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Original

Human Amnion-Derived Mesenchymal Stromal Cell Exosomes Promote Neuroprotection and Neurovascular Remodeling after Cerebral Ischemia

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Background: Ischemic stroke remains a leading cause of death and long-term disability worldwide, and effective neuroprotective therapies applicable to a broad patient population are still limited. Although mesenchymal stromal cell–derived exosomes (MSC-EXO) have emerged as promising cell-free therapeutic agents, evidence regarding exosomes derived from human amnion-derived mesenchymal stromal cells (AMSC-EXO) in cerebral ischemia remains scarce.

Methods: Human AMSC-EXO were isolated from conditioned media of cultured human amnion-derived mesenchymal stromal cells and characterized by nanoparticle tracking analysis and exosomal marker expression. Male mice were subjected to middle cerebral artery occlusion and randomly assigned to receive intravenous AMSC-EXO or vehicle 24 h after ischemia. Neurological function, motor coordination, and spatial working memory were assessed at 3 and 14 days. Post-ischemic neuroinflammation, neuronal degeneration, and endothelial cell proliferation were evaluated by immunohistochemistry and enzyme-linked immunosorbent assay in a blinded manner.

Results: Systemic administration of AMSC-EXO significantly improved neurological outcomes and motor performance after cerebral ischemia and enhanced spatial working memory. AMSC-EXO treatment markedly suppressed microglial activation and reduced the expression of pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin-1β, and interleukin-6, in the ischemic brain. In addition, neuronal degeneration in the cortical infarct border zone was significantly attenuated. At later stages, AMSC-EXO significantly increased the number of proliferating endothelial cells, suggesting a potential involvement in neurovascular remodeling.

Conclusion: Human AMSC-derived exosomes exert neuroprotective and neurovascular restorative effects after cerebral ischemia by suppressing post-ischemic neuroinflammation, reducing neuronal cell death, and promoting endothelial cell proliferation. AMSC-EXO represents a promising, scalable, and cell-free therapeutic strategy for ischemic stroke.

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Introduction

Stroke remains one of the leading causes of death and long-term disability worldwide and represents the second most common cause of long-term care dependency in Japan. Although advances in reperfusion therapies, including intravenous thrombolysis and mechanical thrombectomy, have significantly improved outcomes in patients with acute ischemic stroke caused by large vessel occlusion[1,2]. Nevertheless, only a minority of patients benefit from these treatments due to strict time windows, anatomical constraints, and contraindications, leaving a substantial proportion of patients with permanent neurological deficits[3,4]. Consequently, the development of novel therapeutic strategies that can be applied to a broader patient population and target secondary brain injury remains an urgent unmet medical need. At present, edaravone, a free-radical scavenger, is the only approved neuroprotective agent for ischemic stroke, underscoring the lack of effective pharmacological therapies that directly promote neural repair and functional recovery.

In this context, regenerative medicine–based approaches have gained increasing attention as potential therapies for ischemic stroke. Among these, mesenchymal stem cell (MSC)–based therapies have been extensively investigated for their ability to improve motor and cognitive dysfunction following cerebral ischemia, and several clinical trials have already been conducted[5].

Accumulating evidence now indicates that the therapeutic benefits of MSCs are mediated predominantly by paracrine mechanisms rather than by direct cell replacement[6,7]. Among MSC-derived paracrine factors, extracellular vesicles—particularly exosomes—have emerged as central mediators of tissue repair[8,9]. Exosomes are nanosized (30–150 nm) membrane-bound vesicles containing bioactive cargos such as microRNAs, mRNAs, proteins, and lipids, and are capable of modulating gene expression and signaling pathways in recipient cells. Over the past decade, numerous comprehensive reviews have highlighted the pivotal role of exosomes in intercellular communication and their therapeutic potential in regenerative medicine and neurological disorders[8–10].

