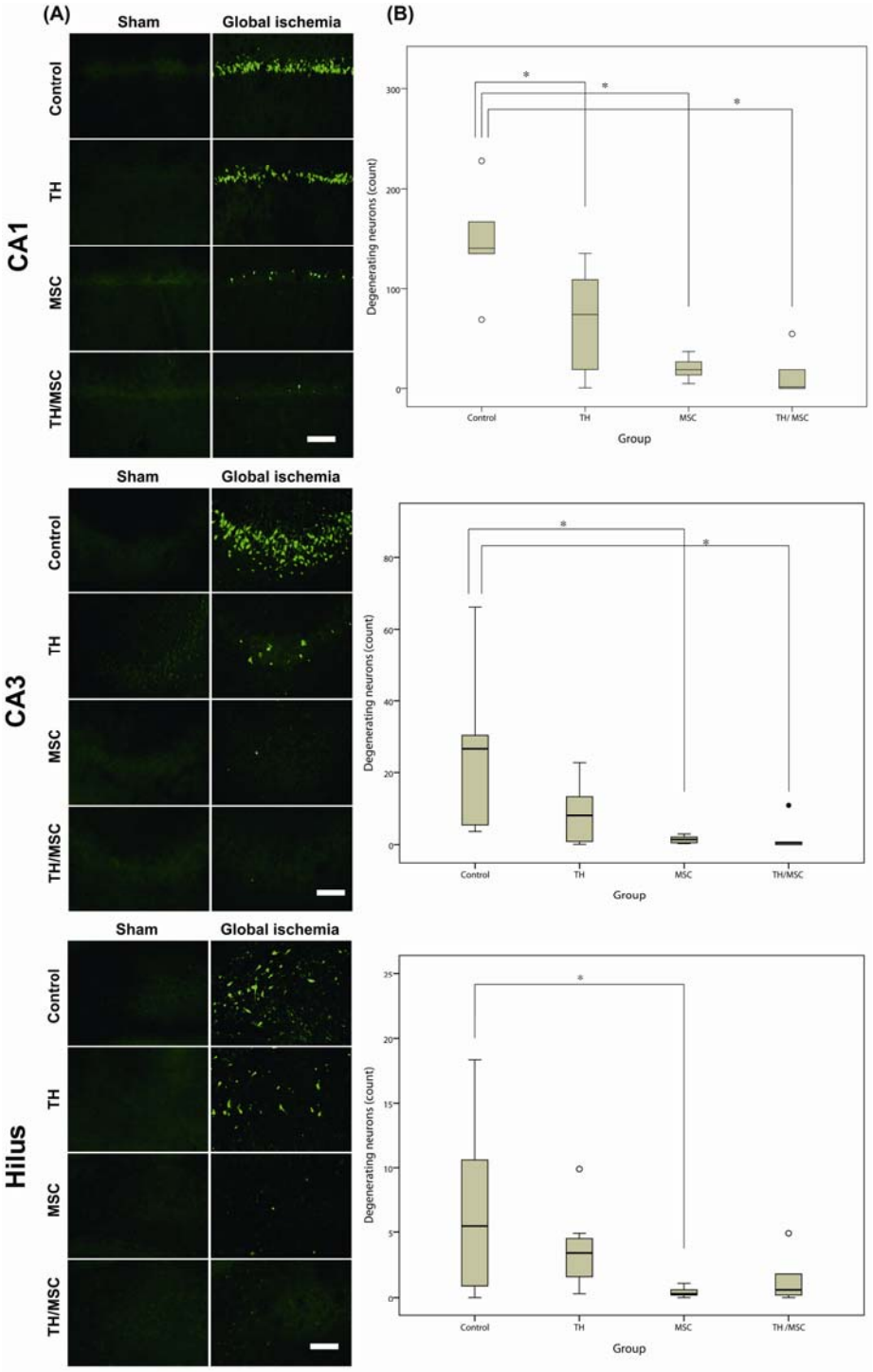

Figure 1. Effect on delayed neuronal death. (A) Transient global cerebral ischemia caused

neuronal death in the hippocampal CA1, CA3, and hilus area 1 wk after the insult, which was shown as FJB(+) neurons in fluorescence images. Sham operation groups did not show any degenerating neuron in any area of hippocampus. Scale bar = 100 μm. (B) Box whisker plot shows the quantification of neuronal degeneration in the hippocampus. MSC administered group had significantly less neuronal damage after global cerebral ischemia compared to placebo controlled group in whole areas of hippocampus, while TH did only in CA1. TH: therapeutic induction of hypothermia, MSC: mesenchymal stem cells, TH/MSC: combined application of TH and MSC. * Statistically significant result in post hoc analysis, ◦ outlier case.

