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Neuroprotective and Neuroregenerative Effect of Secreted Factors from Human Adipose Stem Cells in a Rat Stroke Model

뇌졸중 백서 모델에서 지방유래 줄기세포

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ABSTRACT

Neuroprotective and Neuroregenerative Effect of Secreted Factors from Human Adipose Stem Cells in a Rat Stroke Model

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Stem cell therapy is an emerging paradigm of stroke treatment. Recent evidence shows that stem cells exert their therapeutic effects through the secretion of immune modulatory, neurotrophic factors. Although potential neuroprotective and neuroregenerative mediators of stem cells have been previously suggested in both in vitro and in vivo studies, no study has compared their therapeutic effects with that of a whole stem cell secretome in an in vivo stroke model. Therefore, the objective of this study is to assess the neuroprotective and neuroregenerative effect of selected recombinant factors (RFs), detected in human adipose stem cell (hASC)-conditioned medium (CM), in a rat ischemic stroke model.

The CM was prepared by culturing human adipose stem cells for 72h in serum-free Dulbecco's modified Eagle medium (DMEM). Multi-protein analysis using multiplex bead array and ELISA were used to detect secreted factors in the CM. Ischemic stroke was induced in Sprague-Dawley rats using 2 h transient middle cerebral artery occlusion (MCAO). In experiment 1, the vehicle (DMEM), concentrated CM, and selected RFs mixed with DMEM were administered intracerebroventricularly to each group (n = 14, 15, and 16, respectively) at 1 hr after reperfusion. Rats were sacrificed 24 h after MCAO. In experiment 2, the appropriate treatment was injected intraventricularly 24 h after reperfusion (n = 5, 4, and 4, respectively). All animals received daily single intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg) from day 1 to day 3. Then, all animals exercised on a rat treadmill during 2 weeks and were sacrificed 17 day after reperfusion.

IL-6, VEGF, HGF, and BDNF were detected in hASC-CM. In the experiment 1, the CM and RF groups both showed significantly better sensorimotor neurological test scores than the control group at 24 h post-MCAO. The infarct volume was significantly lower in both the CM and RF groups than in the control group. The number of TUNEL-positive apoptotic cells were reduced, whereas HSP70 expression was enhanced in the peri-infarct area in both the CM and RF groups. Moreover, hASC-CM and RFs reduced IκB phosphorylation and influenced bcl-2 and bax protein expression. In experiment 2, the RF group showed significantly better recovery of sensorimotor deficits at day 10, and increased neuronal proliferation in the perilesional area at day 17 after MCAO.

Our results suggest that RFs, selected from hASC-CM, may exert an immediate neuroprotective effect in an ischemic stroke rat model that is comparable to those effect of full hASC-CM. The therapeutic effects of the RFs may be mediated by an anti-inflammatory mechanism and cell apoptosis inhibition. In addition, the selected RFs may also exert a neuroregenerative effect when administered after acute phase of stroke . Hence, treatment with RFs can be considered a feasible substitute for stem cell therapy after stroke.

Keywords: stroke; stem cells; neuroprotection; rats; recombinant factors

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure 8. RFs increase the neuronal proliferation in the lesion border zone.

LIST OF ABBREVIATIONS

bFGF	basic fibroblast growth factor
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
CM	conditioned media
COX-2	cyclooxygenase-2
DAB	3,3'-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
DCX	doublecortin
DMEM	Dulbecco's modified Eagle medium
ELISA	enzyme-linked immunosorbent assay
GDNF	glial cell line-derived neurotrophic factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
hASC	human adipose-derived stem cell
HGF	hepatocyte growth factor
HSP70	heat shock protein 70
IFN-γ	interferon gamma
IGF-1	insulin-like growth factor 1
iNOS	inducible nitric oxide synthase
JAK/STAT	Janus kinase/signal transducers and activators of transcription
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
OCT	optimal cutting temperature
PBS	phosphate-buffered saline

PDGF-BB platelet-derived growth factor BB

PI3k phosphoinositide-3-kinase

RF recombinant factor

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

TdT terminal deoxynucleotidyl transferase

TNF-α tumor necrosis factor alpha

TUNEL terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling

VEGF vascular endothelial growth factor

INTRODUCTION

Stem cell therapy in stroke

Stem cell therapy is an emerging paradigm of stroke treatment. Various stem cell types, with varying therapeutic mechanisms and time windows, have been investigated for stroke treatment [1]. Although cell replacement was the initial aim of stem cell therapy for stroke, a growing amount of evidence has suggested that transplanted cells exert therapeutic effects by modulating the surrounding microenvironment [2, 3]. This bystander mechanism is known to be mediated by soluble factors, such as growth factors and cytokines, secreted from transplanted cells that may play a role in the protection and restoration of injured tissues [4, 5]. Neuroprotection is a therapeutic mechanism during several hours to days, whereas neuroregeneration is a mechanism persisting several days to weeks, even months, from stroke onset [1]. Accordingly, mesenchymal stem cells, which have several mechanisms of action in stroke including immunomodulation, increased neurogenesis, and angiogenesis, as well as effects on glial cells, have emerged as one of the most promising types of stem cells due to enhanced safety and more favorable ethical and legal profile [6]. Hence, mesenchymal stem cells have been most commonly used in clinical trials for stroke patients [7].

Stem cell secretome and potential therapeutic mediators

Recent studies have shown that stem cell-conditioned media (CM) has therapeutic effects in experimental stroke models [8-10]. Jeon et al. [11] also reported that a cell-free extract derived from human adipose-derived stem cells (hASCs) exerts neuroprotective effects through its protein components rather than nucleic acid or lipid components in stroke models. The biologics secreted by stem cells (secretome) have gained increasing attention for central nervous system repair, and a better understanding of the stem cell secretome may yield an alternative therapeutic option [5, 12] Proteomic analysis revealed the presence of 201 unique gene products, which were classified into different biological processes, such as metabolism, defense response, and tissue differentiation, in the CM of mesenchymal stem cells [13]. Among them, several growth factors, including brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), glial cell line-derived neurotrophic factor (GDNF), and basic fibroblast growth factor (bFGF), as well as some inflammatory cytokines, have been suggested to mediate the neuroprotective and regenerative effects of mesenchymal stem cells in ischemic stroke by activating the survival and anti-inflammatory pathways [12].

Selected recombinant factors from stem cell secretome as a cell-free therapeutic agent in ischemic stroke

Although potential neuroprotective and neuroregenerative mediators of mesenchymal stem cells have been previously suggested in both in vitro and in vivo studies, to our knowledge, no study has compared their therapeutic effects with that of a whole stem cell secretome in an in vivo stroke model. As a treatment, the stem cell secretome raises issues, such as the variation in the secretome profiles, the low concentration of secreted factors, and the biosafety concerns. If the selected mediators have a comparable neuroprotective and neuroregenerative effects with the full stem cell secretome, they may serve as an alternative therapeutic option of stem cell therapy in stroke. Therefore, in the present study, we aimed to assess the neuroprotective and neuroregenerative effects of selected recombinant factors (RFs), detected in human adipose stem cell (hASC)-CM, in comparison with that of hASC-CM in a rat ischemic stroke model.

MATERIALS AND MATHODS

Establishment of animal model and experimental design

The entire study consisted of experiment 1 to investigate the immediate neuroprotective effect and experiment 2 to investigate the long-term neuroregenerative effect of study interventions in a rat ischemic stroke model (Fig 1).

In the experiment 1, 65 male Sprague-Dawley rats (7 weeks old; weight, 220–300 g; Koatech Inc., Gyeonggi-do, Korea) were housed in standard cages with free access to food and water, and a 12:12 h light/dark cycle. After accommodation in the cages for 1 week, the animals were subjected to 2-h transient middle cerebral artery occlusion (MCAO), which was a modification of the reversible occlusion model [14]. In brief, the right common carotid artery was exposed at its bifurcation through a midline incision of the neck, with the animal under inhalation anesthesia using isoflurane. The proximal portions of the common carotid artery and the external carotid artery were ligated with 4-0 silk sutures. The external carotid artery was cut after clamping the internal carotid artery. Thereafter, a 0.39 mm silicon coated monofilament nylon suture (Doccol, Sharon, MA, USA) was inserted into the stump of the external carotid artery and the internal carotid artery clamp was removed. The suture was advanced 19–20 mm into the internal carotid artery from the bifurcation site. Mild resistance indicated that the suture was properly lodged in the anterior cerebral artery, and therefore blocked blood flow to the middle cerebral artery. Body temperature was maintained at 37°C during surgery using a

heating blanket (Homeothermic Blanket Control Unit, Harvard Apparatus, Holliston, MA, USA). For temporary MCAO, reperfusion was achieved by withdrawing the suture gently after 2 h. The rat MCAO model has been established by measuring cerebral blood flow using laser Doppler flowmetry (Peri Flux System 5000, Perimed, Stockholm, Sweden). All animals received 1 mg/kg of meloxicam subcutaneously prior to the surgery to reduce pain.

In the experiment 1, 53 rats were randomized into 3 different experimental groups following surgery (mortality = 12 rats): control, CM-treated, and RF-treated. The appropriate treatment was injected intraventricularly 1 h after reperfusion. All animals were sacrificed 24 h after reperfusion. A total of 8 rats died during or after the intervention (4 in the control, 2 in the CM, and 2 in the RF groups), and therefore a total of 45 rats (14 in the control, 15 in the CM, and 16 in the RF groups) were included in the analysis.

In the experiment 2, 32 male Sprague-Dawley rats were subjected to 2-h MCAO, which was the same as in the experiment 1. Twenty-eight rats were randomized into 3 different experimental groups following surgery (mortality = 4 rats): control, CM-treated, and RF-treated. The appropriate treatment was injected intraventricularly 24 h after reperfusion. To label dividing cells, all animals received daily single intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg) from day 1 to day 3. After that, all animals exercised on a rat treadmill at moderate intensity (10 to 17 m/min) for 30 min per day, 5 days per week during 2 weeks. All animals were sacrificed 17 day after reperfusion. A

total of 4 rats died during or after the intervention (1 in the control, 2 in the CM, and 1 in the RF groups), 11 rats died during the treadmill training period (1 in the control, 7 in the CM, and 3 in the RF groups), and therefore a total of 13 rats (5 in the control, 4 in the CM, and 4 in the RF groups) were included in the analysis.

