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Human Umbilical Cord Mesenchymal Stem Cells Preserve Adult Newborn Neurons and Reduce Neurological Injury after Cerebral Ischemia by Reducing the Number of Hypertrophic Microglia/Macrophages

Willie Lin[1], Yogi Chang-Yo Hsuan[1], Mao-Tsun Lin[2], Ting-Wei Kuo[3], Cheng-Hsien Lin[1], Yu-Chin Su[1], Ko-Chi Niu[4], Ching-Ping Chang[2,3,5], and Hung-Jung Lin[3,6]

Abstract

Microglia are the first source of a neuroinflammatory cascade, which seems to be involved in every phase of stroke-related neuronal damage. Two weeks after transient middle cerebral artery occlusion (MCAO), vehicle-treated rats displayed higher numbers of total ionized calcium-binding adaptor molecule 1 (Iba-1)-positive cells, greater cell body areas of Iba-1-positive cells, and higher numbers of hypertrophic Iba-1-positive cells (with a cell body area over 80 μm^2) in the ipsilateral ischemic brain regions including the frontal cortex, striatum, and parietal cortex. In addition, MCAO decreased the number of migrating neuroblasts (or DCX- and 5-ethynyl-2′-deoxyuridine-positive cells) in the cortex, subventricular zone, and hippocampus of the ischemic brain, followed by neurological injury (including brain infarct and neurological deficits). Intravenous administration of human umbilical cord–derived mesenchymal stem cells (hUC-MSCs; 1×10^6 or 4×10^6) at 24 h after MCAO reduced neurological injury, decreased the number of hypertrophic microglia/macrophages, and increased the number of newborn neurons in rat brains. Thus, the accumulation of hypertrophic microglia/macrophages seems to be detrimental to neurogenesis after stroke. Treatment with hUC-MSCs preserved adult newborn neurons and reduced functional impairment after transient cerebral ischemia by reducing the number of hypertrophic microglia/macrophages.

Keywords

stroke, microglia, umbilical cord mesenchymal stem cells, cell therapy

Introduction

Ischemic stroke, a focal cerebral insult, leads to adverse neurological complications or behavioral disorders. Over the next decades, the prevalence of ischemic stroke is expected to increase.[1] To date, the only approved therapy, vessel recanalization by recombinant tissue plasminogen activator, is limited by a narrow time window[1] and serious complications

[1] Meridigen Biotech Co., Ltd., Neihu, Taipei City, Taiwan
[2] Department of Medical Research, Chi Mei Medical Center, Tainan City, Taiwan
[3] Department of Biotechnology, Southern Taiwan University of Science and Technology, Tainan City, Taiwan
[4] Department of Hyperbaric Oxygen, Chi Mei Medical Center, Tainan City, Taiwan
[5] The Ph.D. Program for Neural Regenerative Medicine, Taipei Medical University, Taipei City, Taiwan
[6] Department of Emergency Medicine, Chi Mei Medical Center, Tainan City, Taiwan

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Corresponding Authors:
Ching-Ping Chang, Department of Medical Research, Chi Mei Medical Center, No. 901, Zhonghua Rd., Yongkang, Tainan City 710, Taiwan.
Hung-Jung Lin, Department of Emergency Medicine, Chi Mei Medical Center, No. 901, Zhonghua Rd., Yongkang, Tainan City 710, Taiwan.
Emails: jessica@stust.edu.tw; jessica.cpchang@gmail.com; and 790001@mail.chimei.org.tw

such as hemorrhage.[2] Consequently, alternative strategies are in high demand. Among these strategies, transplantation of human umbilical cord–derived mesenchymal stem cells (hUC-MSCs) via intravenous administration seems promising.[3] hUC-MSCs improve ischemic stroke outcomes by enhancing neurite remodeling, neurogenesis, and angiogenesis.[4] However, the neuroprotective mechanisms underlying hUC-MSC therapy remain unclear.

Cerebral ischemia transforms the morphology of microglia or macrophages from the resting ramified phenotype into the hypertrophic (or activated amoeboid) phenotype.[5] Moreover, following ischemic stroke, neuroblasts generated in the subventricular zone (SVZ) also migrate into the striatum and cerebral cortex.[6] In rodents, microglial activation is detrimental to newly formed hippocampal neurons.[6] In an ischemic stroke animal model, resting ramified microglia promote neurogenesis,[7] whereas activated hypertrophic microglia impair neurogenesis.[8,9] Thus, hUC-MSC therapy may preserve adult newborn neurons and reduce neurological injury after cerebral ischemia by reducing the number of hypertrophic microglia/macrophages.

To test our hypothesis, we first quantified changes in the total numbers of microglia/macrophages (or ionized calcium-binding adaptor molecule 1 [Iba-1]-positive cells) in the rat brain following a transient ischemic stroke. Then, we used the cell body areas of Iba-1-positive cells in the ischemic brain region to determine the numbers of hypertrophic or amoeboid microglia.[10] In addition, the number of doublecortin (DCX)- and bromodeoxyuridine-specific cells in the brain represents the number of newborn neurons.[11–13] Therefore, in the present study, we ascertained whether hUC-MSCs administered intravenously improved the outcomes of ischemic stroke by modulating the number of both hypertrophic microglia/macrophages and newborn neurons.

Materials and Methods

Transient Middle Cerebral Artery Occlusion (MCAO) Model and Sham Surgery

Adult, male, Sprague-Dawley rats (300 to 350 g, BioLASCO Taiwan Co., Ltd., Taipei, Taiwan) were used in this study. The rats were housed in groups of 4 at an ambient temperature of 22 $\pm$ 1 °C with a 12-h light–dark cycle according to the guidelines of the Institutional Animal Care and Use Committee (approval no. 104042801) at Chi Mei Medical Center. Pellet rat chow and tap water were available ad libitum. The rats were anesthetized with sodium pentobarbital (25 mg/kg, intraperitoneally; Sigma-Aldrich, St. Louis, MO, USA) and a mixture containing ketamine (4.4 mg/kg, intramuscularly [i.m.]; Nankuang Pharmaceutical, Taipei, Taiwan), atropine (0.02633 mg/kg, i.m.; Sintong Chemical, Taoyuan, Taiwan), and xylazine (6.77 mg/kg, i.m.; Bayer AG, Leverkusen, Germany). Experimental stroke was induced by transient MCAO as previously described.[14] We have standardized the MCAO model, with stroke rats showing $\geq$80% reduction in regional cerebral blood flow during a 1 h occlusion period as determined by laser Doppler (OxyFlo™, Oxford Optronix Ltd., Oxford, UK). Focal cerebral ischemia was maintained for 1.5 h. The incision was closed, and 1 cm of the nylon suture was left protruding, so it could be withdrawn to allow reperfusion. After surgery, anesthesia was discontinued, and the rat was allowed free access to food and water. For sham surgery, each animal underwent the same surgical procedures without MCAO and received the same postoperative care.

Isolation of hUC-MSCs and Culture of hUC-MSCs

hUC-MSCs were obtained from Meridigen Biotech Co., Ltd. (Taipei, Taiwan). The participants and their families were informed of the experiment and signed informed consent forms. The study protocol was approved by the Ethics Committee for Clinical Research at the Chi Mei Medical Center (institutional review board no. 10405-008).