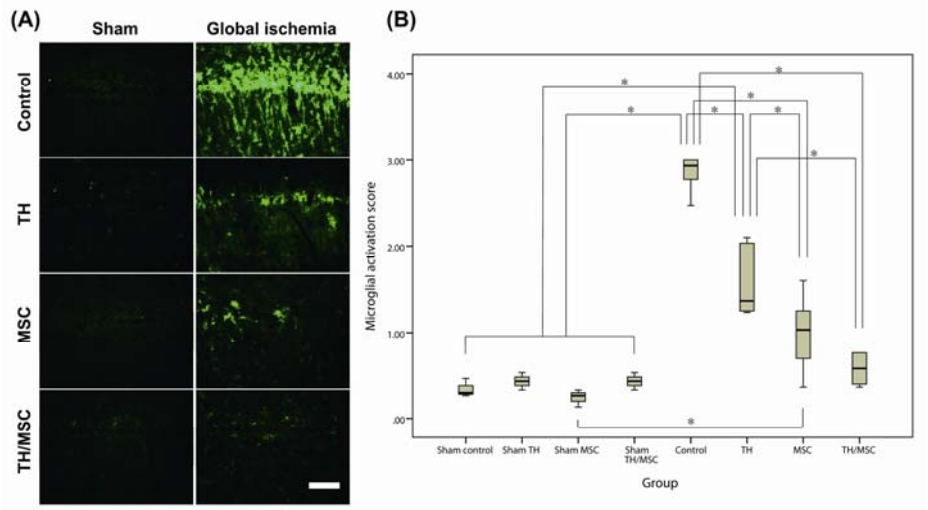

Figure 2. Effect on hippocampal microglial activation. (A) Microglial activation was observed in the hippocampus 1 wk after the insult, which was shown as CD11b stained microglia in fluorescence images. There were few activated microglia observed in sham operated groups. Scale bar = 100 μm. (B) Box whisker plot shows the quantified microglial activation score in hippocampus, which was based on the number, morphology and intensity of CD11b stained microglia. MSC administration and TH/MSC groups showed significantly less microglial activation than placebo control and TH groups. TH group showed less microglial activation score than only control group and even showed higher score than sham groups or MSC administered groups. TH: therapeutic induction of hypothermia, MSC: mesenchymal stem cells, TH/MSC: combined application of TH and MSC. * Statistically significant result in post hoc analysis.

3. *Transient global cerebral ischemia*-induced BBB disruption

ANOVA showed a significant difference in IgG leakage among 8 sham operation and ischemia groups ($p<0.001$). Post hoc analysis revealed: no significant difference among 4 sham operation groups (control, TH, MSC, and TH/MSC with sham operation); the differences between sham groups and control (1.19 ± 0.06; 1.25 ± 0.09; 1.08 ± 0.02; 1.09 ± 0.03 vs. 3.08 ± 0.19, $p<0.001$ for all comparisons), between control and TH (3.08 ± 0.19 vs. 1.55 ± 0.09, $p<0.001$), between control and MSC (3.08 ± 0.19 vs. 1.73 ± 0.18, $p<0.001$), and between control and TH/MSC (3.08 ± 0.19 vs. 1.18 ± 0.05, $p<0.001$) (Figure 3).