The experimental protocol was approved by the Institutional Animal Care and Use Committee of our hospital (IACUC number: 15-0040-C1A1).

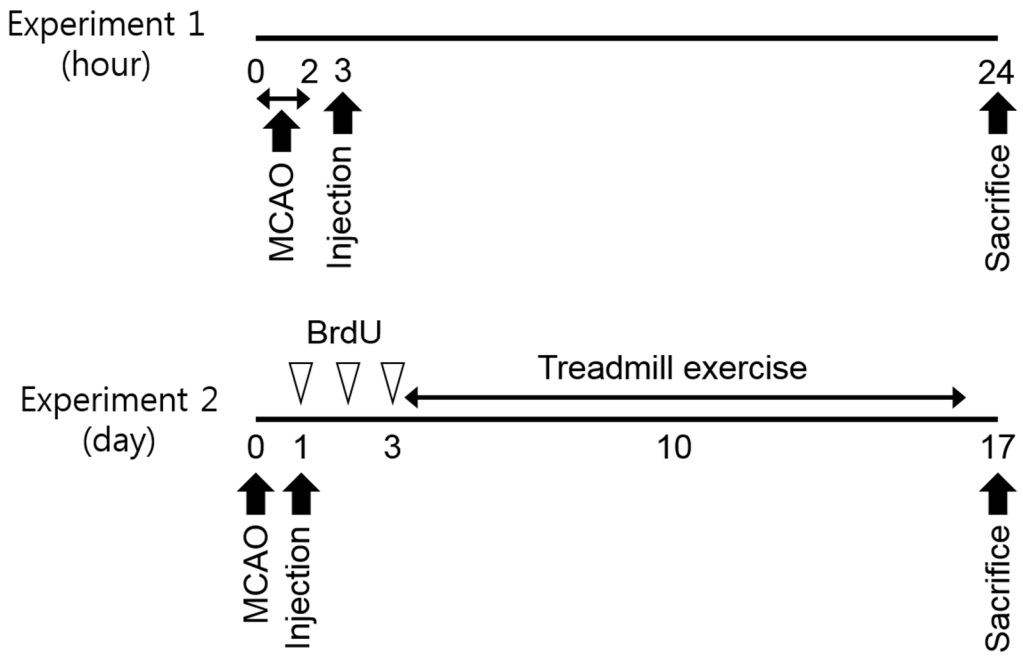


Figure 1. Schematic illustration of the experimental design.

Preparation and analysis of CM

hASCs (Cat. No: ADMSC-004, CEFO Co. Ltd., Seoul, Korea) were grown to 90–100% confluency in 100 mm petri dishes in Dulbecco's modified Eagle medium (DMEM; Welgene Inc., Daegu, Korea). The stem cells from passage 5 were used in this study. After the growth medium was removed and the cells were rinsed 3 times with phosphate-buffered saline (PBS), CM was prepared by culturing the stem cells on 100 mm petri dishes for 24, 48, and 72 h in serum-free DMEM. Five independent samples of each CM were prepared for the analysis. The concentrations of inflammatory cytokines, including IL-1b, 2, 4, 5, 6, 10, 12p70, and 13; granulocyte-macrophage colony-stimulating factor (GM-CSF); interferon gamma (IFN-γ); and tumor necrosis factor alpha (TNF-α); as well as growth factors, including BDNF, IGF-1, VEGF, HGF, bFGF, and platelet-derived growth factor BB (PDGF-BB), were analyzed in the CM at 24, 48, and 72 h. All the secreted factors, except for BDNF and IGF-1, were analyzed using a multiplex protein analysis (Bio-Plex 2000, Bio-Rad, Hercules, CA, USA). The levels of BDNF and IGF-1 were analyzed using an enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) according to the manufacturer's protocol.

For intraventricular injection, the 72 h CM was concentrated by centrifugation for 90 min at 2739 g, using Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA, USA). The protein concentration in the CM sample, measured by the Bradford assay, was increased from 3.076 to 286.903 μg/ml (~95-fold). The concentrated CM was immediately frozen and stored until needed.

Intraventricular injection

Under inhalation anesthesia, an intraventricular injection was administered to each rat using a 10 μl Hamilton syringe on a stereotaxic frame. To reach the lateral ventricle of the lesioned hemisphere, the needle was inserted through a burr hole at 0.8 mm posterior to the bregma, 1.8 mm lateral to the midline, and 3.7 mm below the pial surface. A volume of 10 μl was injected over 10 min using a microdialysis syringe pump (CMA 402, CMA Microdialysis, Stockholm, Sweden) according to group: vehicle (DMEM only), concentrated CM, and RFs mixed with DMEM. For the RF group, 4 factors were selected from the CM analysis: recombinant human BDNF (PeproTech #450-02, Rocky Hill, NJ, USA), VEGF (PeproTech #100-20), HGF (PeproTech #100-39), and IL-6 (PeproTech #200-06). These factors (0.2 μg each) were mixed with 10 μl of DMEM at room temperature 10 min prior to injection.

Behavioral tests

Behavioral tests included the Garcia scale test, foot fault test, and Rotarod test. The Garcia scale is a neurological test for sensorimotor deficits, with a maximum score of 18 points [15]. The scale contains 6-items: spontaneous activity, symmetry in the movement of limbs, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch. The foot fault test assesses locomotor function on a horizontal ladder (120 cm

long, 13 cm wide, 30 cm high, and 3 cm space between two ladder beams) by counting the number of paw misplacements per 10 steps on each side [16]. The mean of 3 trials was calculated. The Rotarod test evaluates motor coordination and balance on a rotating cylinder by measuring the maximum rpm [16]. After a 5 minute adaptation period, the speed of the cylinder is increased from 2 to 40 rotations per minute (rpm) over a period of 20 minutes. The highest rpm was recorded by repeating 3 times.

In the experiment 1, the Garcia scale was measured at baseline, 1 h, and 24 h after reperfusion. Other tests were evaluated at baseline and 24 h after reperfusion. In the experiment 2, all tests were evaluated at baseline, day 1, day 3, day 10, and day 17 from transient MCAO.

Infarct volume

In the experiment 1, infarct volume was measured in 5 rats in each experimental group in the experiment 1. After decapitation under inhalation anesthesia, the rat brains were carefully removed and sectioned coronally into 7 slices (2 mm-thickness) from the frontal pole. The infarcted areas were identified using 2, 3, 5-triphenyl tetrazolium chloride staining and scanned into digital images [17]. The infarcted volume was calculated by a blinded researcher using ImageJ software (NIH, Bethesda, MD, USA) based on the trapezoidal rule. Both the corrected infarct volume, excluding brain edema [18], and the ratio of the infarct volume to the volume of the contralateral hemisphere [19]

were estimated.

Immunohistochemistry

In the experiment 1, the detection of heat shock protein 70 (HSP70), known to be expressed in an ischemic penumbra [20], was performed using immunohistochemical staining to evaluate the change in the lesion border zone according to experimental group in the experiment 1. The rats were transcardially perfused with 50 mL of heparinized saline, followed by 50 mL of 4% paraformaldehyde. Brains were removed and fixed in 4% paraformaldehyde for 24 h, cryoprotected in a series of the cold sucrose solutions of increasing concentration, and frozen in OCT compound. Then, rat brains were sectioned into 10 μm coronal sections at 2 mm-intervals from the frontal pole and were mounted on slides. Six brain sections of each animal were stained using the Discovery XT automated immunohistochemistry stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Detection was performed using the Ventana Chromo Map Kit (Ventana Medical Systems). CC1 standard (pH 8.4 buffer containing Tris/Borate/EDTA) was used for antigen retrieval. Inhibitor D (3% H2O2, endogenous peroxidase) was blocked for 4 min at 37°C. Slides were incubated with an anti-HSP70 mouse monoclonal antibody (1: 5000; Abcam, ab47455) for 32 min at 37 °C, then with a secondary antibody (OmniMap anti-Mouse HRP) for 20 min at 37 °C. Slides were incubated in DAB + H_2O_2 substrate for 8 min at 37 °C. Reaction buffer (pH 7.6 Tris buffer) was used as a washing solution. The area of

HSP70 expression was measured, using ImageJ software, as the ratio to the whole brain area in the 6 brain sections by a blinded researcher. The area was defined from binary images based on an automated or manually adjusted threshold, after converting the color images into an 8-bit grayscale. The number of analyzed animals was 5 in the control, 5 in the CM, and 6 in the RF groups.

In the experiment 2, rat brains were prepared using the same method of experiment 1 for BrdU, and doublecortin (DCX), the marker of immature neurons, immunohistochemical staining. The brains were sectioned into 10 μm coronal sections at 400 μm-intervals between 6 mm and 8 mm from the frontal pole to include the subventricular zone and were mounted on slides. After fixation in 3.7% paraformaldehyde for 15 min, the brain sections were rinsed twice with ice cold PBS. For tissue permeabilization, the sections were incubated for 10 min with PBS containing 0.25% Triton X-100 and rinsed 3 times with PBS for 5 min. The sections were additionally incubated in 1.5 M HCL for 30 min in room temperature for DNA denaturation and then rinsed twice with PBS for 5 min. After blocking of unspecific binding of the antibodies by incubating in 1% BSA in PBS with 0.05% Tween 20 for 30 min, the sections were incubated with mouse monoclonal anti-BrdU antibody (1:100; Cell signaling, #5292) and rabbit polyclonal anti-DCX antibody (1:1000; Abcam, #ab18723). After washing 4 times with PBS for 5 min, they were treated with secondary antibodies against anti-mouse Alexa Fluor® 594 (1:200; Abcam, #ab150112) and anti-rabbit Alexa Fluor® 488 (1:200; Abcam, #ab150061). After washing 4 times with PBS for 5 min in dark, they were

incubated on DAPI for 1min for counter staining. The immunopositive cells for $BrdU^+$ and $BrdU^+/DCX^+$ were counted in 5 fields in the perilesional areas under ×200 magnification by a blinded researcher in each brain section. The number of analyzed animals was 5 in the control, 4 in the CM, and 4 in the RF groups.