Umbilical cord tissue was harvested under sterile conditions and digested with collagenase in a 37 °C incubator. The digestion was terminated with culture medium. The cells were then incubated in a humidified incubator with 5% CO_2 at 37 °C for 3 d, at which point, the culture medium was replenished, and the nonadherent cells (hUC-MSCs) were suspended in CryoStor® CS 10 (STEMCELL Technologies, Vancouver, BC, Canada) and cryopreserved in a vapor phase liquid nitrogen tank for long-term storage.

hUC-MSC Identification, Differentiation, and Preparation

Flow cytometry analysis revealed the expression of specific surface antigens on the hUC-MSCs; the hUC-MSCs were positive for CD44, CD73, CD90, CD105, and ephrin type-A receptor 2 and negative for CD34, CD45, CD19, CD11b, and Human Leukocyte Antigen- antigen D Related (HLADR). Antibodies for flow cytometry were obtained from BD Biosciences (San Diego, CA, USA). hUC-MSCs were also analyzed for their ability to differentiate into osteoblasts, adipocytes, and chondrocytes. After the hUC-MSCs reached 80% confluence, they were freshly harvested for an animal study. Briefly, hUC-MSCs were trypsinized for 2 min at 37 °C and neutralized by complete culture medium. The cell suspension was centrifuged at 300 relative centrifugal force (RCF) for 5 min. After the final wash, cell viability was assessed using 0.4% trypan blue in a Neubauer counting chamber to exclude dead cells and to ensure an adequate number of vital cells for transplantation. The supernatant was aspirated, and the cell pellet was resuspended in normal saline (NS). The final concentration was adjusted to 1×10^6 cells/mL or 4×10^6 cells/mL. Each umbilical cord (35 mL volume) contained 1.2×10^9 cells. The viability of these cord cells was above 95%.

Experimental Groups

The animals were randomly divided into experimental groups. The sham surgery group received an intravenous dose of NS (1 mL/kg of body weight) or hUC-MSCs (4×10^6 cells/mL) 24 h after sham surgery (sham + NS or sham + hUC-MSCs 4×10^6), and the MCAO group received an intravenous dose of NS (MCAO + NS) or hUC-MSCs (4×10^6 cells/mL/kg of body weight) 24 h after surgery (MCAO + hUC-MSCs 4×10^6). The stroke animals were intravenously injected with NS or hUC-MSCs suspended in NS 24 h after stroke.

Functional Outcome

Modified neurologic severity scores (mNSSs)[15] were used to assess the motor, sensory, reflex, and balance function of all rats at 1 d before surgery and at 1, 7, and 14 d after surgery. Baseline readings at 1 d before surgery were used as the internal controls. All behavioral experiments were performed between 9 AM and 2 PM.

We used an inclined plane system with a microcontroller to determine the limb motor function of the rats.[16] The rat was placed, facing right and then left, perpendicular to the scope of a 20×20 cm^2 rubber, ribbed surface of an inclined plane starting at an angle of 55°. The angle was increased or decreased in 5° increments to determine the maximal angle at which a rat could hold onto the plane.

The adhesive removal test was used to analyze both forepaw sensitivity, including the presence of neglect, and forepaw motor impairments.[17,18] The rats were placed in a transparent box for 1 min for habituation. Next, the rat was removed from the box, and 1 strip of adhesive tape was attached to each of the rat's paws. The rat was then placed into the box again. For a total of 120 s, the investigators calculated the time to either contact or remove the adhesive tape.

A modified forelimb foot fault placing test was used to examine forelimb function.[19,20] If the rats inaccurately placed a forelimb, the limb would fall through one of the openings in the grid. These mistakes were considered foot faults. The rats displayed better forelimb function if they made fewer errors during this test.[21]

Assessment of Infarct Size

Triphenyl tetrazolium chloride (Sigma-Aldrich) staining procedures were used to assess cerebral infarct size.[22] To avoid the impact of liquefaction and atrophy after infarction, we modified previous methods used for quantification of infarction volume in rats subjected to MCAO and used these methods to assess infarct size in the present study.[23,24] We defined the infarct area as the normally stained (red color) area in the contralateral hemisphere minus the normally stained area in the ipsilateral hemisphere. Infarction volume was calculated as the sum of the infarct area across all brain sections, multiplied by the section thickness (1 mm). The corrected infarct volume (CIV) was calculated by the following equation:

$$\mathrm{CIV} = (\mathrm{LT} - \mathrm{RNIA}) \times d,$$

where LT is the area of the left (i.e., contralateral) hemisphere, RNIA is the right (i.e., ipsilateral) hemisphere (noninfarcted area), and d is the slice thickness (1 mm).

Randomization and Full Blinding

The animals were housed 4 per cage and identified by a number printed on the base of the tail. In each cage, 2 rats were randomly assigned to a vehicle group and the others to an MSC treatment group. Two individuals who were responsible for the functional outcome measurements were the only 2 experimenters blinded to the treatments among those working on the animals (single blind). These individuals used the cage and animal codes to recognize individuals and to report repeated measurements on data collection forms.

Assessment of Endogenous Stem Cell Proliferation

To evaluate cell proliferation, the rats received an intraperitoneal injection of a cell proliferation–specific marker 5-ethynyl-2′-deoxyuridine (EdU, 10 mg/kg; Invitrogen Life Technologies, Rockford, IL, USA) once daily, starting at day 1 after stroke and continuing for 14 d.[25] The animals were euthanized at 14 d post-MCAO and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS).

Histology and Immunohistochemistry

Coronal paraffin sections (10 μm) were washed twice with PBS and incubated with primary antibodies at 4 °C overnight and with secondary antibodies in blocking solution at room temperature for 2 h. For cell proliferation analysis, the sections were incubated with antidoublecortin (DCX; Cell Signaling Technology, Inc., Danvers, MA, USA) and/or antineuronal nuclei (NeuN; Millipore Corporation, Billerica, MA, USA) antibodies. Immunoreactivity was visualized using Alexa Fluor 568–conjugated goat antimouse IgG (Invitrogen Life Technologies, Rockford, IL, USA) or Alexa Fluor 568–conjugated goat antirabbit IgG (Invitrogen Life Technologies, Rockford, IL, USA) secondary antibodies. Labeling for EdU was performed using a Click-iT® EdU Alexa Fluor® 488 Imaging Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Inc., Eugene, OR, USA). The primary and secondary antibodies used for immunohistochemical staining are listed in Tables 1 and 2.

The sections were mounted with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA), and fluorescence signals were detected with a Carl Zeiss upright fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) at excitation/emission wavelengths of 535/565 nm (rhodamine, red) and 470/505 nm (fluorescein isothiocyanate (FITC), green). A digital camera linked to a computer running Axioscope version 4 (Carl Zeiss) was used to capture images.

For quantification of the total numbers of Iba-1-positive cells, every tenth 5-μm-thick section corresponding to coronal

Table 1. Antibodies Used for Immunofluorescence Staining.

Antibody	Antigen	Host	Company	Catalog Number	Dilution
Primary antibody					
NeuN	Neuron	Mouse	Millipore	MAB377	1:200
Doublecortin (DCX)	Neuroblast	Rabbit	Cell Signaling	4604	1:200
Iba-1	Microglia	Rabbit	GeneTex	GTX100042	1:200
Secondary antibody (conjugation)					
Goat antirabbit IgG (Alexa Fluor 568)	Rabbit IgG	Goat	Invitrogen	A11011	1:400
Goat antimouse IgG (Alexa Fluor 568)	Mouse IgG	Goat	Invitrogen	A11004	1:400

Table 2. Immunohistochemistry Staining Kits.