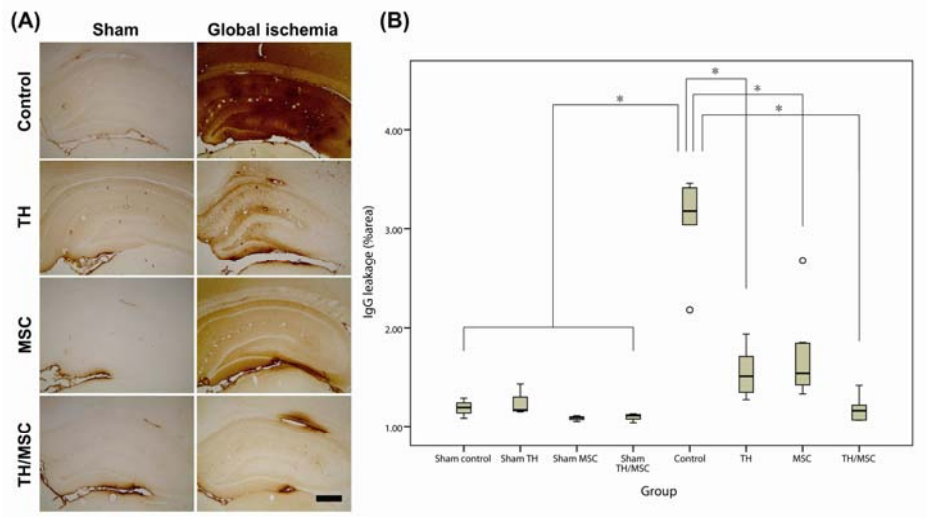

Figure 3. Effect on blood brain barrier (BBB) damage. (A) BBB disruption was observed in the hippocampus 1 wk after the insult, which was shown as IgG stained area stained in low magnification photomicrographs. Sham groups showed sparse IgG staining in the hippocampus. At 1 wk post-ischemia, the entire hippocampus was intensely stained with IgG-immunoreactivity indicating that substantial BBB damage has occurred in placebo controlled

group. Scale bar = 500 μm. (B) Box whisker plot shows the quantified IgG stained area in hippocampus which reflects BBB disruption and leakage. TH, MSC, and TH/MSC groups all showed significantly less damage in BBB than placebo controlled group. TH: therapeutic induction of hypothermia, MSC: mesenchymal stem cells, TH/MSC: combined application of TH and MSC. * Statistically significant result in post hoc analysis, ◦ outlier case.

4. *Transient global cerebral ischemia*-induced neutrophil infiltration

ANOVA failed to show a significant difference in MPO(+) cell count among 8 sham operation and ischemia groups ($p=0.052$). However, the difference was shown after excluding the extreme outlier cases (one in TH and another in MSC group, $p<0.001$), and post hoc analysis revealed: no significant difference among 4 sham operation groups (control, TH, MSC, and TH/MSC with sham operation); the differences between sham groups and control (0.27 ± 0.03; 1.70 ± 0.87; 1.07 ± 0.15; 0.57 ± 0.07 vs. 18.32 ± 3.05, $p<0.001$ for all comparisons), between control and TH (18.32 ± 3.05 vs. 2.85 ± 0.85, $p<0.001$), between control and MSC (18.32 ± 3.05 vs. 2.8 ± 0.60, $p<0.001$), and between control and TH/MSC (18.32 ± 3.05 vs. 2.38 ± 0.50, $p<0.001$) (Figure 4).

5. *Transient global cerebral ischemia* -induced oxidative injury

Significant difference in 4HNE intensity was found among 8 sham operation and ischemia groups through ANOVA ($p<0.001$). Post hoc analysis showed: no significant difference among 4 sham operation groups (control, TH, MSC, and TH/MSC with sham operation); the differences between sham groups and control (173.00 ± 1.02; 172.59 ± 0.32; 171.79 ± 0.40; 173.12 ± 0.46 vs. 214.34 ± 2.11, $p<0.001$ for all comparisons), between sham groups and TH

(173.00 ± 1.02; 172.59 ± 0.32; 171.79 ± 0.40; 173.12 ± 0.46 vs. 192.48 ± 1.87, p<0.001 for all comparisons), between control and TH (214.34 ± 2.11 vs. 192.48 ± 1.87, p<0.001), between control and MSC (214.34 ± 2.11 vs. 179.61 ± 1.99, p<0.001), between control and TH/MSC (214.34 ± 2.11 ± 2.1 vs. 175.55 ± 0.50, p<0.001), between TH and MSC (192.48 ± 1.87 vs. 179.61 ± 1.99, p<0.001), and between TH and TH/MSC (192.48 ± 1.87 vs. 175.55 ± 0.50, p<0.001) (Figure 5).