TUNEL staining

In experiment 1, DNA fragmentation in cells undergoing apoptosis was detected in the cryosections of brain tissue (10 μm) (same as immunohistochemical staining for HSP70), by using terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL), according to the manufacturer's recommendations (ApopTag® peroxidase kit, Cat. No: S7100, Millipore, Schwalbach, Germany) in the experiment 1. After tissue preparation, endogenous peroxidase activity was quenched by placing sections into a solution of 3% H_2O_2 in PBS for 5 min. Equilibration buffer was applied, and sections were incubated with reaction buffer containing TdT enzyme at 37°C for 1 h. After washing with stop/wash buffer, the sections were treated with anti-digoxigenin conjugate for 30 min, and color was subsequently developed with peroxidase substrate for 5 min. The nuclei were counterstained with 1% methyl green for 10 min.

TUNEL-positive cells with apoptotic nuclear features were counted manually in 5 random microscopic fields in the perilesional areas, which were identified by HSP70 expression, under ×400 magnification by a blinded researcher. The number of analyzed

animals were 5 in the control, 5 in the CM, and 6 in the RF groups.

Western blot

In the experiment 1, rats were euthanized in a chamber pre-charged with carbon dioxide (4 in the control, 5 in the CM, and 5 in the RF group), and cortical samples were obtained from the perilesional area. Isolated tissues were homogenized with 350 μl lysis buffer (Pro-Prep protein extract solution, iNtRON biotechnology, Seoul, Korea) on ice. Samples of the homogenate were placed on ice for at least 1 h. After 1 h, each sample of the homogenate was centrifuged at 4 °C, 15000 rpm, for 30 min, and the upper solution of the tube, without any surface residual, was obtained. The Bradford assay was used to measure the protein concentration. An equal amount of sample (40 μg) was electrophoresed on an SDS-PAGE (Mini-PROTEAN, Bio-Rad, Zurich, Switzerland) 10% Tri-glycine gel. After the electrophoresis protein bands were transferred to polyvinylidene fluoride membranes membranes using a Mini-PROTEAN apparatus, they were blocked for 1 h at room temperature with 5 % bovine serum albumin. Thereafter, they were incubated with rabbit anti-phospho-Akt (Ser 473) (1:1000; Cell Signaling, #4060), Akt (1:10000; Cell Signaling, #4691), bcl-2 (1:5000; Cell Signaling, #2870), bax (1:1000; Cell Signaling, #14796), iNOS (1:200; Abcam, ab15323), COX-2 (1:1000; Cell Signaling, #12282), β-actin (1:10000; Abcam, #ab8227), mouse anti-phospho-IκB (Ser 32/36) (1:500; Cell Signaling, #9246), and IκB (1:1000; Cell Signaling, #4812).

Horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Cell Signaling) and anti-mouse IgG (1:2000, Cell Signaling) were used as secondary antibodies. The chemiluminescence of the secondary antibody was developed with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Biosciences, Piscataway, NJ, USA). Normalized optical densities were measured with ImageJ software for quantification, with β-actin used for normalization. The p-Akt and p-IκB levels were expressed as the ratio to Akt and IκB, respectively.

Statistical analysis

Values are expressed as means ± standard error of the mean. The differences among the 3 experimental groups were evaluated using the Kruskal-Wallis test. For comparison between the 2 groups, the Mann-Whitney test was used. The changes in the behavioral tests in each group were also evaluated using the Wilcoxon signed rank test. The estimated number of animals per group was 5 for each statistical analysis. A *P*-value less than 0.05 was considered significant.

RESULTS

Experiments 1

hASC-CM and RFs ameliorate neurologic deterioration after experimental stroke

IL-6, VEGF, HGF, and BDNF were detected in the CM (Table 1). The concentrations of the detected factors increased with an increase in culture time. These factors were selected for intraventricular injection in the RF group.

There was no significant difference in the weight between the groups (Fig 2). The Garcia scale score was significantly different between the groups at 24 h after reperfusion (P = 0.029). In particular, both CM and RF groups showed better scores as compared to the control group (P = 0.029 and P = 0.034, respectively). Although the number of foot faults on the left side increased after MCAO in all groups, there was no significant difference in these values between groups. In contrast, the maximum rpm on the Rotarod significantly decreased after MCAO only in the control group (P = 0.003), and the RF group showed significantly better results as compared to the control group at 24 h after reperfusion (P = 0.038).

Table 1. Factors detected in the hASC-CM (pg/ml)

	24 h	48 h	72 h
IL-6	6.73 ± 1.40	91.27 ± 29.06	293.86 ± 73.51
VEGF	163.76 ± 8.24	376.78 ± 18.22	682.50 ± 40.78
HGF	2.52 ± 0.42	4.75 ± 0.40	42.04 ± 14.30
BDNF	Not detected	89.00 ± 10.88	255.80 ± 58.57

IL-1b, 2, 4, 5, 10, 12p70, and 13; GM-CSF; IFN-γ; TNF-α; IGF-1; bFGF; and PDGF-BB were not detected.

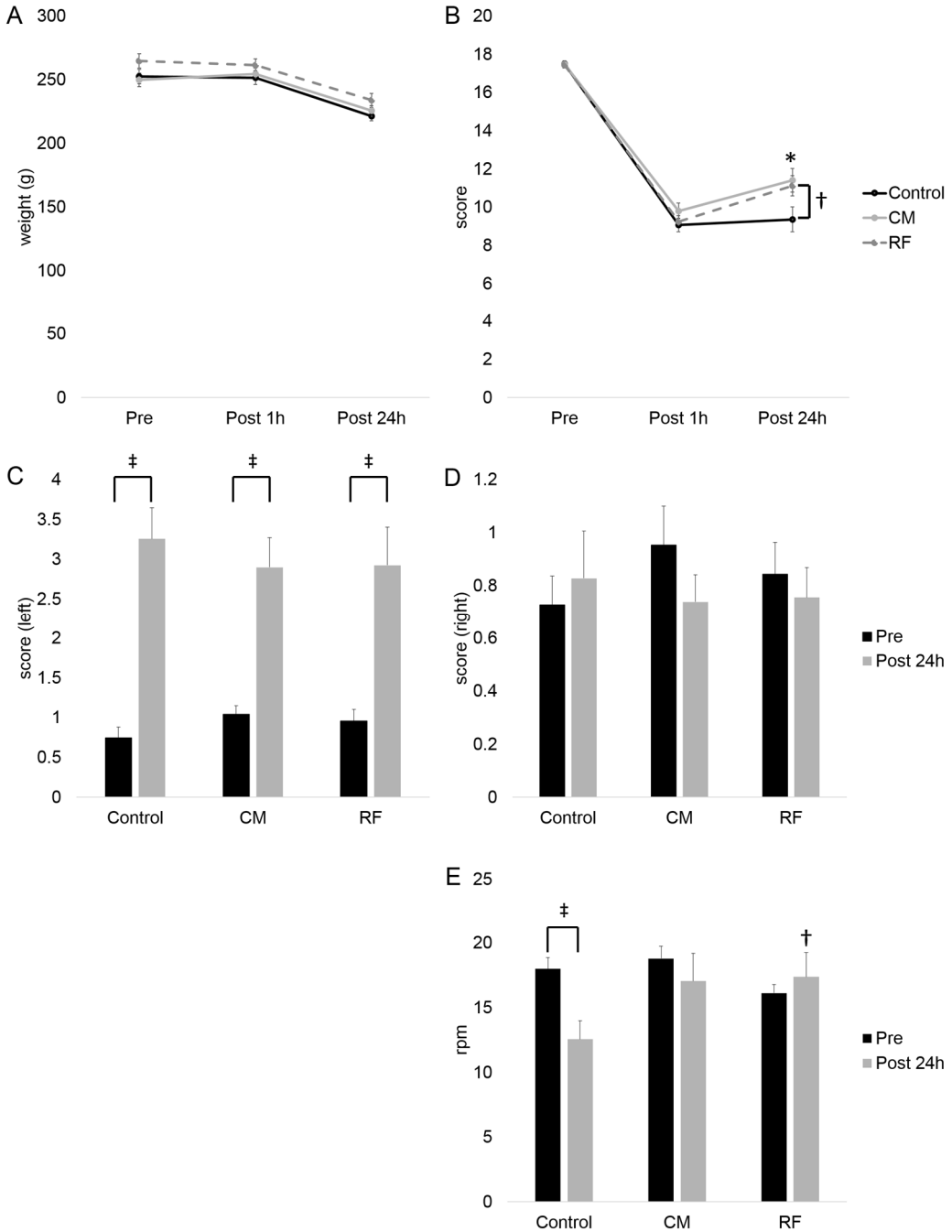


Figure 2. hASC-CM and RFs ameliorate neurologic deterioration after experimental

stroke. Following MCAO, an intraventricular injection was administered to each rat after 1 h of reperfusion according to experimental group: vehicle (DMEM), concentrated CM, and RFs mixed with DMEM. The weight (A) and Garcia scale scores (B) were evaluated at baseline, 1 h, and 24 h after reperfusion. The number of foot faults on the left (C) and right (D) sides during a horizontal ladder walk, and the maximum rpm on the Rotarod (E) were evaluated at baseline and 24 h after reperfusion.

Note: n = 14 in the control, 15 in the CM, and 16 in the RF groups; *$P < 0.05$, among the 3 groups by the Kruskal-Wallis test; †$P < 0.05$, compared to the control group by the Mann-Whitney test; ‡$P < 0.05$, between before and after 24 h by the Wilcoxon signed rank test.

hASC-CM and RFs reduce the infarct volume in the ischemic brain

The corrected infarct volumes were 258.86 ± 62.20, 62.28 ± 25.85, and 90.79 ± 38.62 mm^3 in the control, CM, and RF groups, respectively (P = 0.048) (Fig 3). The ratios of the infarct volume to the volume of the contralateral hemisphere were 35.90% ± 8.10%, 8.74% ± 3.69%, and 11.80% ± 5.05% in the control, CM, and RF groups, respectively (P = 0.036). Between-group comparisons indicated that the corrected infarct volume was significantly lower in the RF group than in the control group (P = 0.036); however, the difference in the infarct volume was only marginally significant in the CM group, as compared to that in the control group (P = 0.056). The ratio of the infarct volume to the volume of the contralateral hemisphere was significantly lower in both the CM and RF groups than in the control group (P = 0.032, respectively).