Kit	Company	Catalog Number
DNA Fragmentation Assay Kit (terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling kit)	Clontech	630108
EdU (5-ethynyl-2′-deoxyuridine)	Invitrogen	E10415
Click-iT® EdU Alexa Fluor® 488 Imaging Kit	Invitrogen	C10337
N-Histofine® Simple Stain MAX PO (MULTI)	Nichirei Biosciences	414151F
AEC Substrate Pack	BioGenex	HK092-5K

Abbreviations: MULTI, this kit can be used in rat and mouse species; AEC, 3-amino-9-ethylcarbazole.

coordinates 4.2 mm anterior to bregma to 5.3 mm posterior to bregma was obtained and incubated in 2 mol/L hydrogen chloride for 30 min, rinsed in 0.1 mol/L boric acid (H_3BO_3; pH 8.5) for 3 min at room temperature, and then incubated with a rabbit anti-Iba-1 antibody (GeneTex Inc., San Antonio, TX, USA) at 4 °C overnight. After the sections were washed with PBS, each section was incubated with N-Histofine Simple Stain MAX PO (Nichirei Biosciences Inc., Tokyo, Japan) as a secondary antibody at room temperature for 60 min. Binding of the primary antibody was visualized using 3-amino-9-ethylcarbazole (AEC) in acetate buffer (BioGenex, San Ramon, CA, USA). The numbers of Iba-1-positive cells were counted throughout the cortical, striatal, SVZ, hippocampal, and hypothalamic fields of the ischemic core and boundary area. Images of the red–brown–immunohistochemistry staining of immune cells were captured with a 20× objective (numerical aperture [N.A.] 0.5) using an upright microscope system (Carl Zeiss) and counted using Axio Vision Image analysis software V4.8.1 (Carl Zeiss).

We modified the methodology of Resende et al.[10] to assess the cell body (soma) size of microglia. Sequences of 10 immunostained sections per region per rat were examined with a 100× oil immersion objective (N.A. 1.4) and Zen Software V2.3 (Carl Zeiss). Each microglial cell body was outlined using a contour tool and expressed in square micrometer. The averaged microglial cell body areas were calculated for the frontal cortex (4.2 mm anterior to bregma to 1.7 mm anterior to bregma), striatum (1.7 mm anterior to bregma to 0.8 mm posterior to bregma), and parietal cortex (1 mm posterior to bregma to 4.5 mm posterior to bregma). As shown in Fig. 1C, any Iba-1-positive cell with a cell body area above 80 μm^2 was defined as a hypertrophic or amoeboid microglial cell. An observer blinded to the exposure groups quantified the total number of activated microglia, determined by a cell body area above 80 μm^2, as well as the total number of resting ramified microglia (with a cell body area below 80 μm^2). The counts obtained from 3 different images per section were averaged. The results are expressed as a percentage of activated microglia or total number of microglia in the analyzed regions of interest.

For quantification of neuronal apoptosis, coronal cryosections (10 μm thick) from the animals of each group were stained with a terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL) assay kit (Clontech, Palo Alto, CA, USA). The sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

Negative controls without the primary antibody revealed no positive signals (data not shown). Two independent investigators evaluated all immunohistochemical staining. The total numbers of NeuN-positive cells and NeuN/DAPI/TUNEL triple-labeled cells were calculated in 5 coronal sections from each rat, counted for at least 10 rats per group, and expressed as the mean number of cells per section.

Statistics

Two-way analysis of variance (ANOVA) with Tukey's post hoc test or Bonferroni post hoc test was used to analyze the percentage of infarct area and behavioral performance, respectively. Histological measures were analyzed using one-way ANOVA with Bonferroni posttests. All data are expressed as the mean ± standard deviation. Statistical significance was considered $P < 0.05$ according to standard conventions and was indicated by single symbols (* and +).

Results

hUC-MSCs Attenuate Infarct Volume

Compared with the sham + NS group or sham + hUC-MSC 4×10^6 group, the MCAO + NS group had significantly larger cerebral infarct volumes (Fig. 2). However, compared with the MCAO + NS group, the MCAO + hUC-MSC 1×10^6 and MCAO + hUC-MSC 4×10^6 groups had lower cerebral infarct