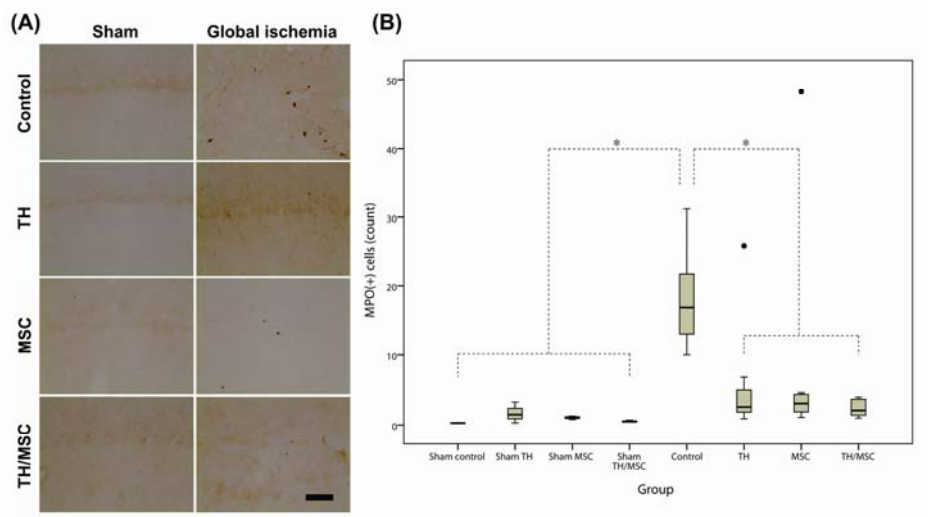

Figure 4. Effect on hippocampal neutrophil infiltration. (A) Light photomicrographs from coronal sections of the rat hippocampus demonstrated neutrophil infiltration by myeloperoxidase (MPO) staining 1 wk after global cerebral ischemia. MPO is a heme protein synthesized during myeloid differentiation that constitutes the major component of neutrophil azurophilic granules. Few MPO(+) cells were observed in sham operated groups. Scale bar = 50 μm. (B) Box whisker plot shows the count of MPO(+) cells in hippocampus. There was no statistical difference in the number of MPO(+) cells among the experimental groups. However, statistical significance was shown after the reduction of extreme outlier cases (case containing the value 3 times or more interquartile range farther from quartiles, black dots in the plot) with the result of post hoc analysis showing higher MPO(+) cells count in placebo controlled group than in other groups. TH: therapeutic induction of hypothermia, MSC: mesenchymal stem cells,

TH/MSC: combined application of TH and MSC. * Statistically significant result in post hoc analysis after the reduction of extreme outlier cases, • extreme outlier case.