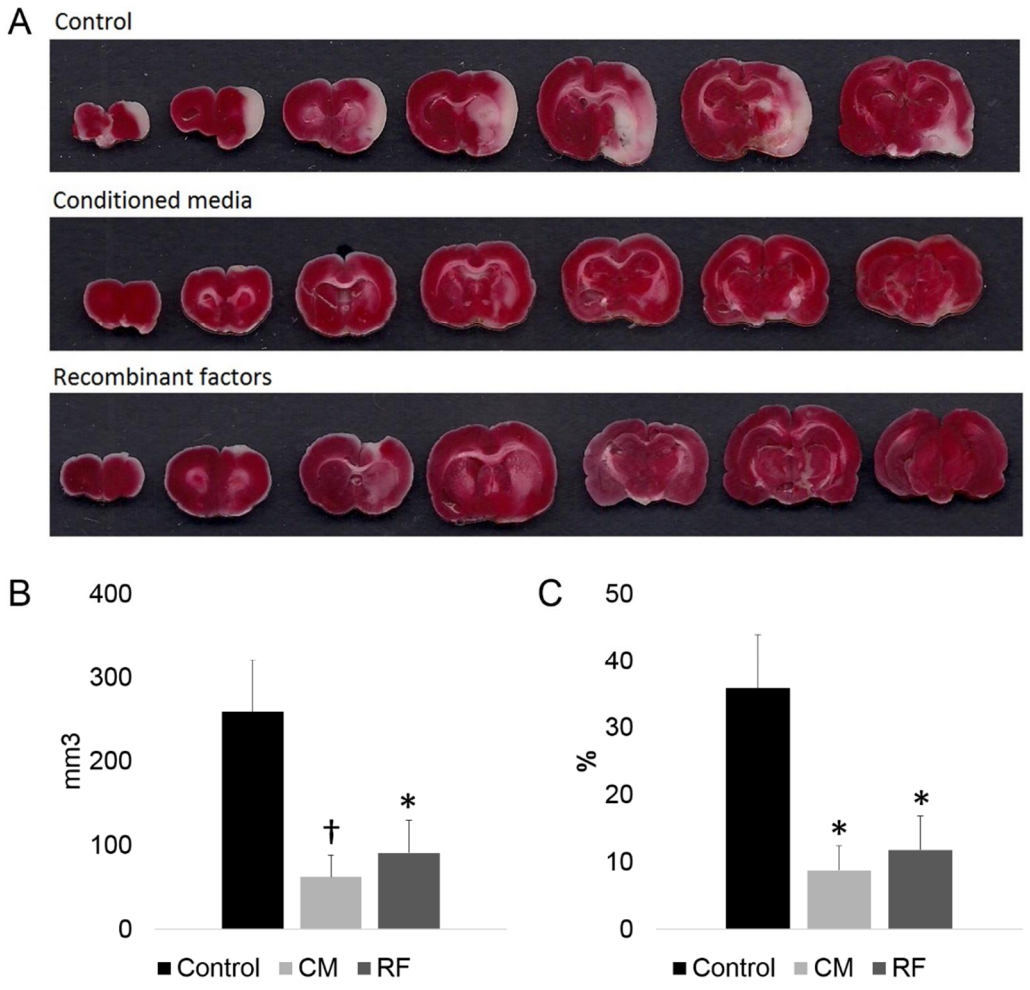


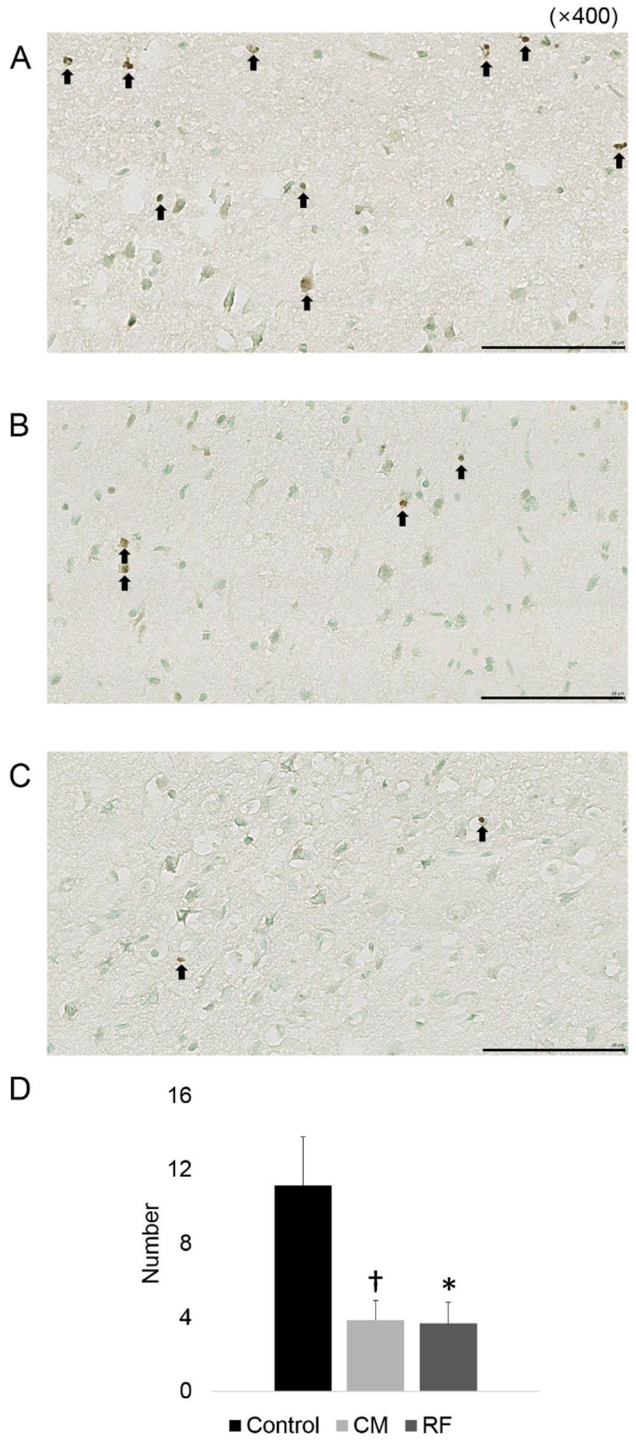
Figure 3. hASC-CM and RFs reduce the infarct volume in the ischemic brain. The rat brains were sectioned coronally into 7 slices (2 mm-thickness) from the frontal pole, and the infarcted areas were identified using 2, 3, 5-triphenyl tetrazolium chloride staining (A). Both the corrected infarct volume (B) and the ratio of the infarct volume to the volume of the contralateral hemisphere (C) were estimated using scanned digital images after 24 h of reperfusion.

Note: n = 5 in each group; *$P < 0.05$, †$P = 0.056$, compared to the control group by the Mann-Whitney test.

hASC-CM and RFs reduce cell apoptosis and enhance HSP 70 expression in the lesion border zone

There was a significant difference in the number of TUNEL-positive apoptotic cells in the peri-infarct area among the experimental groups ($P = 0.008$) (Fig 4). The RF group showed a significantly lower number of such cells as compared to the control group ($P = 0.030$). The number of such cells in the CM group was fewer than that in the control group, with borderline significance ($P = 0.056$).

HSP70 expression was mostly detected in the capillaries and bipolar cells in the ipsilateral perilesional cortex (Fig 5). A greater number of neuronal cells expressed HSP70 immunoreactivity in the CM and RF groups than in the control group. There was a significant difference in the area expressing HSP70 among the groups ($P = 0.028$). This area was significantly larger in the RF group than in the control group ($P = 0.004$). Although the difference between the CM and control groups was not significant ($P = 0.095$), the area was larger in the CM group than in the control group.


(×400)
A
B
C
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16
12
8
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†
*
Control
CM
RF

Figure 4. hASC-CM and RFs reduce cell apoptosis in the lesion border zone. DNA fragmentation in cells undergoing apoptosis was detected using TUNEL staining after 24 h of reperfusion. The arrows indicate the apoptotic cells (brown staining) in the perilesional area of the control (A), CM (B), and RF (C) groups (×400; scale bar, 100 μm). (D) A number of apoptotic cells were counted manually in 5 random microscopic fields in the perilesional areas.

Note: n = 5 in the control, 5 in the CM, and 6 in the RF group; **P* < 0.05, †*P* = 0.056 compared to the control group by the Mann-Whitney test.

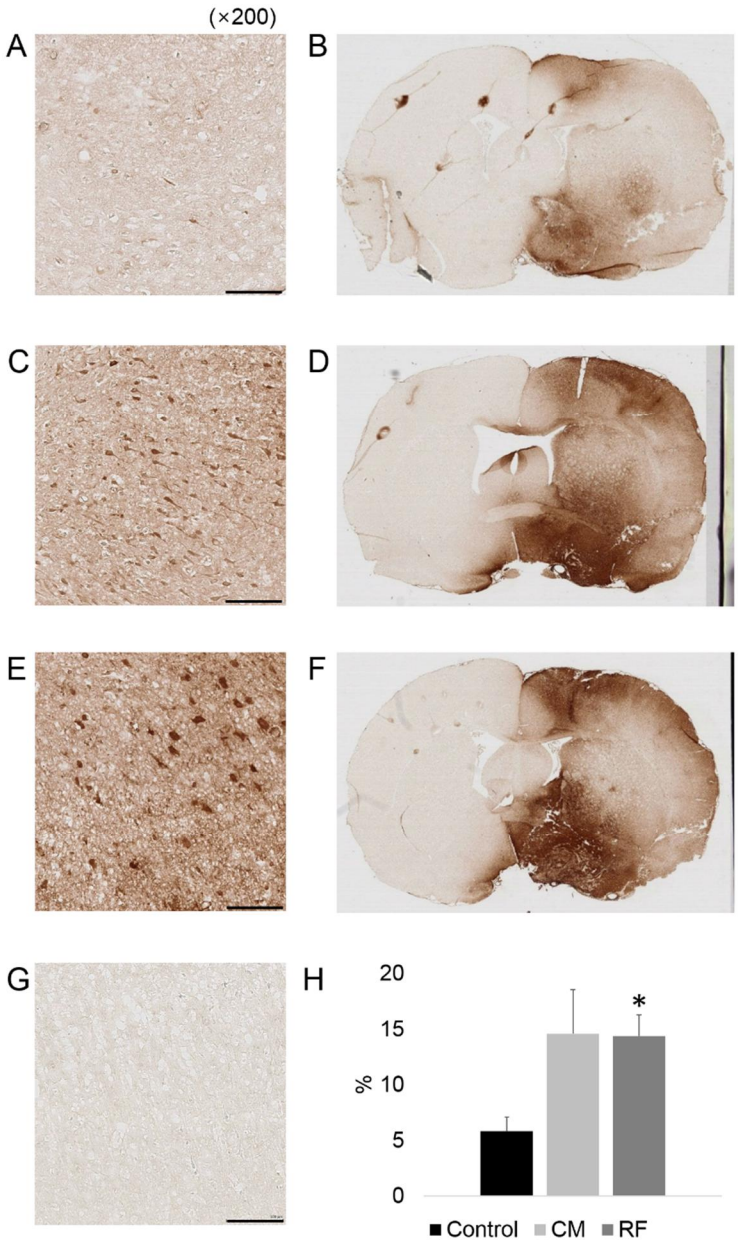

Figure 5. hASC-CM and RFs enhance HSP70 expression in the lesion border zone. The detection of HSP70 was performed using immunohistochemical staining to evaluate the