MSCs can be isolated from various tissues, including bone marrow, adipose tissue, dental pulp, umbilical cord blood, and the amniotic membrane. Among these sources, amnion-derived MSCs (AMSCs) represent a particularly attractive option for exosome production. AMSCs can be obtained non-invasively and in large quantities from discarded amniotic tissue, involve minimal ethical concerns, and exhibit high proliferative capacity and low immunogenicity. The amnion contains a high density of MSCs, enabling efficient and cost-effective large-scale production of exosomes under standardized culture conditions. These features have been highlighted in recent reviews as key advantages for clinical translation of perinatal tissue–derived exosome therapies.

In contrast, most existing reviews and preclinical investigations predominantly address exosomes derived from bone marrow–, adipose tissue–, or umbilical cord–derived MSCs, with relatively little emphasis on AMSC-EXO in the context of cerebral ischemia. This is largely due to historical research trends, as bone marrow–, adipose tissue–, and umbilical cord–derived MSCs were the first to be extensively characterized and widely used in experimental studies, whereas amnion-derived MSCs represent a relatively newer cell source. Consequently, the neuroprotective efficacy and underlying mechanisms of AMSC-EXO in ischemic stroke have not yet been fully elucidated.

Therefore, in the present study, we aimed to address this gap in the literature by investigating the therapeutic effects of systemically administered human AMSC-EXO in an experimental model of cerebral ischemia, with particular focus on post-ischemic neuroinflammation, neuronal survival, endothelial cell proliferation, and functional recovery. By evaluating a scalable, ethically favorable, and cell-free therapeutic strategy, this study seeks to further clarify the translational potential of AMSC-EXO for ischemic stroke.

Materials and Methods

Ethics Statements

All experiments involving the care or use of animals were conducted in accordance with the guidelines and regulations of the National Institutes of Health, and were approved by the Animal Experiments Ethical review of Nippon Medical School (approval number: 2021-013). The experimental protocols dealing with human subjects were approved by Central Ethics Committee of the Nippon Medical School Foundation (approval number: A-2020-024)[11]. Animals were randomly assigned to various experimental groups using a lottery box. All neurological outcome assessments were performed in a blinded manner. In addition, the animal experiments conducted in this study were in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines[12].

Cell Culture and Exosome Isolation

Human amnion-derived mesenchymal stem cells (hAMSCs) were provided by Kaneka Corporation (Osaka, Japan). Human fetal membranes were aseptically obtained from pregnant patients undergoing cesarean delivery, and written informed consent was obtained from all donors prior to participation in the study. hAMSCs were isolated and cultured as previously described[13]. Cultured cells were harvested and cryopreserved in liquid nitrogen until further use. hAMSCs from passages 1 to 5 were used for all experiments. For experimental use, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) without supplements at a final concentration of 1×10^6 cells/mL. Cell viability was assessed using the trypan blue dye exclusion method.

For exosome collection, hAMSCs were cultured at 37 °C until approximately 80% confluence. The culture medium was then replaced with serum-free medium, and cells were incubated for an additional 48 hours. The conditioned medium was collected and subjected to ultracentrifugation-based pellet-down procedures to isolate exosomes. The resulting pellet was resuspended in phosphate-buffered saline and defined as hAMSC-derived exosomes (AMSC-EXO). The size distribution and particle concentration of isolated exosomes were analyzed using a NanoSight nanoparticle tracking analysis system (Malvern Panalytical, Malvern, UK). Exosome identity was further confirmed by enzyme-linked immunosorbent assay (ELISA) detection of established exosomal surface markers.

Animal Model and Exosome Administration

Male C.B-17/Icr-+/+Jcl mice (8 weeks old) were used in this study. Mice were fasted overnight before and after surgery but were allowed free access to tap water. Anesthesia was induced with 5% isoflurane and maintained with 1.5% isoflurane in a gas mixture of 70% N_2O and 30% O_2 under spontaneous respiration. Rectal temperature was continuously monitored and maintained at 37 ± 0.5 °C throughout the surgical procedure.

Under inhalation anesthesia, a skin incision was made in the left temporal region, and the distal portion of the middle cerebral artery was directly exposed and permanently occluded by electrocoagulation to establish a permanent middle cerebral artery occlusion (pMCAO) model, as previously described[14].