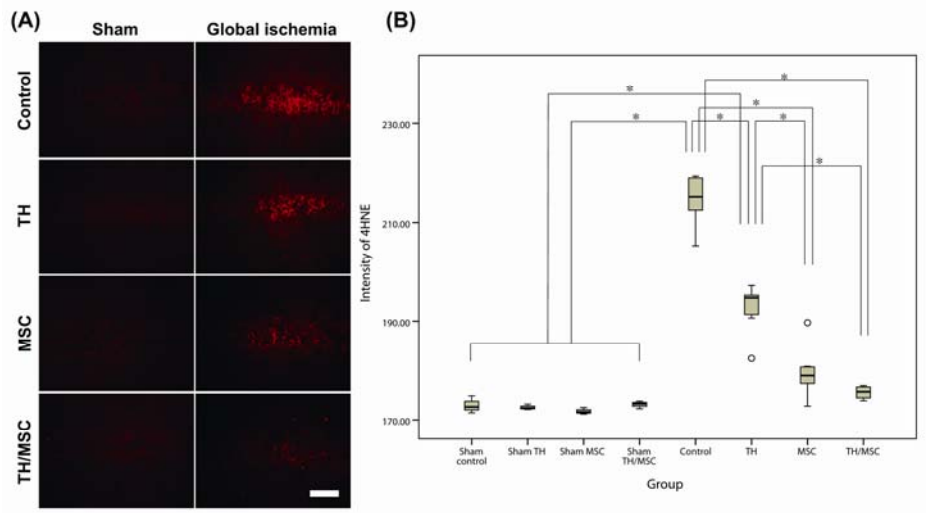

Figure 5. Effect on oxidative injury. (A) Neuronal oxidative injury was detected in the hippocampus 1 wk after the insult, which was shown as 4HNE stained neurons in fluorescence images. Sham operated brain section showed almost no 4HNE stained neurons. Scale bar = 20 μm. (B) Box whisker plot shows the quantified intensity of 4HNE staining in the hippocampal CA1 area, which was calculated from image processing software, ImageJ. MSC administration and TH/MSC groups showed significantly less 4HNE intensity than placebo control and TH groups. TH group showed less 4HNE intensity than only control group and even showed higher intensity than sham groups or MSC administered groups. TH: therapeutic induction of hypothermia, MSC: mesenchymal stem cells, TH/MSC: combined application of TH and MSC. * Statistically significant result in post hoc analysis, ◦ outlier case.

6. *Transient global cerebral ischemia* -induced behavioral impairment

Significant difference in the result of behavioral analysis was found among 1 normal and 4 ischemia groups through ANOVA ($p<0.001$). Post hoc analysis showed the differences between control and normal animal group (153.00 ±

18.17 vs. 46.25 ± 26.84, p=0.001), between control and TH (153.00 ± 18.17 vs. 49.00 ± 12.98, p<0.001), between control and MSC (153.00 ± 18.17 vs. 48.50 ± 11.48, p<0.001), between control and TH/MSC (153.00 ± 18.17 vs. 42.17 ± 9.78, p<0.001) (Figure 6).

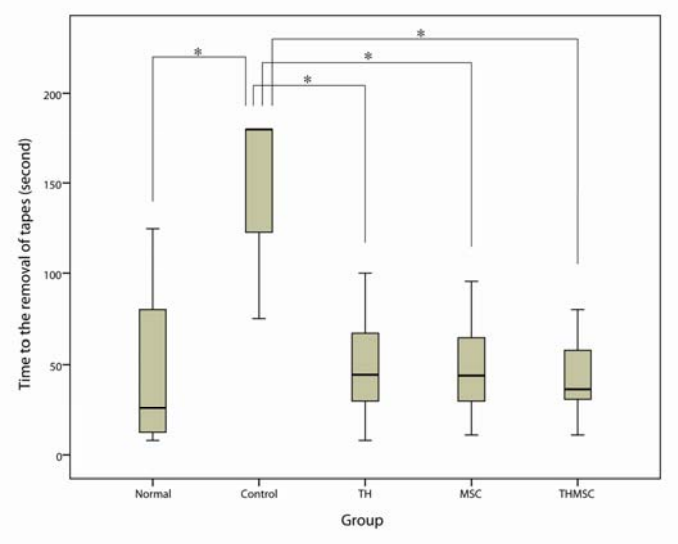

Figure 6. Effect on behavioral impairment. Box whisker plot shows the quantified impairment in behavior of global cerebral ischemia induced animals by tape removal test, which measures the time to remove small adhesive tapes on both foreclaws of the animals. TH, MSC, and TH/MSC groups took significantly less time to remove the tapes than placebo controlled group. * Statistically significant result in post hoc analysis.

IV. Discussion

Our results provide proof-of-principle that administration of MSC under hypothermia after transient global cerebral ischemia-reperfusion can prevent neuronal death. This reduction in cell death was associated with a near-complete suppression of the cellular brain inflammatory response, oxidative stress, BBB disruption.

The results of FJB stain in MSC treatment groups, which showed a marked decrease in the count of dysfunctional neurons, suggest that the administration of MSC may have prominent neuroprotective effect on global cerebral ischemia. This correlates well with the results of prior studies that demonstrated the neuroprotective effect of MSC on various focal or global cerebral ischemia models.[22, 23, 33-38] The results in TH group also showed marked survival of hippocampal neurons, as in prior studies which suggested the neuroprotection of TH.[39-43] One of the most remarkable findings was that MSC showed similar or higher degree of neuroprotection against ischemia-related neuronal death in whole region of hippocampus, compared with TH. None of the similar previous studies directly compared the efficacy of stem cell therapy in the treatment of global cerebral ischemia with that of TH, the current standard treatment option. Furthermore, not less and even higher degree of neuronal survivals were observed in combined TH and MSC application group than in TH or MSC single treatment group, which implies that there may be no negative interaction between TH and MSC treatment. These facts provide strong evidence for the use of MSC as a substitute or a complement of standard therapy in global cerebral ischemia.