change in the lesion border zone. HSP70 expression was mostly detected in the capillaries and bipolar cells in the ipsilateral perilesional area of the control (A, B), CM (C, D), and RF (E, F) groups (×200; scale bar, 100 μm), and was not detected in the contralateral cortex (G). The ratio of the area of HSP70 expression to the whole brain area was measured in each brain section (H).

Note: n = 5 in the control, 5 in the CM, and 6 in the RF group; *$P < 0.05$, compared to the control group by the Mann-Whitney test.

hASC-CM and RF reduce IκB phosphorylation and influence bcl-2 and box protein expression

The ratio of phospho-IκB to total IκB was significantly different among the experimental groups (P = 0.018) (Fig 6). IκB phosphorylation was lower in both the CM and RF groups than in the control group (P = 0.016, respectively). The bcl-2 and bax protein expression was significantly different between the groups (P = 0.016, respectively). Bcl-2 expression was greater in the CM and RF groups than in the control group (P = 0.016 and P = 0.032, respectively); whereas, bax expression was lower in the CM and RF groups than in the control group (P = 0.016 and P = 0.032, respectively). There was no significant difference in the ratio of phospho-Akt to total Akt between the groups. Although iNOS and COX-2 expressions were relatively low in the CM and RF groups, respectively, the differences in the expressions were not significant.

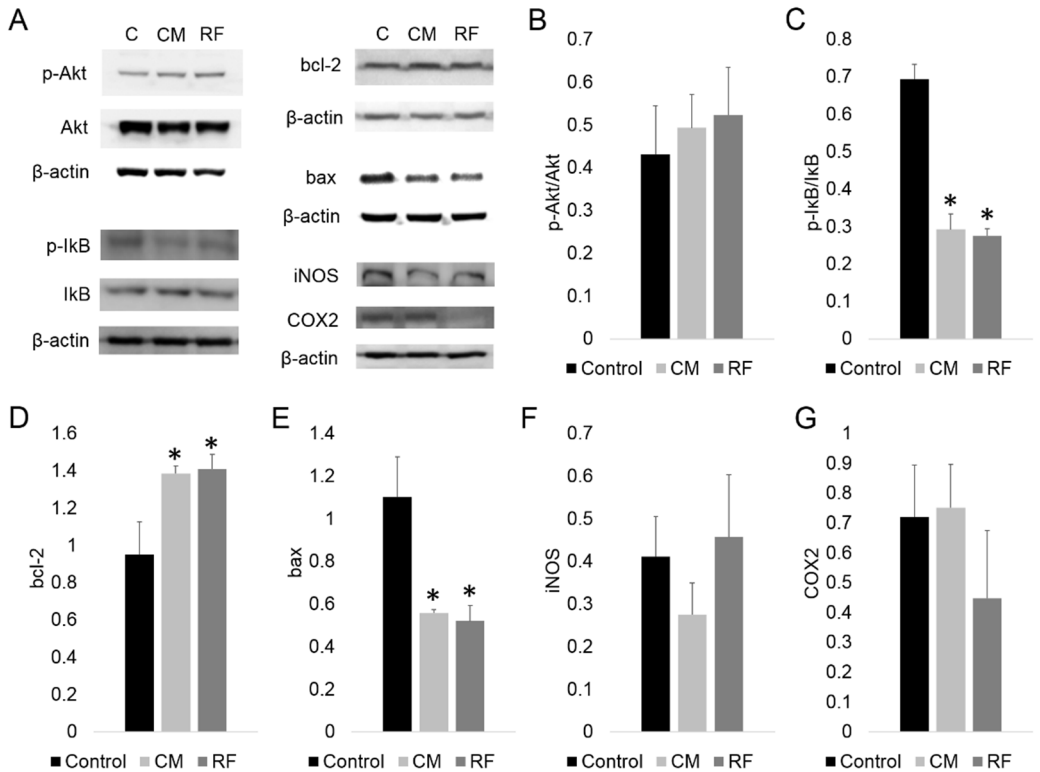

Figure 6. hASC-CM and RF reduce IκB phosphorylation and influence bcl-2 and box protein expression. The cortical samples were obtained from the perilesional area after 24 h of reperfusion and subjected to Western blot analysis (A) for detecting p-Akt/Akt (B), p-IκB/ IκB (C), bcl-2 (D), bax (E), iNOS (F), and COX-2 (G). The band density was normalized to β-actin.

Note: n = 4 in the control, 5 in the CM, and 5 in the RF group; **P* < 0.05, compared to the control group by the Mann-Whitney test.

Experiments 2

RFs improve the recovery of sensorimotor deficits

There was no significant difference in the weight between the groups until day 17 (Fig 7). The Garcia scale score was significantly different between the groups at day 10 (P = 0.031). In particular, the RF group showed better score as compared to the control group at day 10 (P = 0.016). Both foot fault test and Rotarod test showed no significant difference between the groups throughout the experimental period.

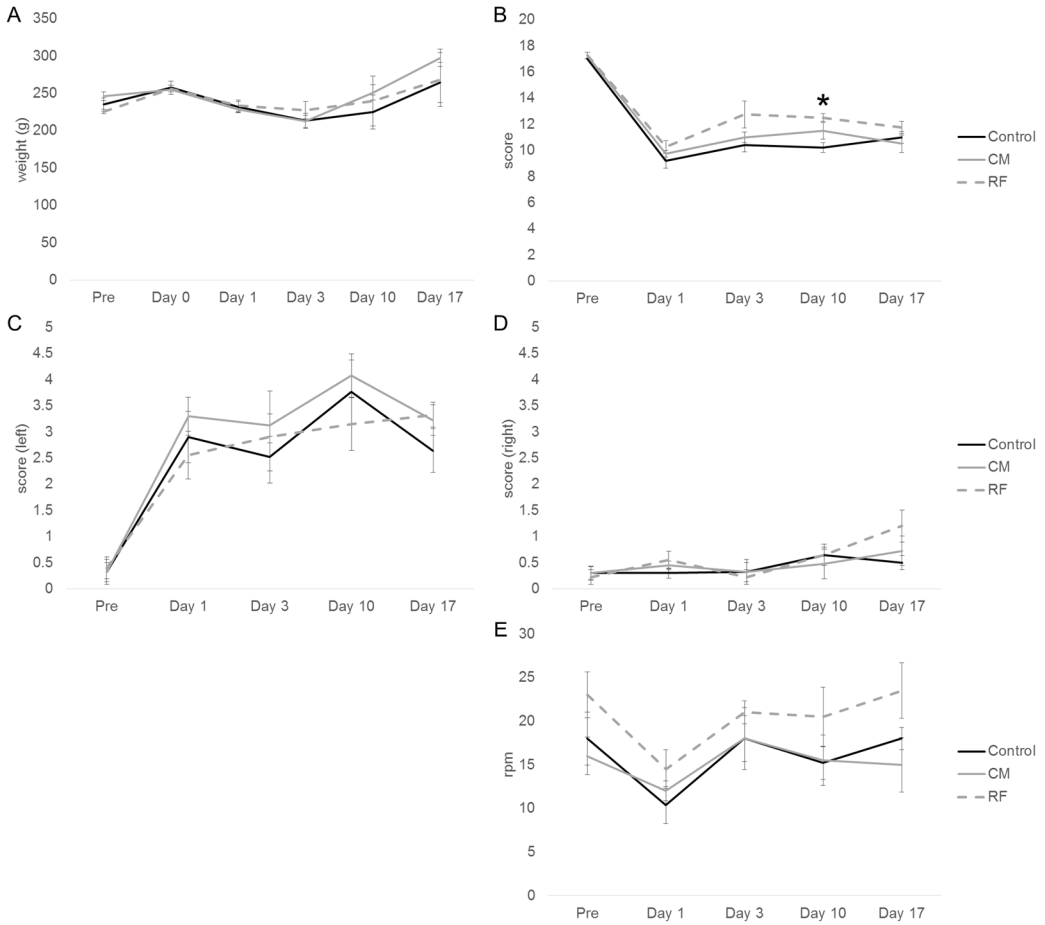

Figure 7. RFs improve the recovery of sensorimotor deficits. Following MCAO, an intraventricular injection was administered to each rat after 24 h of reperfusion according to experimental group: vehicle (DMEM), concentrated CM, and RFs mixed with DMEM. The weight (A), Garcia scale scores (B), number of foot faults on the left (C) and right sides (D) during a horizontal ladder walk, and the maximum rpm on the Rotarod (E) were evaluated from baseline to day 17.

Note: n = 5 in the control, 4 in the CM, and 4 in the RF groups; *$P < 0.05$, among the 3

groups by the Kruskal-Wallis test.

RFs increase the neuronal proliferation in the lesion border zone

Fig 8 shows the results of immunohistochemistry for BrdU and DCX in the perilesional areas. Although the number of $BrdU^+$ cells and $BrdU^+/DCX^+$ cells was greater in the CM and RF groups than the control group, the difference between the groups was marginally significant only in the number of $BrdU^+/DCX^+$ cells ($P = 0.074$). Between-group comparisons indicated that the number of $BrdU^+/DCX^+$ cells was significantly higher in the RF group than in the control group ($P = 0.016$).