Animals were randomly assigned to the following experimental groups: (I) vehicle-treated group (phosphate-buffered saline, PBS) and (II) AMSC-derived exosome–treated group (AMSC-EXO). At 24 hours after cerebral ischemia, vehicle or AMSC-EXO (10 μg) was administered intravenously. All animals were randomized to treatment groups, and all surgical procedures, exosome administration, behavioral assessments, and outcome analyses were performed in a blinded manner.

Behavioral Tests

Neurological function was evaluated at 3 and 14 days after ischemia by an investigator blinded to the experimental groups. Hemiparesis and abnormal posture were assessed using a previously described neurological scoring system[15]. For assessment of motor weakness, the right hind limb of each mouse was gently extended using round-tipped forceps, and the flexor response was scored as follows: normal, 0; slight deficit, 1; moderate deficit, 2; and severe deficit, 3. Postural abnormalities were evaluated by suspending the mouse by the tail and scoring forelimb flexion and body twisting as follows: normal, 0; slight twisting, 1; marked twisting, 2; and marked twisting with forelimb flexion, 3. Motor coordination and balance were evaluated using the rotarod test with a rotarod apparatus (Model 7750; Ugo Basile, Gemonio, Italy). The rotating speed was initially set at 4 rpm and gradually accelerated to 40 rpm over 5 minutes, with incremental increases every 30 seconds. Each mouse underwent three consecutive trials, and the mean latency to fall (in seconds) was calculated. Mice that repeatedly fell during preoperative training were returned to the rod until they were able to maintain balance for 150 seconds. Spatial working memory was assessed using the spontaneous alternation Y-maze test (Muromachi Kikai, Tokyo, Japan), as previously described[16,17]. Briefly, mice were placed in the start arm and allowed to explore the maze freely for 15 minutes for habituation. After a 1-hour interval, mice were reintroduced into the start arm and allowed to explore the maze for 8 minutes. The sequence and number of arm entries were recorded by an observer blinded to the treatment conditions. The percentage of spontaneous alternation was calculated as the ratio of successive entries into all three arms to the total number of arm entries.

Immunohistochemistry

Mice were deeply anesthetized and transcardially perfused with heparinized saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS) at 3 or 14 days after cerebral ischemia. Brains were rapidly removed, post-fixed, and cryoprotected, and coronal brain

sections (20 μm thickness) were prepared using a cryostat. For immunohistochemical analysis, sections were incubated in PBS containing 10% goat serum (Thermo Fisher Scientific) to block nonspecific binding. To evaluate neuroinflammation at 3 days after ischemia, sections from the cortical ischemic border zone (ischemic border zone; IBZ) adjacent to the infarct core were incubated overnight at 4 °C with rabbit polyclonal antibodies against ionized calcium-binding adapter molecule 1 (Iba-1; 1:250, FUJIFILM Wako) and tumor necrosis factor-α (TNF-α; 1:100, R&D Systems, Minneapolis, MN, USA). Sections were then incubated with a biotinylated goat anti-polyvalent secondary antibody (Thermo Fisher Scientific) for 1 hour at room temperature, followed by an avidin–biotin–peroxidase complex (Vector Laboratories, Newark, CA, USA) for 30 minutes. Immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB; Vector Laboratories). Neuronal degeneration was assessed using Fluoro-Jade C (FJC) staining with a ready-to-dilute staining kit for identifying degenerating neurons (Biosensis, Thebarton, SA, Australia), according to the manufacturer's instructions. To evaluate cell proliferation and angiogenesis at 14 days after ischemia, immunofluorescence double staining was performed in the cortical IBZ. Sections were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) and blocked using a commercial blocking solution (1:9; UKB80, KAC, Kyoto, Japan), followed by overnight incubation at 4 °C with a rabbit polyclonal antibody against Ki67 (1:2,500, Abcam, Cambridge, UK) and a mouse monoclonal antibody against CD31 (1:500, Abcam). Sections were subsequently incubated with Alexa Fluor 430–conjugated goat anti-rabbit IgG or Alexa Fluor 594–conjugated goat anti-mouse IgG (Molecular Probes, Waltham, MA, USA) for 1 hour at room temperature in the dark. Sections were mounted using Vibrance Antifade Mounting Medium containing DAPI (Vector Laboratories). All images were acquired using an all-in-one fluorescence microscope (BZ-X800; KEYENCE, Osaka, Japan). For quantitative analysis, the number of positively stained cells was counted in five randomly selected fields (0.5 mm^2 per field) within the cortical ischemic border zone for each section by an investigator blinded to the experimental groups.