Various mechanisms have been suggested for the effect of stem cells on

neuronal damage, such as a cell replacement, direct neuroprotection, immunomodulation or enhancement of neurotrophic factor activity.[18, 19, 22, 23, 44-47] In particular, the result of Ohtaki et al.[22] is concordant with ours, with respect to the suggestion of microglial activation as an underlying mechanism. The CD11b antigen is expressed not only by microglia, but also by activated blood-borne macrophages.[48] Since macrophages infiltrate brain after ischemia, the reduction in CD11b immunoreactivity may reflect reduced macrophage infiltration in addition to reduced microglial activation.[49-51] Brain inflammation has been recognized as a potential target for cerebral ischemia treatment for several years, and various approaches have been tried to suppress aspects of post-ischemic brain inflammation. These approaches include hypothermia, inhibition of matrix metalloproteinases, antibody-mediated inhibition of neutrophil infiltration, lipopolysaccharide preconditioning, and the use of minocycline.[51-54] Minocycline has recently been shown to be a very potent poly ADP ribose polymerase (PARP) inhibitor,[55] and this may be the mechanism by which minocycline suppresses microglial activation. Results of CD11b staining which showed prominent decrease of microglial activation in MSC administered groups suggest that neuroprotection shown in those groups is due to the suppression of microglial activation. However, the possibility of a direct neuroprotective effect cannot be entirely excluded on the basis of the studies completed. Moreover, statistically significant difference between single TH treatment group and MSC administered groups suggests the possible superiority of MSC treatment over current standard therapeutic option, TH. Future studies will address this point and also examine outcome after much longer survival times.

The result of IgG stain revealed that the extent of IgG stained area markedly decreased in MSC administered group to the similar level shown in sham operation group, while prominent extent of IgG staining was observed in placebo control group. This suggests that neuroprotection of MSC may be

associated with the restoration or protection from BBB disruption, which is one of the most important mechanisms related with ischemic neuronal injury.[56] Prevention of BBB permeability has been suggested as an action mechanism of TH on global cerebral ischemia,[57] but not as that of stem cell administration before. In addition, prominent BBB disruption shown in placebo control may be a clue to explain how systemically administered MSC can reach the brain. MSC may be imported to the brain owing to increased BBB permeability following ischemic injury, which cannot directly pass BBB in normal condition.

The result of MPO immunostaining failed to show the significant difference among the experimental groups in statistical analysis, ANOVA. However, we could easily deduce from box-whisker plot that the insignificance was mostly due to just one extreme outlier case in MSC treatment group. Consequently, same analysis after reduction of extreme outlier cases showed strong statistical significance as expected. This implies that there is a trend of less neutrophil infiltration in TH, MSC, and TH/MSC groups than in placebo control group. The result of more MPO(+) cells in placebo control group than in sham operation groups is concordant with the fact that tissue injury begins with an inflammatory reaction after the interruption of cerebral blood flow, which requires the recruitment and infiltration of leukocytes including polymorphonuclear neutrophils.[58, 59] This result correlates with that of IgG stain, which showed marked decrease of BBB disruption in TH, MSC, and TH/MSC groups. Administered MSC is thought to decrease BBB disruption and influx of immune cells, which can cancel or slow ongoing neuronal injury after global cerebral ischemia.

Significant amounts of oxygen free radicals are generated during cerebral ischemia/reperfusion, and oxidative stress plays an important role in brain damage after stroke.[60] Our 4HNE staining also produced concordant result

which showed significantly more oxidative injury in placebo control group than in sham operation groups. The result in TH and MSC applied groups suggests that oxidative injury after cerebral ischemia is reduced by TH or MSC. Especially, the result of significantly lower 4HNE intensity in MSC treated groups than in TH group implies that antioxidant property of MSC after cerebral ischemia/reperfusion may be greater than that of TH.