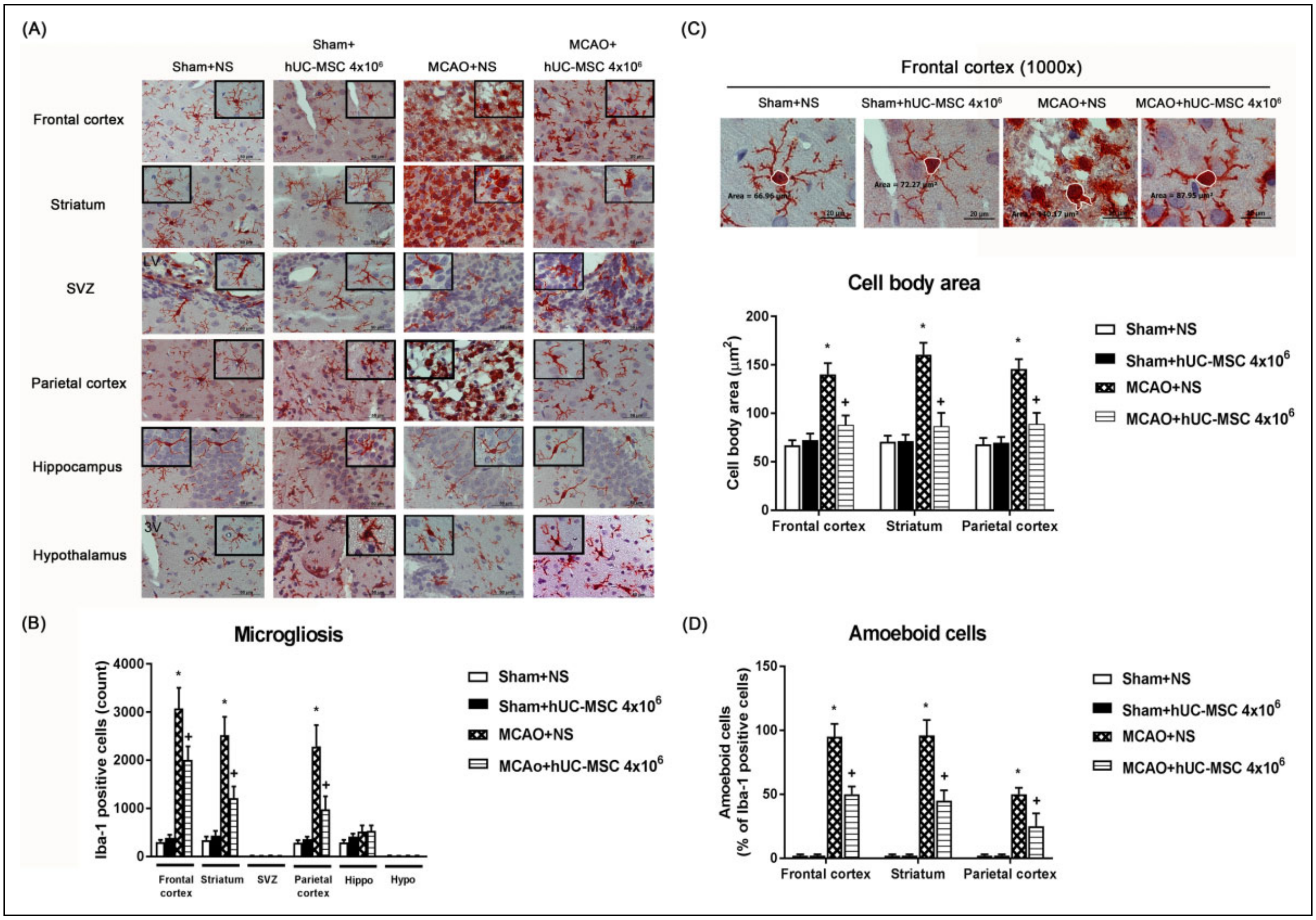

Figure 1. Effect of human umbilical cord mesenchymal stem cells (hUC-MSCs) 4×10^6 on the numbers of ionized calcium-binding adaptor molecule 1 (Iba-1)-positive cells, cell body area, and percentage of amoeboid microglia in the frontal cortex, striatum, parietal cortex, and other regions of the different groups of rats at 14 d postinjury. (A) Representative Iba-1 immunohistochemical images show resting (ramified morphology) or activated (hypertrophic morphology) microglial phenotypes. (B) The total counts of Iba-1-positive cells in the frontal cortex, striatum, parietal cortex, and other regions of each group. (C) The total cell body areas of Iba-1-positive cells in the frontal cortex, striatum, and parietal cortex of the middle cerebral artery occlusion (MCAO) + normal saline (NS) group were significantly higher than those of the sham + NS group or sham + hUC-MSC 4×10^6 group. Increased cell body areas of total Iba-1-positive cells in the ipsilateral brain region following MCAO can be significantly reduced by hUC-MSCs. (D) The percentage of amoeboid (or hypertrophic) microglia/macrophages (with an average cell body area above $140 \pm 12\ \mu m^2$) of the MCAO + NS group was significantly higher than that of the sham + NS group or sham + hUC-MSC group. Again, hUC-MSC therapy significantly attenuated the accumulation of amoeboid Iba-1-positive cells in the frontal cortex, striatum, and parietal cortex of injured brains. *$P < 0.001$, MCAO + NS versus sham + NS. +$P < 0.001$, MCAO + hUC-MSCs 4×10^6 versus MCAO + NS. The data are presented as the mean $\pm$ standard deviation ($n = 10$ for each group).

volumes (Fig. 2). The results revealed that hUC-MSC therapy attenuated MCAO-induced cerebral infarct. However, the beneficial effects of hUC-MSCs 4×10^6 on brain infarct reduction were not superior to those of hUC-MSCs 1×10^6.

hUC-MSCs Promote Neurological Motor Functional Recovery

Compared with the sham + NS group, the MCAO + NS group had a lower maximal angle (in the inclined test), a higher error ratio (in the forelimb foot fault placing test), a higher mNSS, and a higher number of seconds (in the adhesive removal test; Fig. 3). However, compared with the MCAO + NS group, the MCAO + hUC-MSC 1×10^6 group or MCAO + hUC-MSC 4×10^6 group had a higher maximal angle, a lower error ratio, a lower mNSS, and a lower number of seconds. The results revealed that hUC-MSC therapy attenuated ischemic stroke–induced neurological motor deficits. The beneficial effects of hUC-MSCs persisted up to day 14 poststroke. In the following studies, only the 4×10^6 hUC-MSC dosage was used.

hUC-MSCs Reduce Neuronal Apoptosis and Loss

Compared with the sham + NS group, the MCAO + NS group exhibited significantly lower numbers of neurons (or NeuN-positive cells) as well as significantly higher numbers of apoptotic neurons (or NeuN + TUNEL-positive cells) in ipsilateral brain regions including the cortex and striatum (Fig. 4). However,

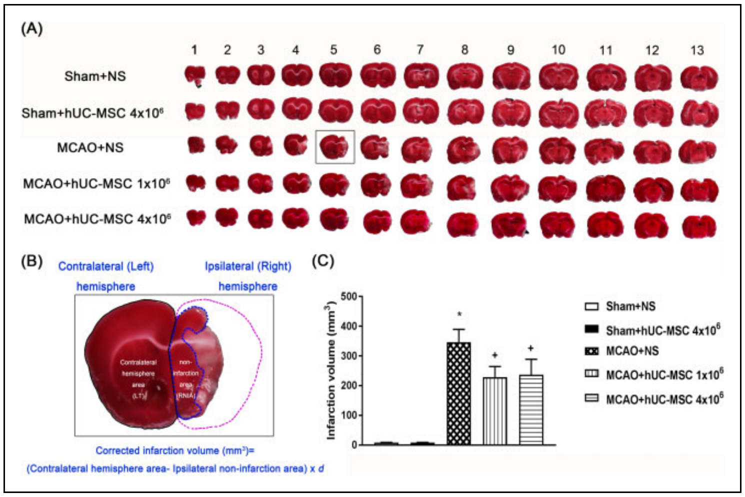

Figure 2. Mesenchymal stem cells therapy attenuates cerebral infarct caused by MCAO in different groups of rats. (A) Representative triphenyl tetrazolium chloride–stained brain sections of sham + NS, sham + hUC-MSC 4 $\times$ 10^6, MCAO + NS, MCAO + hUC-MSC 1 $\times$ 10^6, and MCAO + hUC-MSC 4 $\times$ 10^6 rats. In the frame in (B), the icon with the continuous solid line is the framed one in (A). The blue dotted line denotes the noninfarction area in the ipsilateral hemisphere, whereas the pink dotted line denotes the area of brain tissue that should normally appear (reversed horizontally from the contralateral hemisphere). The equation in blue indicates how the infarction area was acquired. (C) The bar graphs demonstrate brain infarction volumes in different groups of rats on day 14. The values are mean $\pm$ standard deviation ($n = 10$ for each group); **P* < 0.05 for MCAO + NS group versus sham + NS group; +*P* < 0.05 for MCAO + hUC-MSC 4 $\times$ 10^6 or 1 $\times$ 10^6 group versus MCAO + NS group. Sham + NS, sham operation rats that received normal saline injection; sham + hUC-MSC 4 $\times$ 10^6, sham operation rats that received an injection of 4 $\times$ 10^6 hUC-MSC; MCAO + NS, MCAO rats that received an injection of normal saline; MCAO + hUC-MSC 1 $\times$ 10^6, MCAO rats that received an injection of 1 $\times$ 10^6 hUC-MSC; and MCAO + hUC-MSC 4 $\times$ 10^6, MCAO rats that received an injection of 4 $\times$ 10^6 hUC-MSC. Please see the definitions of the group abbreviations in the Fig. 1 legend.

compared with the MCAO + NS group, the MCAO + hUC-MSC 4 $\times$ 10^6 group had significantly higher numbers of neurons and significantly lower numbers of apoptotic neurons in ipsilateral brain regions (Fig. 4). The results revealed that hUC-MSC therapy reduced MCAO-induced neuronal loss and neuronal apoptosis.