Enzyme-Linked Immunosorbent Assay of Brain Tissue Lysates

Brain tissue samples were collected from the entire ischemic hemisphere at 3 days after cerebral ischemia. The tissues were homogenized, and protein extracts were prepared using standard lysis procedures. Total protein concentrations were determined using a bicinchoninic acid (BCA) protein assay to allow normalization of cytokine levels. The concentrations of pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), in brain tissue lysates were quantified using a Mouse IL-1β/IL-1F2 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), a Mouse IL-6 Quantikine ELISA Kit (RayBiotech, Peachtree Corners, GA, USA), and a TNF-α Quantikine ELISA Kit (RayBiotech), in accordance with the manufacturers' instructions. dColorimetric absorbance was measured at 450 nm using a microplate reader. Cytokine concentrations were calculated from standard curves and normalized to total protein content.

Statistical Analysis

All data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test was used for comparisons of infarct volume, edema volume, rotarod performance, Y-maze spontaneous alternation rate, immunohistochemical cell counts, and ELISA data. Neurological deficit scores were analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. A p value of less than 0.05 was considered statistically significant. Non-significant differences are not shown. All statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, La Jolla, CA, USA).

Results

Characterization of AMSC-EXO

Exosomes were isolated from the conditioned medium of human amniotic mesenchymal stromal cells as described in the Methods. Transmission electron microscopy revealed round, membrane-bound extracellular vesicles with a typical exosomal morphology (**Figure 1A**). Nanoparticle tracking analysis demonstrated that the isolated vesicles had a mean diameter of approximately 100 nm consistent with the characteristic size of exosomes and a particle concentration of approximately 2.5×10^6 particles/mL (**Figure 1B**). These results confirm the successful isolation and characterization of AMSC-EXO.

AMSC-EXO Delivery Improves Neurological Outcomes and Enhanced Motor and Spatial Cognitive Functions

We investigated whether administration of AMSC-EXO improves neurological outcomes following ischemic in-

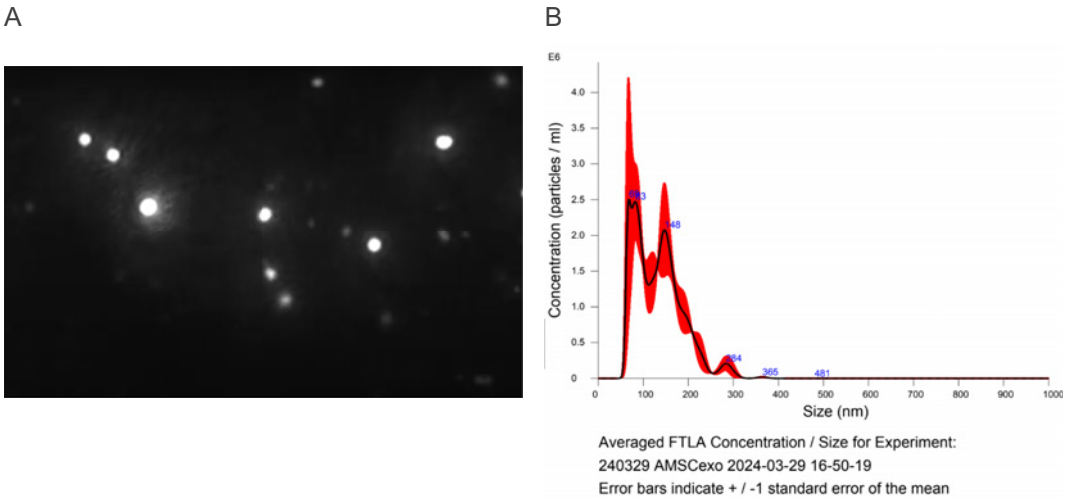

Figure 1 Characterization of AMSC-EXO
(A) Representative transmission electron microscopy image showing the typical morphology of AMSC-EXO. (B) Nanoparticle tracking analysis demonstrating a mean particle diameter of approximately 100 nm and a concentration of approximately 2.5×10^6 particles/mL.