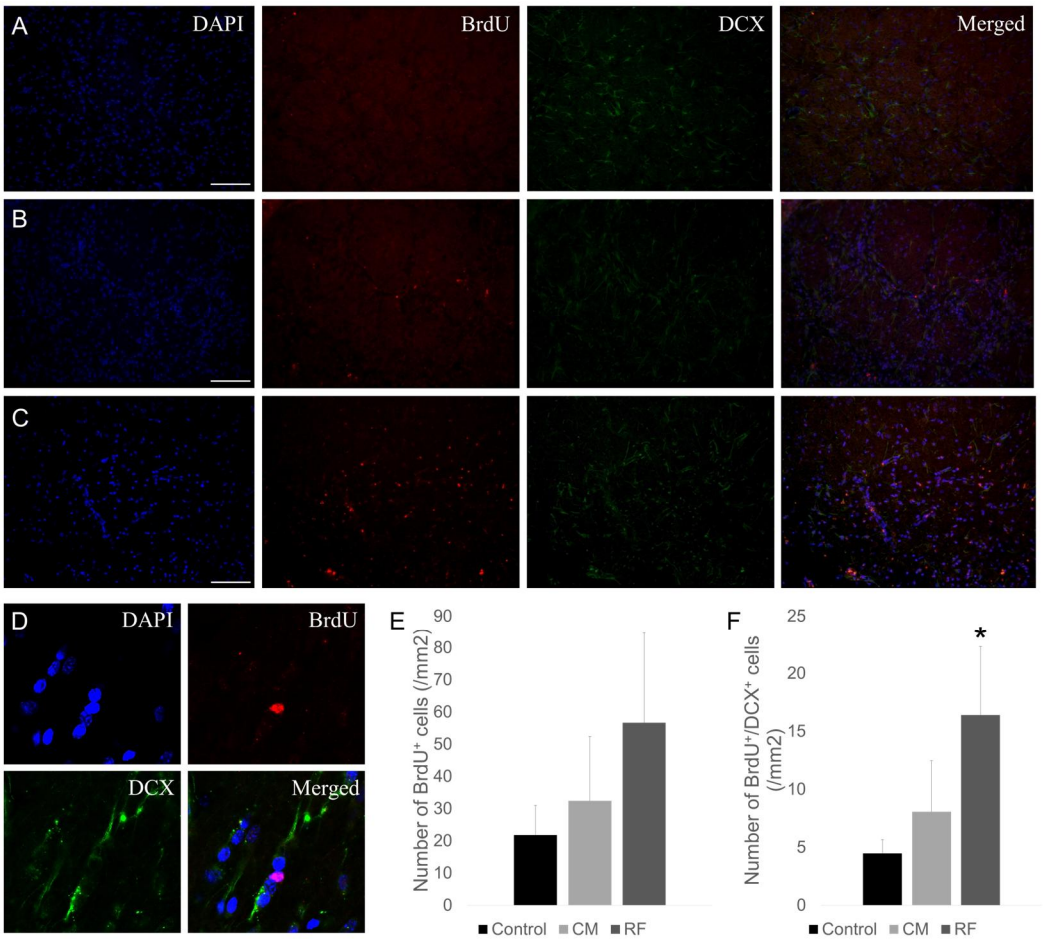

Figure 8. RFs increase the neuronal proliferation in the lesion border zone. The detection of BrdU+ and DCX+ cells was performed using immunohistochemical staining to evaluate the neuronal proliferation in the lesion border zone of the control (A), CM (B), and RF (C) groups (×200; scale bar, 100 μm) at 17 day after MCAO . The representative image shows a BrdU staining of nuclei with DCX staining of cytoplasm (D). The number of $BrdU^+$ (E) and $BrdU^+/DCX^+$ cells (F) were counted in 5 fields in each brain sections.

Note: n = 5 in the control, 4 in the CM, and 4 in the RF group; *$P < 0.05$, compared to the control group by the Mann-Whitney test

DISCUSSION

The hASC-CM and selected RFs, administered intraventricularly 1 h after reperfusion injury, exerted neuroprotective effects in an ischemic stroke rat model. Both the CM and RF groups demonstrated better neurological outcomes and a reduced infarct volume as compared to the control group. Moreover, reduced cell apoptosis and enhanced HSP70 expression, which indicates a promising prognosis for neuronal survival [20], in the peri-infarct area were observed in the CM and RF groups. As the results were comparable between the RF and CM groups, selected RFs may be considered to exert a comparable neuroprotective effect to hASC-CM, and may even harness the therapeutic potential of hASCs. In addition, the selected RFs, administered intraventricularly 24 h after reperfusion injury, improved the recovery of sensorimotor deficits and increased the neuronal proliferation in the perilesional areas after treadmill exercise in the stroke rat model. Although the results were preliminary, the selected RFs may also exert a neuroregenerative effect for ischemic stroke.

The profile of stem cell secretome has been widely investigated using cytokine arrays and liquid chromatography with mass spectrometry in previous studies [21, 13, 22, 23]. The secretome contains factors related to neuroprotection and angiogenesis [23-25], and has demonstrated neuroprotective effects in both in vitro [22, 23, 26] and in vivo models [8-10]. Therefore, the stem cell secretome has been suggested as a potential cell-free treatment that may overcome the limitations and risks associated with cell-based therapeutics [5, 24]. However, as a treatment, the stem cell secretome raises several

issues, such as the variation in the secretome profiles between studies and the lack of standardized methods for its production [27]. The development of secretome therapy may be difficult due to the very low concentration of the secreted factors. In the present study, the estimated concentration of the detected factors in the concentrated CM (~95-fold) was about 4.0-64.8 ng/ml. On the other hand, the concentration of each recombinant factor was 20 μg/ml. Moreover, human stem cells are required for secretome use, and the safety aspect of using cell-based therapeutics remains a concern.

The neuroprotective and neuroregenerative mediators in the stem cell secretome have been assessed via measurement or inhibition of selected factors in most studies. VEGF [28-30], HGF [28, 31], BDNF [32, 33], and IL-6 [29, 30], which were detected in the hASC-CM in the present study, are the mediators secreted from mesenchymal stem cells. Nakano et al. [22] reported that individual trophic factors contained in the bone marrow stromal cell-CM, including IGF-1, HGF, VEGF, TGF-β1, or their combinations, were effective at promoting neuronal survival and neurite outgrowth in vitro, but at a lesser extent than that promoted by the CM itself. Although these factors have been assessed individually for their neuroprotective and neuroregenerative effects in in vivo ischemic stroke models [19, 34-41], the simultaneous administration of several factors may be necessary to harness the therapeutic potential of stem cells [5]. However, no study has assessed the therapeutic effect of multiple secreted factors from human stem cells in an in vivo stroke model until now.

BDNF is one of the most abundant growth factors in the brain. BDNF acts through

tropomyosin-related kinase receptor type B, has major roles in supporting neuronal survival and function in the normal adult brain, and has been shown to mediate neuroprotective and neuroregenerative effects in models of various neurological diseases [42]. In rat models of ischemic stroke, intraventricular BDNF has been found to reduce the infarct size and apoptosis in the penumbra [39], and ameliorate neurological deficits [34]. The intravenous infusion of BDNF also improved functional recovery by increasing neuronal remodeling [37] and by upregulating the binding densities of excitatory glutamate receptors [43] after cortical ischemia in rats. As BDNF is an intriguing candidate for the development of neurological disease indications, peptide analogues of BDNF have been developed with molecular weights that enable the crossing of the blood-brain barrier [44].

VEGF is a growth factor that stimulates the formation of blood vessels; it also exerts neuroprotective effects directly via neural cells and indirectly via brain perfusion [45]. VEGF has been found to be a neuroprotective mediator of stem cells in most studies [28-30]. In fact, previous studies have reported that intraventricularly delivered VEGF decreased the infarct size in ischemic stroke models [36, 19, 38]. The effect of VEGF administration has been suggested to involve anti-apoptosis [38] and anti-inflammatory mechanisms [46]. Although the therapeutic use of VEGF should be carefully considered, as it could aggravate tissue damage through an increase in blood-brain-barrier leakage [45], it may serve as an essential component for harnessing the therapeutic potential of stem cells for ischemic stroke.

In addition, HGF, which was originally discovered as a mitogen of hepatocytes, exerts protective effects on multiple organs, including the brain, via anti-apoptotic and anti-inflammatory signals [47]. HGF reportedly plays an important role in the therapeutic potency of stem cells, particularly hASCs [31, 48]. HGF treatment reduced infarct volume, apoptotic neuronal cell death, and glial scar formation, and enhanced neurogenesis, angiogenesis, and synaptogenesis after ischemic stroke [35, 49]. In addition, acute HGF treatment after stroke induced long-term neuroprotection and neurological recovery [50].

IL-6 is part of the family of IL-6-type cytokines that are involved in the regulation of the acute-phase response to injury and infection [51]. IL-6 induces a signal via glycoprotein 130, which then activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway and the mitogen-activated protein kinase (MAPK) cascade. Although upregulated IL-6 is considered to increase harmful mediators and lead to the aggravation of cerebral ischemic damage via inflammatory cascades [52], there is no direct evidence showing detrimental effect of IL-6 in acute phase of cerebral ischemia. IL-6 is also involved in the controlling the level of acute inflammatory responses by decreasing the pro-inflammatory and increasing anti-inflammatory cytokines [53]. On the other hand, IL-6 has an aspect of a neurotrophic cytokine which reduces excitotoxic neuronal damage against NMDA-mediated injury and protects neurons against apoptosis [53]. Despite the ambivalent roles of IL-6 between inflammatory and neurotrophic aspects, its neuroprotective effect after ischemic brain injury has been reported in several

studies [40, 41, 54, 55]. Moreover, mesenchymal stem cell-induced neuronal recovery and immune modulation are reportedly mediated by the secretion of IL-6 and VEGF [29]. The role of IL-6 after ischemic stroke may depend on the stages of cerebral ischemia and the pathologic context. Further studies are warranted to elucidate the precise role of IL-6 over a longer period after cerebral ischemia.