hUC-MSCs Promote Neuron Proliferation

Compared with the MCAO + NS group, the MCAO + hUC-MSC 4 $\times$ 10^6 group had significantly higher numbers of proliferating neurons (or NeuN- and EdU-positive cells) in ipsilateral brain regions including the frontal cortex, SVZ, parietal cortex, and hippocampus (Fig. 5). Double immunofluorescence staining revealed that treatment with hUC-MSCs significantly augmented the numbers of NeuN-EdU-positive cells in the cerebral cortex, SVZ, and hippocampus in rats 14 d after stroke (Fig. 5). The results revealed that hUC-MSC therapy promoted neuron proliferation in ipsilateral injured brain regions.

hUC-MSCs Promote the Proliferation of Neuroblasts

Again, compared with the MCAO + NS group, the MCAO + hUC-MSC 4 $\times$ 10^6 group had significantly higher numbers of proliferating neuroblasts (or DCX-EdU-positive cells) in ipsilateral brain regions including the cortex, striatum, SVZ, parietal cortex, and hippocampus (Fig. 6). The results revealed that hUC-MSC therapy stimulated neuroblast proliferation in various brain regions.

hUC-MSCs Attenuate Microglial Activation

Immunohistochemistry revealed that compared with the sham + NS group, the MCAO + NS group had higher numbers of both total Iba-1-positive cells (microglia or macrophages; Fig. 1B) and larger cell body areas (Fig. 1C) in the ipsilateral frontal cortex, striatum, and parietal cortex (Fig. 1). As shown in Fig. 1C, the average cell body areas for the ramified microglia and hypertrophic microglia were 75 to 85 μm^2 and 140 to 150 μm^2, respectively. Based on the assumption that any Iba-1-positive cell with a cell body area above 80 μm^2 was a hypertrophic microglial cell, the MCAO + NS group had a significantly higher percentage of hypertrophic or amoeboid Iba-1-positive cells in the ipsilateral frontal cortex, striatum, and parietal cortex than the sham + NS group (Fig. 1D). MCAO-induced accumulation of total Iba-1-positive cells, larger cell body areas of Iba-1-positive cells, and accumulation of hypertrophic Iba-1-positive cells in the ischemic brain regions were all attenuated by hUC-MSCs but not by NS therapy (Fig. 1).

Discussion

MSCs Derived from Human Umbilical Cord Tissue or Cord Blood Are Expected to Be a Promising Tool for Acute Stroke Therapy

Different routes of administration include intraperitoneal, intravenous, and intraarterial injections. Because of its convenience, intravenous is the most common method in

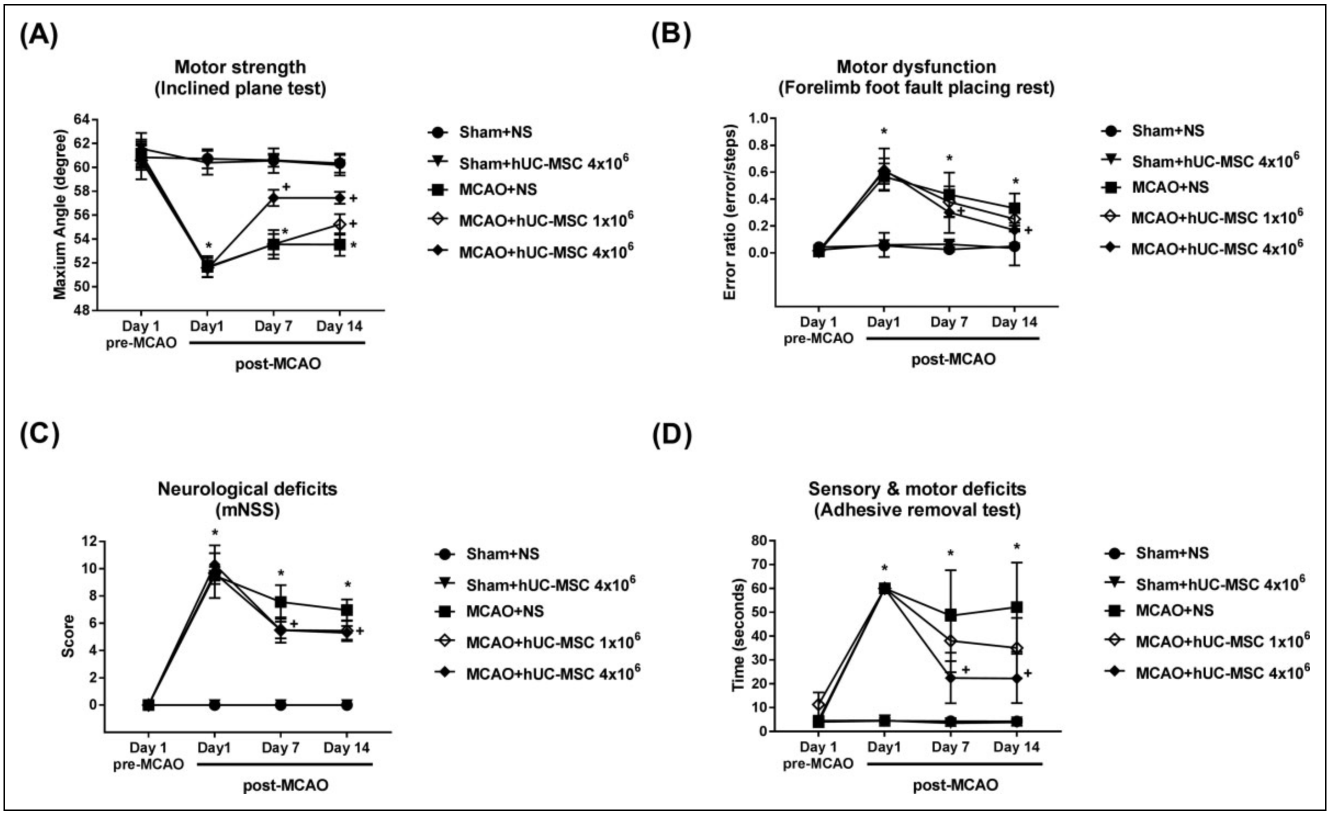

Figure 3. Mesenchymal stem cells therapy attenuates neurological motor deficits caused by MCAO in rats. Neurological motor function was evaluated by (A) an inclined plane test, (B) forelimb foot fault placing test, (C) modified neurological severity score (mNSS), and (D) adhesive removal test. The data are shown as the mean $\pm$ standard deviation values of 10 rats for each group. *$P < 0.05$ for MCAO + NS group versus sham + NS group; +$P < 0.05$ for MCAO + hUC-MSC 1×10^6 group or MCAO + hUC-MSC 4×10^6 group versus MCAO + NS group. Please see the defined abbreviations for the different groups in the Fig. 1 legend.

preclinical and clinical settings. In 2001, intravenous administration of human umbilical cord blood cells (hUCBCs) produced the greatest recovery when administered at 24 h poststroke in rodents.[26] In 2012, Boltze and colleagues[27] further demonstrated that hUCBCs administered within a 72-h time window attenuated neurological dysfunction, brain atrophy, and glial scarring in stroke rats. In the present study, MSCs derived from human umbilical cords at 24 h poststroke attenuated neurological impairment (evidenced by neurological deficits, cerebral infarct, and neuronal apoptosis) in rats. These 3 independent laboratories have demonstrated the beneficial effects of hUCBCs or hUC-MSCs administered within 72 h after MCAO. In this study, we investigated the functional outcome of single treatment with hUC-MSCs after MCAO only. Compared with single injection, repeated administration of human umbilical cord blood–derived MSCs did not elicit significant improvements in reducing stroke injury.[28] They proposed that elucidation of the therapeutic time window, rather than repeated administration of stem cells, would facilitate hUC-MSCs therapy in clinical application. Intravenous delivery of hUC-MSCs, in addition to induction of angiogenesis, neurogenesis, and anti-inflammatory effects,[29,30] may improve the outcomes of ischemic stroke by enhancing cerebrovascular function.[31] Of course, this needs further verification.