jury (**Figure 2**). Neurological deficits and motor performance were assessed at 3 and 14 days after permanent middle cerebral artery occlusion (pMCAO). At 3 days after ischemia, AMSC-EXO treatment significantly ameliorated postural abnormalities compared with vehicle treatment ($p < 0.05$, $n = 5$; **Figure 2A**), while hindlimb paralysis showed a trend toward improvement that did not reach statistical significance ($n = 5$; **Figure 2B**). At 14 days after ischemia, motor coordination, as evaluated by the rotarod test, was significantly improved in the AMSC-EXO–treated group relative to the vehicle group ($p < 0.05$, $n = 5$; **Figure 2C**). In addition, spatial working memory assessed using the Y-maze test was significantly enhanced in the AMSC-EXO group ($p < 0.05$, $n = 8$; **Figure 2D**). Collectively, these findings indicate that AMSC-EXO administration provides functional neurological benefits following cerebral ischemia, leading to improved motor performance and enhanced spatial cognitive function.

Suppressed Inflammatory Cytokines and Post-Ischemic Microglial Activation in the Ischemic Brain

To elucidate the mechanisms underlying the beneficial effects of AMSC-EXO on neurological outcomes after ischemic injury, we examined its anti-inflammatory effects by assessing microglial activation and inflammatory cytokine expression in the ischemic brain (**Figure 3**). Microglial activation was evaluated in the cortical infarct border zone (IBZ) using ionized calcium-binding adaptor molecule 1 (Iba-1), a well-established marker of activated microglia following ischemic brain injury. At 3 days after permanent middle cerebral artery occlusion (pMCAO), the number of Iba-1–positive cells in the IBZ was significantly reduced in the AMSC-EXO–treated group compared with the vehicle group ($p < 0.05$, $n = 7$; **Figure 3B, left**), indicating marked suppression of post-ischemic microglial activation. To further characterize the anti-inflammatory effects of AMSC-EXO, we assessed inflammatory cytokine expression in both the cortical IBZ and the ischemic hemisphere. Immunohistochemical analysis revealed a significant reduction in the number of TNF-α–positive cells in the IBZ of AMSC-EXO–treated mice compared with vehicle-treated controls ($p < 0.05$, $n = 6$; **Figure 3B, right**). Consistently, quantitative analysis of the ischemic hemisphere demonstrated that the levels of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α, were markedly decreased in the AMSC-EXO group relative to the vehicle group ($p < 0.01$, $n = 6$; **Figure 4**). Collectively, these findings indicate that AMSC-EXO administration robustly attenuates post-ischemic neuroinflammation by suppressing microglial activation and reducing pro-inflammatory cytokine expression in the ischemic brain.

Reduced Neuronal Cell Death after Ischemia

To assess the neuroprotective effects of AMSC-EXO administration in the pMCAO model, Fluoro-Jade C (FJC) staining was performed in the cortical infarct border zone (IBZ) at 3 days after ischemia. Quantitative analysis of FJC-positive degenerating neurons in the IBZ (**Figure 5A**)