The neuroprotective effects of the hASC-CM and the selected RFs appear to be mediated by an anti-inflammatory mechanism and cell apoptosis inhibition. NF-κB, a redox-sensitive transcription factor, mediates the transcription of various inflammatory genes involved in the pathophysiology of cerebral ischemic/reperfusion injury [56, 57]. As IκB phosphorylation leads to the activation and migration of NF-κB into the nucleus [58], reduced IκB phosphorylation indicates the suppression of the NF-κB pathway by hASC-CM and RFs in the present study. Moreover, enhanced HSP70 expression in the peri-infarct area may contribute to this suppression, because HSP70 has been reported to bind to the NF-κB:IκB complex, thus preventing IκB phosphorylation by IκB kinase [59]. Although the changes in iNOS and COX-2 expression were not significant, the neuroprotective effect observed in the CM and RF groups is deemed to be mediated, at least in part, via an anti-inflammatory mechanism. Moreover, increased bcl-2 and decreased bax expression, which are well known regulators of cell apoptosis [60], could also be involved in the mechanism of neuroprotection by hASC-CM and selected RFs. In contrast, the PI3k/Akt signaling pathway was not directly involved in this process, although the activation of this pathway is known to prevent neuronal apoptosis after

cerebral ischemic/reperfusion injury [61, 62].

Our study has several limitations. First, hASC-CM was analyzed using the multiple-protein analysis method with selected inflammatory cytokines and growth factors. Considering the numerous factors secreted from stem cells [13], there may be other factors that were not included in our analysis that are responsible for stem cell-mediated neuroprotection and neuroregeneration after ischemic stroke. However, this study showed that some key factors could exert a therapeutic effect similar to that of the whole stem cell secretome, so that it could be used as a therapeutic agent without revealing all the trace elements secreted by the stem cells. Second, the different concentrations of the secreted factors and their temporal variations in the hASC-CM were not considered when preparing the RFs. Although there is no evidence that the temporal variation in the stem cell secretome has an effect on their therapeutic effect, specific temporal variations in the composition of the CM may trigger different effects on the neurons and distinct glial cell populations [63]. The synergistic effects of multiple factors may require specific concentrations and time points of administration to achieve an optimal therapeutic effect. Third, although this study also evaluated the long-term neuroregenerative effect, most results focused on the immediate neuroprotective effect at 24 h after cerebral ischemic/reperfusion injury. The results of neuroregenerative effect were quiet preliminary and suggest no underlying mechanism. Further studies are warranted to elucidate the long-term effect of the RFs in ischemic stroke.

In conclusion, selected RFs, detected in the hASC-CM, exerted a neuroprotective effect

in an ischemic stroke rat model when administered intraventricularly at 1 h after reperfusion. The effect may be comparable to that exerted by the hASC-CM. Moreover, these therapeutic effects exerted by the RFs seem to be mediated by an anti-inflammatory mechanism and cell apoptosis inhibition. In addition, the selected RFs may also exert a neuroregenerative effect when administered intraventricularly at 24 h after reperfusion. Thus, the therapeutic use of RFs can be considered a feasible substitute for stem cell therapy after stroke.

References

[1] Janowski M, Wagner DC, Boltze J. Stem Cell-Based Tissue Replacement After Stroke: Factual Necessity or Notorious Fiction? Stroke. 2015;46:2354-63.

[2] Martino G, Pluchino S. The therapeutic potential of neural stem cells. Nat Rev Neurosci. 2006;7:395-406.

[3] Pluchino S, Zanotti L, Rossi B, Brambilla E, Ottoboni L, Salani G et al. Neurosphere-derived multipotent precursors promote neuroprotection by an immunomodulatory mechanism. Nature. 2005;436:266-71.

[4] Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006;98:1076-84.

[5] Drago D, Cossetti C, Iraci N, Gaude E, Musco G, Bachi A et al. The stem cell secretome and its role in brain repair. Biochimie. 2013;95:2271-85.

[6] Dulamea AO. The potential use of mesenchymal stem cells in stroke therapy--From bench to bedside. J Neurol Sci. 2015;352:1-11.

[7] Bang OY. Clinical Trials of Adult Stem Cell Therapy in Patients with Ischemic Stroke. J Clin Neurol. 2016;12:14-20.

[8] Egashira Y, Sugitani S, Suzuki Y, Mishiro K, Tsuruma K, Shimazawa M et al. The conditioned medium of murine and human adipose-derived stem cells exerts neuroprotective effects against experimental stroke model. Brain Res. 2012;1461:87-95.

[9] Cho YJ, Song HS, Bhang S, Lee S, Kang BG, Lee JC et al. Therapeutic effects of human adipose stem cell-conditioned medium on stroke. J Neurosci Res. 2012;90:1794-802.

[10] Tsai MJ, Tsai SK, Hu BR, Liou DY, Huang SL, Huang MC et al. Recovery of neurological function of ischemic stroke by application of conditioned medium of bone marrow mesenchymal

stem cells derived from normal and cerebral ischemia rats. J Biomed Sci. 2014;21:5.

[11] Jeon D, Chu K, Lee ST, Jung KH, Ban JJ, Park DK et al. Neuroprotective effect of a cell-free extract derived from human adipose stem cells in experimental stroke models. Neurobiol Dis. 2013;54:414-20.

[12] Teixeira FG, Carvalho MM, Sousa N, Salgado AJ. Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration? Cell Mol Life Sci. 2013;70:3871-82.

[13] Sze SK, de Kleijn DP, Lai RC, Khia Way Tan E, Zhao H, Yeo KS et al. Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. Mol Cell Proteomics. 2007;6:1680-9.

[14] Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke. 1989;20:84-91.

[15] Garcia JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. Stroke. 1995;26:627-34; discussion 35.

[16] Rogers DC, Campbell CA, Stretton JL, Mackay KB. Correlation between motor impairment and infarct volume after permanent and transient middle cerebral artery occlusion in the rat. Stroke. 1997;28:2060-5; discussion 6.

[17] Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM. Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke. 1986;17:1304-8.

[18] Belayev L, Khoutorova L, Deisher TA, Belayev A, Busto R, Zhang Y et al. Neuroprotective effect of SolCD39, a novel platelet aggregation inhibitor, on transient middle cerebral artery occlusion in rats. Stroke. 2003;34:758-63.

[19] Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A et al. VEGF-induced neuroprotection,

neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest. 2003;111:1843-51.

[20] Popp A, Jaenisch N, Witte OW, Frahm C. Identification of ischemic regions in a rat model of stroke. PLoS One. 2009;4:e4764.

[21] Liu CH, Hwang SM. Cytokine interactions in mesenchymal stem cells from cord blood. Cytokine. 2005;32:270-9.

[22] Nakano N, Nakai Y, Seo TB, Yamada Y, Ohno T, Yamanaka A et al. Characterization of conditioned medium of cultured bone marrow stromal cells. Neurosci Lett. 2010;483:57-61.

[23] Hsieh JY, Wang HW, Chang SJ, Liao KH, Lee IH, Lin WS et al. Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis. PLoS One. 2013;8:e72604.

[24] Kim HO, Choi S-M, Kim H-S. Mesenchymal stem cell-derived secretome and microvesicles as a cell-free therapeutics for neurodegenerative disorders. Tissue Engineering and Regenerative Medicine. 2013;10:93-101.

[25] Bronckaers A, Hilkens P, Martens W, Gervois P, Ratajczak J, Struys T et al. Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. Pharmacol Ther. 2014;143:181-96.

[26] Wang X, Zhao Z, Gong J, Zhou S, Peng H, Shatara A et al. Adipose stem cells-conditioned medium blocks 6-hydroxydopamine-induced neurotoxicity via the IGF-1/PI3K/AKT pathway. Neurosci Lett. 2014;581:98-102.

[27] Pawitan JA. Prospect of stem cell conditioned medium in regenerative medicine. Biomed Res Int. 2014;2014:965849.

[28] Chang CP, Chio CC, Cheong CU, Chao CM, Cheng BC, Lin MT. Hypoxic preconditioning enhances the therapeutic potential of the secretome from cultured human mesenchymal stem cells in experimental traumatic brain injury. Clin Sci (Lond). 2013;124:165-76.

[29] Huang P, Gebhart N, Richelson E, Brott TG, Meschia JF, Zubair AC. Mechanism of mesenchymal stem cell-induced neuron recovery and anti-inflammation. Cytotherapy. 2014;16:1336-44.

[30] Lahiani A, Zahavi E, Netzer N, Ofir R, Pinzur L, Raveh S et al. Human placental eXpanded (PLX) mesenchymal-like adherent stromal cells confer neuroprotection to nerve growth factor (NGF)-differentiated PC12 cells exposed to ischemia by secretion of IL-6 and VEGF. Biochim Biophys Acta. 2015;1853:422-30.

[31] Ikegame Y, Yamashita K, Hayashi S, Mizuno H, Tawada M, You F et al. Comparison of mesenchymal stem cells from adipose tissue and bone marrow for ischemic stroke therapy. Cytotherapy. 2011;13:675-85.

[32] Wei X, Du Z, Zhao L, Feng D, Wei G, He Y et al. IFATS collection: The conditioned media of adipose stromal cells protect against hypoxia-ischemia-induced brain damage in neonatal rats. Stem Cells. 2009;27:478-88.

[33] Zhao L, Wei X, Ma Z, Feng D, Tu P, Johnstone BH et al. Adipose stromal cells-conditional medium protected glutamate-induced CGNs neuronal death by BDNF. Neurosci Lett. 2009;452:238-40.

[34] Schabitz WR, Schwab S, Spranger M, Hacke W. Intraventricular brain-derived neurotrophic factor reduces infarct size after focal cerebral ischemia in rats. J Cereb Blood Flow Metab. 1997;17:500-6.

[35] Tsuzuki N, Miyazawa T, Matsumoto K, Nakamura T, Shima K, Chigasaki H. Hepatocyte growth factor reduces infarct volume after transient focal cerebral ischemia in rats. Acta Neurochir Suppl. 2000;76:311-6.

[36] Harrigan MR, Ennis SR, Sullivan SE, Keep RF. Effects of intraventricular infusion of vascular endothelial growth factor on cerebral blood flow, edema, and infarct volume. Acta

Neurochir (Wien). 2003;145:49-53.