$CD34^+$ or $CD34^-$ MSCs Equally Attenuate Neurological Injury in Stroke Rats

Cells administered intravenously home to the site of injury through a less invasive route.[32] However, cells delivered intravenously have the disadvantages of accumulating in the lungs and spleen, requiring high cell numbers, inducing possible systemic effects, and requiring blood–brain barrier permeability (thus limiting the time window).[32,33] As shown in Fig. 2, the beneficial effects of hUC-MSCs in reducing neurological injury are not superior to those of 1×10^6 hUC-MSCs. Most likely, 4×10^6 hUC-MSCs delivered intravenously pass through the lungs before they are distributed throughout the body; potentially, the same numbers of cells are distributed throughout the body by injections of both 4×10^6 and 1×10^6 hUC-MSCs. Umbilical cord blood–derived MSCs exit the lungs faster than bone marrow–derived MSCs.[34] A low dose of peripheral transplanted stem cells plus mannitol administration may enhance their therapeutic effects.[35]

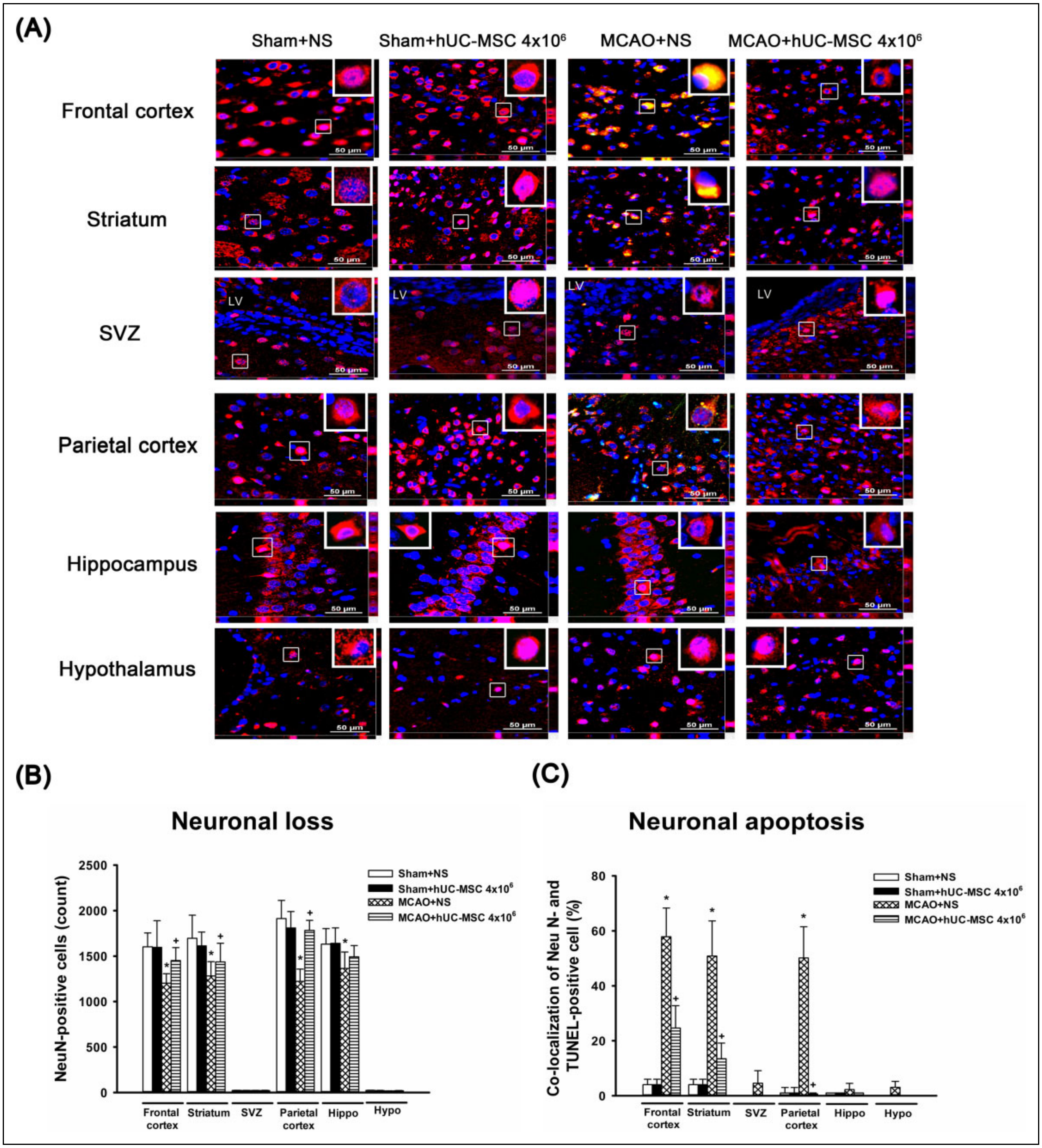

Figure 4. Mesenchymal stem cells therapy attenuates neuronal loss and apoptosis caused by MCAO in various brain regions of different groups of rats. (A) The upper panels depict representative antineuronal nuclei (NeuN; red) + 4,6-diamidino-2-phenylindole (DAPI; blue) or NeuN-TUNEL (green)-DAPI triple staining for sham + NS, sham +hUC-MSC 4 $\times$ 10^6, MCAO + NS, and MCAO + hUC-MSC 4 $\times$ 10^6 rats. The data are presented as the mean $\pm$ standard deviation. The values of the numbers of NeuN-TUNEL-DAPI-positive cells (B) and the numbers of colocalized NeuN- and TUNEL-positive cells (C) in the different brain regions are depicted. The data were obtained 14 d after MCAO or sham operation (n = 10 for each group). *P < 0.01, MCAO + NS group versus the sham + NS group; +P < 0.05, MCAO + hUC-MSC 4 $\times$ 10^6 group versus MCAO + NS group. Please see the defined abbreviations for the different groups in the Fig. 1 legend.