One of the most important findings in our study was that MSC treated groups showed better or equivalent results compared to TH group in all analyses. Moreover, there was no evidence of negative interaction observed in combined application of TH and MSC group. This suggests the possibility of clinical application of MSC as an alternative or complement treatment option for global cerebral ischemia. Another interesting point of our results was that combined MSC and TH treatment group had a better value than single MSC treatment group, with respect to the mean value in most analyses, despite insignificance in statistical comparison. Considering that our study was not powered enough to compare combined MSC and TH treatment group with single MSC treatment group, this may indicate an additive or synergistic effect on global cerebral ischemia. This trend is also concordant with the study of Saito et al.[61] which showed an enhanced stemness of neural stem cells with moderate low temperature in an in-vitro condition, and suggests that in-vivo moderate low temperature may also enhance the effect of exogenously administered stem cells. Further in vivo study focused to the comparison of the efficacies of combined TH/MSC treatment and sole MSC treatment on global cerebral ischemia may be necessary.

Results of behavioral analysis confirms that various effects of MSC and TH shown in our histologic findings correlate with real function, which means that administration of MSC can improve neurologic outcome after transient global cerebral ischemia. This may be a clue for clinical application of MSC

to global cerebral ischemia, along with the experimental setting used in our experiments which designed to compare the effect of MSC with that of current standard therapy.

There were a few limitations in our study. First, we did not directly identify administered MSC in brain of the animals. However, it has been shown in many researches that intravenously administered human MSC can be placed in the brain of experimental animals passing through BBB.[23, 33, 34, 37, 62] Also, our result of IgG staining suggests the possible mechanism of how systemically administered cells can pass BBB. Second, we did not clarify whether the effects of MSC shown in our results were due to the replacement of lost neurons by the cells differentiated from administered MSC or due to the neuroprotective effect of MSC mediated by expression of various cytokines or growth factors. Considering that our experiment used xenograft model, paracrine effect may explain the results better, which is concordant with the findings from many other studies showing the effect of MSC in repairing central nervous system injury.[63]

V. Conclusion

Administration of MSC after transient global cerebral ischemia has a prominent protective effect on delayed hippocampal neuron death comparing with TH, current standard treatment option, which is associated with suppression of BBB disruption, hippocampal microglial activation, neutrophil infiltration, and reperfusion oxidative injury. The present results also suggest combined treatment of MSC and hypothermia warrants a potential therapeutic strategy for intervention of global cerebral ischemia after cardiac arrest.

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ABSTRACT (IN KOREAN)

일과적 전뇌허혈 후의 지연된 신경세포사멸에 대한 지방기원 중간엽줄기세포 투여와 치료적 저체온 유도의 효과

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정 태 녕

연구배경: 전뇌허혈은 심정지로부터의 성공적 소생 후 발생하는 불량한 예후의 가장 중요한 요인이다. 다양한 실험실적, 임상적 연구를 통해 전뇌허혈로 인한 신경손상을 줄임에 있어 뛰어난 효능을 보인 치료적 저체온유도(12~24 시간 동안 중심체온을 32~34℃로 유지하는 것)는 현재 표준 심정지 후 치료 중 하나로 권장되고 있다. 그러나 치료적 저체온유도는 권장 온도까지의 유도나 재가온 시 권장 속도 유지의 난이도 등으로 인해 그 사용에 제한이 있어 이에 대한 대체 혹은 보조 치료 방법이 필요한 실정이다. 이에 현재 표준 치료인 저체온 유도와의 비교를 포함하여 줄기세포 투여가 일과성 전뇌허혈 후 신경학적 회복에 미치는 영향을 보고자 하였다.

연구방법: 실험용 쥐에 7 분간 일과성 전뇌허혈을 유발한 이후 4 가지의 조작군(위약통제군, 치료적 저체온유도군, 인간 중간엽줄기세포 투여군, 치료적 저체온 유도군 및 중간엽줄기세포 투여 병용군) 및 동일한 조작의 모의수술군 4 군으로 무작위 배치하였다. 전뇌허혈 유발 7 일 후 실험동물의 뇌를 적출하여 해마 신경세포 손상을 관찰하기 위해 Fluoro Jade B 염색을, 미세아교세포의 활성을 보기 위해

CD11b 면역염색을, 혈액뇌장벽 손상을 확인하기 위해 IgG 면역염색을, 호중성구 침착을 보기 위해 MPO 면역염색을, 그리고 산화손상을 확인하기 위해 4HNE 면역염색을 각각 시행하였다. 운동 기능을 측정하기 위해 실험 동물이 양발에 붙은 접착 테이프를 떼어내는데 소요되는 시간을 측정하였다.