[37] Schabitz WR, Berger C, Kollmar R, Seitz M, Tanay E, Kiessling M et al. Effect of brain-derived neurotrophic factor treatment and forced arm use on functional motor recovery after small cortical ischemia. Stroke. 2004;35:992-7.

[38] Kaya D, Gursoy-Ozdemir Y, Yemisci M, Tuncer N, Aktan S, Dalkara T. VEGF protects brain against focal ischemia without increasing blood--brain permeability when administered intracerebroventricularly. J Cereb Blood Flow Metab. 2005;25:1111-8.

[39] Takeshima Y, Nakamura M, Miyake H, Tamaki R, Inui T, Horiuchi K et al. Neuroprotection with intraventricular brain-derived neurotrophic factor in rat venous occlusion model. Neurosurgery. 2011;68:1334-41.

[40] Jung JE, Kim GS, Chan PH. Neuroprotection by interleukin-6 is mediated by signal transducer and activator of transcription 3 and antioxidative signaling in ischemic stroke. Stroke. 2011;42:3574-9.

[41] Feng Q, Wang YI, Yang Y. Neuroprotective effect of interleukin-6 in a rat model of cerebral ischemia. Exp Ther Med. 2015;9:1695-701.

[42] Nagahara AH, Tuszynski MH. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. Nat Rev Drug Discov. 2011;10:209-19.

[43] Muller HD, Hanumanthiah KM, Diederich K, Schwab S, Schabitz WR, Sommer C. Brain-derived neurotrophic factor but not forced arm use improves long-term outcome after photothrombotic stroke and transiently upregulates binding densities of excitatory glutamate receptors in the rat brain. Stroke. 2008;39:1012-21.

[44] Logvinov IO, Antipova TA, Gudasheva TA, Tarasiuk AV, Antipov PI, Seredenin SB. Neuroprotective effects of dipeptide analogue of brain-derived neurotrophic factor GSB-106 in in vitro experiments. Bull Exp Biol Med. 2013;155:343-5.

[45] Lange C, Storkebaum E, de Almodovar CR, Dewerchin M, Carmeliet P. Vascular endothelial growth factor: a neurovascular target in neurological diseases. Nat Rev Neurol. 2016.

[46] Herz J, Reitmeir R, Hagen SI, Reinboth BS, Guo Z, Zechariah A et al. Intracerebroventricularly delivered VEGF promotes contralesional corticorubral plasticity after focal cerebral ischemia via mechanisms involving anti-inflammatory actions. Neurobiol Dis. 2012;45:1077-85.

[47] Nakamura T, Mizuno S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. Proc Jpn Acad Ser B Phys Biol Sci. 2010;86:588-610.

[48] Cai L, Johnstone BH, Cook TG, Liang Z, Traktuev D, Cornetta K et al. Suppression of hepatocyte growth factor production impairs the ability of adipose-derived stem cells to promote ischemic tissue revascularization. Stem Cells. 2007;25:3234-43.

[49] Shang J, Deguchi K, Ohta Y, Liu N, Zhang X, Tian F et al. Strong neurogenesis, angiogenesis, synaptogenesis, and antifibrosis of hepatocyte growth factor in rats brain after transient middle cerebral artery occlusion. J Neurosci Res. 2011;89:86-95.

[50] Doeppner TR, Kaltwasser B, ElAli A, Zechariah A, Hermann DM, Bahr M. Acute hepatocyte growth factor treatment induces long-term neuroprotection and stroke recovery via mechanisms involving neural precursor cell proliferation and differentiation. J Cereb Blood Flow Metab. 2011;31:1251-62.

[51] Heinrich PC, Behrmann I, Haan S, Hermanns HM, Muller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J. 2003;374:1-20.

[52] Huang J, Upadhyay UM, Tamargo RJ. Inflammation in stroke and focal cerebral ischemia. Surg Neurol. 2006;66:232-45.

[53] Suzuki S, Tanaka K, Suzuki N. Ambivalent aspects of interleukin-6 in cerebral ischemia:

inflammatory versus neurotrophic aspects. J Cereb Blood Flow Metab. 2009;29:464-79.

[54] Loddick SA, Turnbull AV, Rothwell NJ. Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. J Cereb Blood Flow Metab. 1998;18:176-9.

[55] Ali C, Nicole O, Docagne F, Lesne S, MacKenzie ET, Nouvelot A et al. Ischemia-induced interleukin-6 as a potential endogenous neuroprotective cytokine against NMDA receptor-mediated excitotoxicity in the brain. J Cereb Blood Flow Metab. 2000;20:956-66.

[56] Williams AJ, Dave JR, Tortella FC. Neuroprotection with the proteasome inhibitor MLN519 in focal ischemic brain injury: relation to nuclear factor kappaB (NF-kappaB), inflammatory gene expression, and leukocyte infiltration. Neurochem Int. 2006;49:106-12.

[57] Buchan AM, Li H, Blackburn B. Neuroprotection achieved with a novel proteasome inhibitor which blocks NF-kappaB activation. Neuroreport. 2000;11:427-30.

[58] Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci. 2005;30:43-52.

[59] Zheng Z, Kim JY, Ma H, Lee JE, Yenari MA. Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke. J Cereb Blood Flow Metab. 2008;28:53-63.

[60] Basu A, Haldar S. The relationship between BcI2, Bax and p53: consequences for cell cycle progression and cell death. Mol Hum Reprod. 1998;4:1099-109.

[61] Lu C, Liu L, Chen Y, Ha T, Kelley J, Schweitzer J et al. TLR2 ligand induces protection against cerebral ischemia/reperfusion injury via activation of phosphoinositide 3-kinase/Akt signaling. J Immunol. 2011;187:1458-66.

[62] Jover-Mengual T, Miyawaki T, Latuszek A, Alborch E, Zukin RS, Etgen AM. Acute estradiol protects CA1 neurons from ischemia-induced apoptotic cell death via the PI3K/Akt pathway. Brain Res. 2010;1321:1-12.

[63] Ribeiro CA, Salgado AJ, Fraga JS, Silva NA, Reis RL, Sousa N. The secretome of bone

marrow mesenchymal stem cells-conditioned media varies with time and drives a distinct effect on mature neurons and glial cells (primary cultures). J Tissue Eng Regen Med. 2011;5:668-72.

Appendix

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Dear Han Gil Seo,

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국문초록

뇌졸중 백서 모델에서 지방유래 줄기세포 분비인자의 신경보호 및 신경재생 효과

줄기세포 치료는 뇌졸중 치료의 새로운 패러다임으로 떠오르고 있다. 최근의 근거에 따르면 줄기세포는 면역조절인자 및 신경영양인자의 분비를 통해 치료 효과를 나타낸다. 비록 줄기세포의 잠재적인 신경보호 및 신경재생성 매개체가 시험관 내 및 생체 내 연구에서 제안된 바 있지만, 생체 내 뇌졸중 모델에서 그 분비인자들의 치료 효과를 전체 줄기세포 분비인자군(secretome)의 치료 효과와 비교한 연구는 없다. 따라서 본 연구의 목적은 허혈성 뇌졸중 백서 모델에서 인간 지방유래 줄기세포(human adipose stem cell; hASC)의 조건화 배지(conditioned media; CM)에서 검출된 선택된 재조합 인자들(recombinant factors; RFs)의 신경보호 및 신경재생 효과를 평가하는 것이다.

CM은 혈청이 없는 Dulbecco's modified Eagle medium (DMEM)에서 72시간 동안 hASC를 배양하여 제조하였다. Multiplex bead array와 ELISA를 이용한 다중 단백질 분석을 통해 CM 내에 포함된 분비인자를 검출할 수 있었다. 허혈성 뇌졸중은 Sprague-Dawley 백서에서 2시간의

일시적인 중간 대뇌 동맥 폐쇠(middle cerebral artery occlusion; MCAO)를 사용하여 유도되었다. 실험 1에서 재관류 1시간 후 매개체(DMEM), 농축된 CM, DMEM과 혼합된 선택된 RFs를 각 그룹(각각 n=14, 15, 16)에 뇌실내로 투여하였다. MCAO 후 24시간 후에 백서를 희생시켰다. 실험 2에서는 재관류 24시간 후 해당하는 중재를 각 군에 시행하였다(각각 n=5, 4, 4). 모든 동물들은 1일부터 3일까지 매일 bromodeoxyuridine (BrdU, 50mg/kg)을 복강 내 주사하였다. 이후, 동물들은 백서 트레드밀을 이용해 2주간 운동을 시행하였고 재관류 17일에 희생되었다.

다중 단백질 분석결과 hASC-CM에서 IL-6, VEGF, HGF, BDNF가 검출되었다. 실험 1에서 CM군과 RF군 모두 MCAO 후 24시간에 대조군보다 유의하게 우수한 감각운동 신경검사 점수를 나타냈다. 뇌경색 부피는 CM군과 RF군 모두 대조군보다 유의하게 작았다. CM군과 RF군에서 TUNEL 양성인 사멸 세포의 수가 감소된 반면, HSP70의 발현은 경색 주위 영역에서 강화되었다. 또한, hASC-CM과 RFs는 IκB 인산화를 감소시키고 bcl-2 및 bax 단백질 발현에 영향을 주었다. 실험 2에서, RF군은 MCAO 후 10일째에 대조군에 비해 감각운동 장애의 회복이 유의하게 양호한 것으로 나타났고, 17일째에 시행한 면역형광염색에서 경색 주변부에서 증가된 신경세포 증식을 보였다.

본 연구결과는 허혈성 뇌졸중 백서 모델에서 hASC-CM에서 선택된

RFs가 전체 hASC-CM와 유사한 뇌졸중 직후의 신경보호 효과를 나타낼 수 있음을 시사한다. RFs의 신경보호 효과는 항염증 기전 및 세포사멸 억제에 의해 매개될 수 있다. 또한, 선택된 RFs는 급성기 이후 투여되었을 때 신경재생 효과를 나타낼 수 있다. 그러므로 RFs를 이용한 치료는 뇌졸중에서 줄기세포 치료의 실현 가능한 대체물로 고려될 수 있을 것이다.

색인 : 뇌졸중, 줄기세포, 신경보호, 백서, 재조합 인자

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