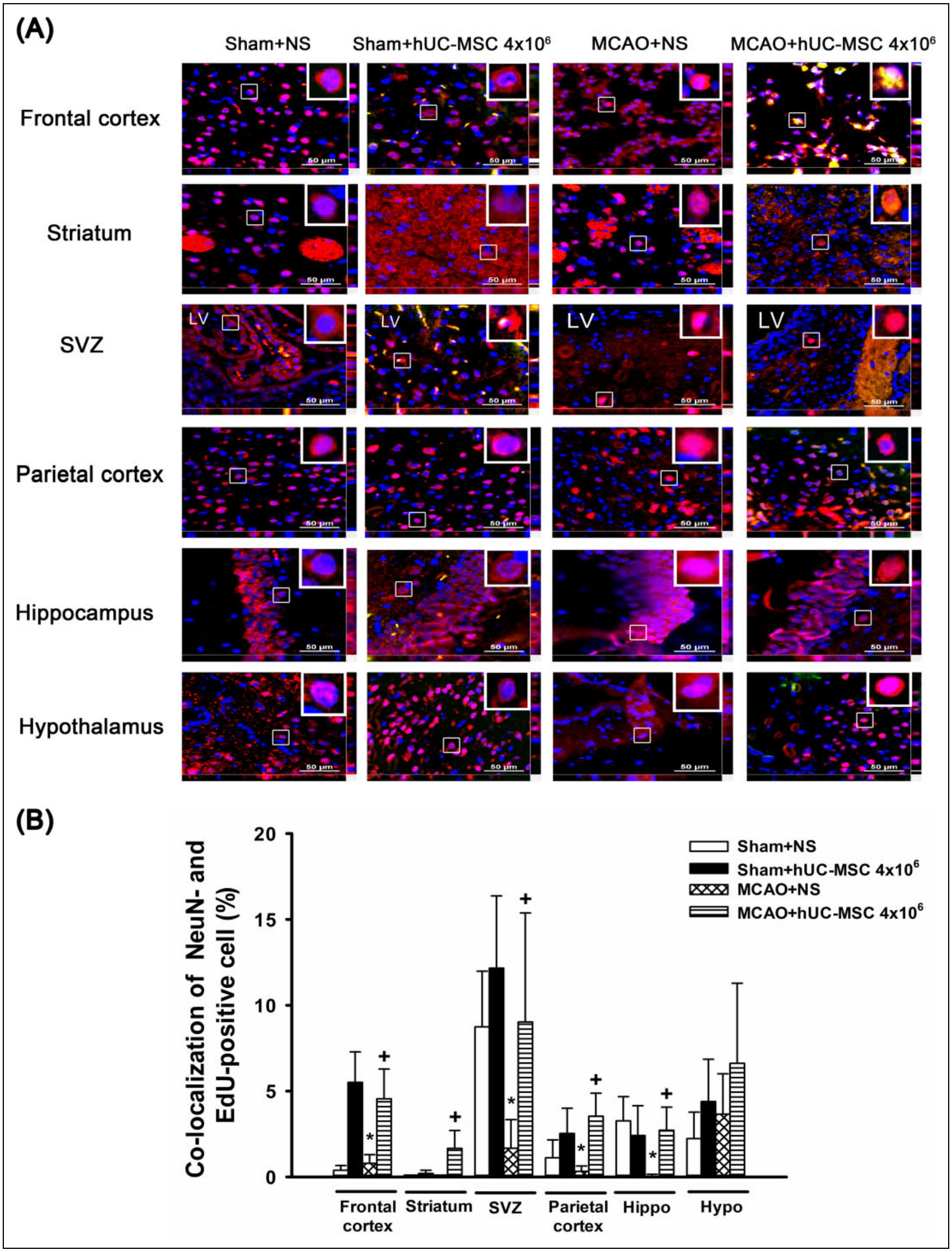

Figure 5. Mesenchymal stem cells therapy increases newly formed cells (or 5-ethynyl-2′-deoxyuridine [EdU]–positive cells) in different groups of rats. Neuronal proliferation in the frontal and parietal cortex, subventricular zone (SVZ), corpus striatum, hippocampus, and hypothalamus was evaluated by EdU (green)–antineuronal nuclei (NeuN; red)–4,6-diamidino-2-phenylindole (DAPI; blue) triple immunofluorescence staining 14 d postischemic stroke. (A) The upper panels depict representative EdU-NeuN-DAPI triple staining for sham + NS, sham + hUC-MSC 4 $\times$ 10^6, MCAO + NS, and MCAO + hUC-MSC 4 $\times$ 10^6 rats. (B) The data are presented as the mean $\pm$ standard deviation of 10 rats for each group. **P* < 0.05 for the MCAO + NS group versus the sham + NS group; +*P* < 0.05 for the MCAO + hUC-MSC 4 $\times$ 10^6 group versus the MCAO + NS group. Please see the Fig. 1 legend for the defined group abbreviations.