결과: Fluoro-Jade B 염색결과 모의수술군에서는 어떠한 손상된 신경세포도 발견되지 않았다. 해마 CA1, CA3, 및 해마문 부위에서 분산분석상 4 군의 조작군간 유의한 통계적 차이를 보였다(각각 p= <0.001, 0.004, 0.03). CA1 부위의 경우 위약통제군과 치료적 저체온유도군 간, 위약통제군과 중간엽줄기세포 투여군 간, 그리고 위약통제군과 저체온유도 및 중간엽줄기세포 투여 병용군 간에서 사후검정상 유의한 통계적 차이를 보였고, CA3 부위의 경우 위약통제군과 중간엽줄기세포 투여군 간, 위약통제군과 저체온유도 및 중간엽줄기세포 투여 병용군 간에서 사후검정상 유의한 통계적 차이를 보였으며, 해마문 부위의 경우 위약통제군과 중간엽줄기세포 투여군 간에서만 사후검정상 유의한 통계적 차이를 보였다. 미세아교세포 활성화 정도 결과에 대한 분산분석상 8 군의 조작군 및 모의수술군 간 유의한 통계적 차이가 있었다($p<0.001$). 사후검정상 모의수술군 4 군 사이에는 유의한 통계적 차이가 없었고, 모의수술군과 위약통제군 간, 모의수술군과 치료적 저체온유도군 간, 중간엽줄기세포 투여 모의수술군과 중간엽줄기세포 투여군 간, 위약통제군과 치료적 저체온유도군 간, 위약통제군과 중간엽줄기세포 투여군 간, 위약통제군과 저체온유도 및 중간엽줄기세포 투여 병용군 간, 저체온 유도군과 중간엽줄기세포 투여군 간, 그리고 저체온 유도군과 저체온유도 및 중간엽줄기세포 투여 병용군 간 유의한 차이를 보였다. IgG 면역염색

결과의 분산분석상 8 군의 조작군 및 모의수술군 간 유의한 통계적 차이가 있었다($p<0.001$). 사후검정상 모의수술군 4 군 사이에는 유의한 통계적 차이가 없었고, 모의수술군과 위약통제군 간, 위약통제군과 치료적 저체온유도군 간, 위약통제군과 중간엽줄기세포 투여군 간, 그리고 위약통제군과 저체온유도 및 중간엽줄기세포 투여 병용군 간 유의한 통계적 차이를 보였다. MPO 염색 결과의 경우 각 군 간 분산분석상 유의한 통계적 차이를 검출하지 못했다($p=0.052$). 4HNE 면역염색 결과의 분산분석상 8 군의 조작군 및 모의수술군 간 유의한 통계적 차이가 있었다($p<0.001$). 사후검정상 모의수술군 4 군 사이에는 유의한 통계적 차이가 없었고, 모의수술군과 위약통제군 간, 위약통제군과 치료적 저체온유도군 간, 위약통제군과 중간엽줄기세포 투여군 간, 그리고 위약통제군과 저체온유도 및 중간엽줄기세포 투여 병용군 간 유의한 통계적 차이를 보였다. 행동기능 측정 결과의 경우 5 군의 정상 및 조작군 간 유의한 통계적 차의를 보였으며, 사후 검정상 위약투여 전뇌허혈군과 나머지 군 간 유의한 차이를 보였다.

결론: 일과적 전뇌허혈 후 중간엽줄기세포 투여는 현재의 표준치료인 치료적 저체온유도와 비교하여 뚜렷한 신경보호효과를 갖는다. 또한 현재의 결과는 추후 심정지 후 전뇌허혈의 치료전략으로써 치료적 저체온유도와 중간엽줄기세포 투여의 병용이 이용될 가능성을 제시한다.

핵심되는 말 : 전뇌허혈, 중간엽줄기세포, 치료적 저체온유도