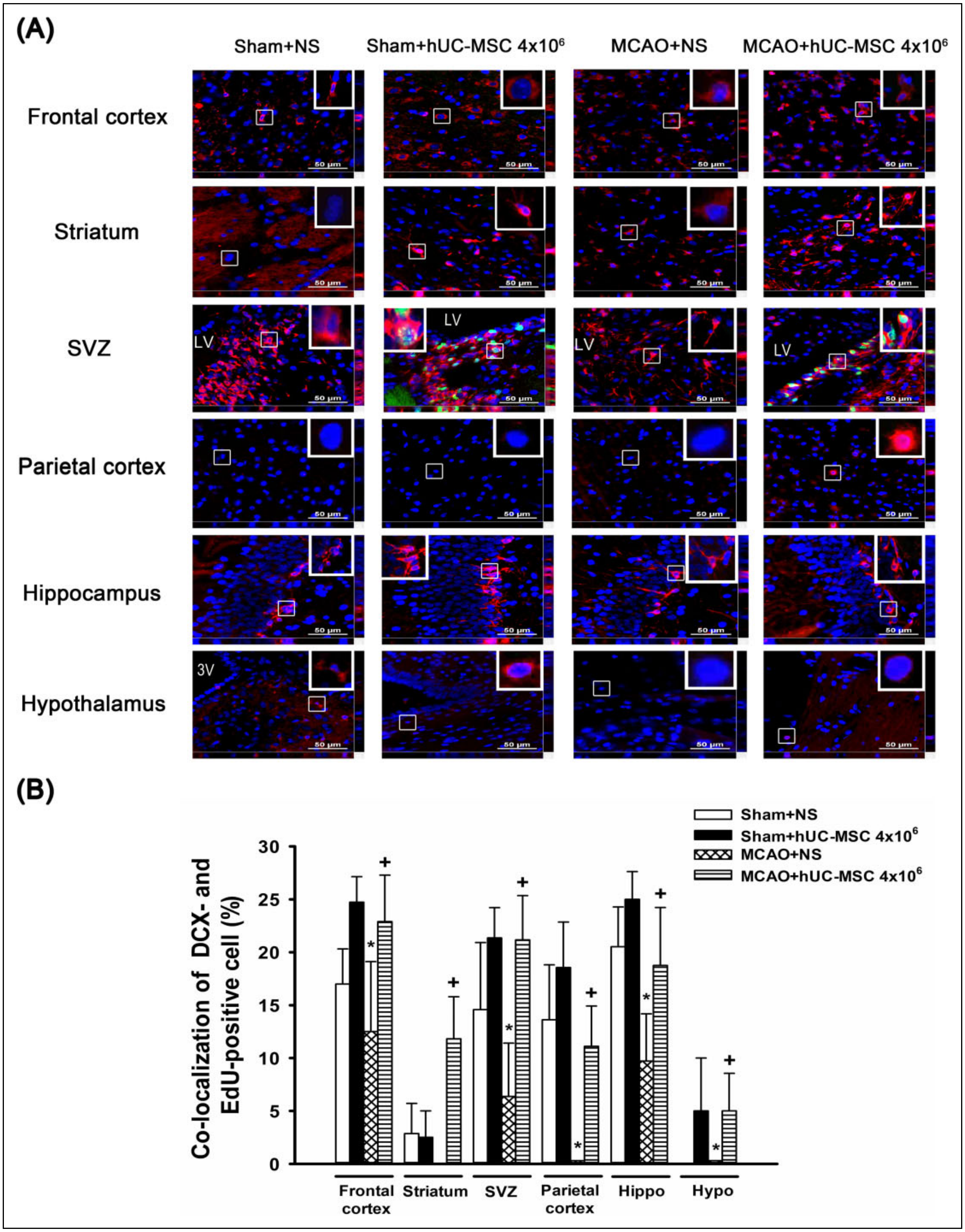

Figure 6. Mesenchymal stem cells therapy stimulates the proliferation of neuroblasts in different brain regions of the different groups of rats. Neuroblast proliferation in the frontal and parietal cortex, subventricular zone (SVZ), corpus striatum, hippocampus, and hypothalamus was evaluated by 5-ethynyl-2′-deoxyuridine (EdU; red)–DCX (green)–4,6-diamidino-2-phenylindole (DAPI; blue) triple immunofluorescence staining 14 d postischemic stroke. (A) The upper panels depict representative EdU-DCX-DAPI triple staining for sham + NS, sham + hUC-MSC 4×10^6, MCAO + NS, and MCAO + hUC-MSC 4×10^6 rats. (B) The data are presented as the mean ± standard deviation values of 10 rats per group. *$P < 0.05$ compared with the sham + NS group; +$P < 0.05$ compared with the MCAO + NS group. Please see the Fig. 1 legend for the definitions of the group abbreviations.

Our hUC-MSCs were negative for the specific surface antigen CD34. Approximately 2% of human umbilical cord blood (hUCB) mononuclear cells (MNCs) are considered stem cells and a majority are $CD34^+$ [27]. In ischemic stroke rats, intravenous injections of hUCB·MNCs·$CD34^+$ or hUCB·MNCs·$CD34^-$ cells equally reduced neurofunctional deficits and diminished lesion volume. Similarly, in the present study, intravenous injection of hUC·MSCs·$CD34^-$ improved ischemic stroke outcomes in rats. Moreover, in vitro, compared with $CD34^-$ cells, $CD34^+$ cells can more easily home to neural tissue.[36]

MSCs Attenuate Neurological Injury in Stroke Rats by Reducing the Number of Hypertrophic Microglia/Macrophages

Microglia/macrophages in the brain undergo transformations in their morphology between ramified and hypertrophic (amoeboid) forms to maintain the homeostasis of the brain.[5] In a rat model of MCAO, hypertrophic or amoeboid microglia are mainly concentrated around peri-lesioned areas,[37] whereas macrophages can shift to the ischemic core.[38] A reduction but not complete loss of blood flow around microglia somata induces morphological activation.[39] Once activated, microglia retract their ramifications and transform into an amoeboid (or hypertrophic) morphology, which may be related to phagocytosis and proinflammatory cytokine production. Both microglia and macrophages increase phagocytic activity at 72 h after a stroke.[40] Hypertrophic microglia produce relatively higher levels of reactive oxygen species and tumor necrosis factor α (TNF-α), whereas macrophages produce higher levels of interleukin-1β (IL-1β).[40] Increased proinflammatory cytokines result in neuronal apoptosis.[41] Indeed, as shown in the present study, all the neurological injury, hypertrophic microglia accumulation, and decreased endogenous neurogenesis occurred 14 d after MCAO in rats that received vehicle solution therapy. Our results further demonstrate that hUC-MSC therapy significantly attenuates MCAO-induced neurological injury via promoting the transformation of hypertrophic microglia with larger somata area (140 ± 11.56 μm^2) into ramified microglia with smaller somata area (85 ± 9.78 μm^2). However, it should be stressed that hUC-MSCs may ameliorate ischemic brain injury by inhibiting both immune cell activation within the brain and immune cell migration into the brain from the periphery.[42]

MSCs Promote Newborn Neuron Migration in the Ischemic Brain by Reducing the Number of Hypertrophic Microglia/Macrophages

The generation of newborn neurons in both the subgranular zone (SGZ) of the hippocampus and the SVZ of the lateral cerebral ventricle is attributed to the constant process of neurogenesis.[43] Newly formed neuroblasts from the SGZ or SVZ migrate to where they are needed to replenish neuronal loss or apoptosis in the adult brain.[44] In rats with ischemic stroke, a large number (>80%) of newly formed neurons die during the first 2 wk after the insult.[45] Indeed, as shown in our present results, 2 wk after MCAO or a sham operation, sham surgeries (with or without hUC-MSC treatment) induced more newly born neurons (or $NeuN^+$/EdU^+ cells) than MCAO alone (Fig. 5). Likewise, the migration of newly formed neuroblasts (or DCX^+/EdU^+ cells) was lowest in MCAO animals (Fig. 6). These findings are in sharp contrast to the existing literature and common understanding.[46–48] These investigators have provided convincing data that neurogenesis occurs reactively after an ischemic stroke but is rare in the steady state. However, our present hypothesis is supported by several reports. For example, physical exercise increases cell proliferation and neurogenesis, while stress reduces this type of cellular response.[49,50] In MCAO rats, microglial activation is detrimental to the survival of newly formed neurons.[6] Interestingly, a recent report has demonstrated that umbilical cord blood plasma shares with the hUC-MSCs the same beneficial effects in inhibiting microglial activation but stimulating neurogenesis in a stroke rat brain.[51] In fact, paracrine signaling is believed to be the most important mediator of MSC therapy in brain injury.[3] Again, the paracrine mechanisms mediating the beneficial effects of MSCs need to be elucidated in future work.

Conclusions

In summary, 14 d after MCAO, rats displayed neurological injury (evidenced by cerebral infarction, neuronal apoptosis, and neurological deficits), followed by accumulation of hypertrophic microglia/macrophages but the reduction of neuron and neuroblast migration in ipsilateral brain regions. Intravenous administration of hUC-MSCs at 24 h after MCAO resulted in a significant recovery in neurological injury, accompanied by a reduction in hypertrophic (or amoeboid) microglia/macrophage accumulation and enhancement of neuron and neuroblast migration in ipsilateral brain regions. In MCAO rats, hypertrophic microglia/macrophages were detrimental to the survival of newly formed neurons. On this basis, it appears that hUC-MSCs might improve neurological injury in ischemic stroke rats by inhibiting hypertrophic microglia/macrophage accumulation and promoting neuroblast migration.

Author Contribution

Authors conceived and designed the experiments (WL, CPC, MTL, and HJL), performed the experiments (TWK, CHL, and YCS), contributed reagents/materials/analysis tools (WL, YCYH, KCN, and CPC), and wrote the manuscript (MTL, CPC, and HJL).

Ethical Approval

This study was approved by the Institutional Animal Care and Use Committee (approval no. 104042801) at Chi Mei Medical Center.

Statement of Human and Animal Rights

When not undergoing surgery (as described in the Materials and Methods section), the rat was given postoperative care and allowed free access to food and water.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Drs. Willie Lin, Yogi Chang-Yo Hsuan, Cheng-Hsien Lin, and Yu-Chin Su are employees of Meridigen Company.

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ORCID iD

Ching-Ping Chang http://orcid.org/0000-0003-0890-9414

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