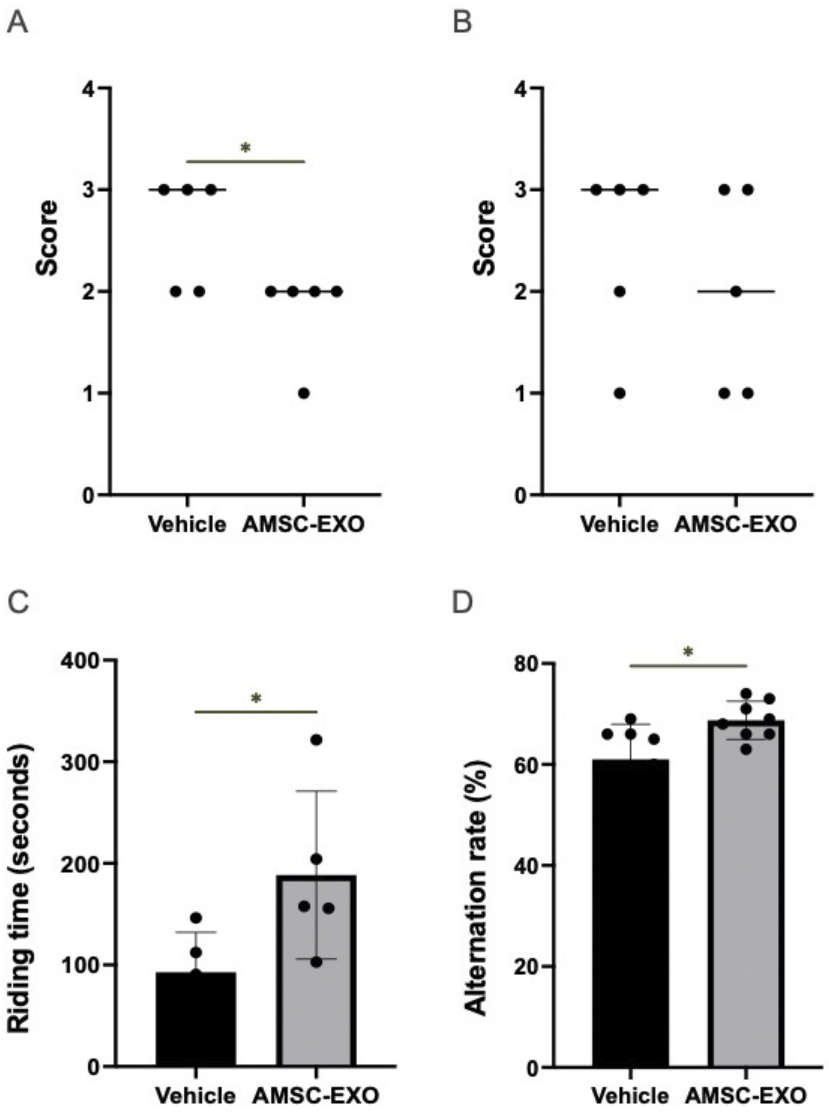

Figure 2 AMSC-EXO improves neurological outcomes after cerebral ischemia
Neurological function was evaluated at 3 and 14 days after permanent middle cerebral artery occlusion (pMCAO). At 3 days after ischemia, postural abnormalities were significantly ameliorated in the AMSC-EXO–treated group compared with the vehicle group ($p<0.05$, n =5; A), while hindlimb paralysis showed a tendency toward improvement (n = 5; B). At 14 days after ischemia, motor coordination assessed by the rotarod test was significantly enhanced in the AMSC-EXO group ($p<0.05$, n = 5; C). Spatial working memory evaluated by the Y-maze test exhibited a significant improvement in the AMSC-EXO–treated group ($p<0.05$, n = 8; D).

demonstrated a significant reduction in the number of FJC-positive cells in the AMSC-EXO–treated group compared with the vehicle group ($p < 0.05$, n = 7; **Figure 5B**). These findings indicate that AMSC-EXO administration confers neuroprotective effects by attenuating neuronal cell death in the post-ischemic brain.

Enhanced Endothelial Cell Proliferation after Ischemia

To further confirm the proliferative effect of AMSC-EXO on cerebral microvessels, double immunofluorescence staining for Ki67 and CD31 was performed in the cortical infarct border zone (IBZ) at 14 days after ischemia (**Figure 6A**). Quantitative analysis revealed a significant increase in the number of Ki67/CD31 double-positive endothelial cells in the IBZ of the AMSC-EXO–treated group compared with the vehicle group ($p < 0.05$; AMSC-EXO, n = 3; vehicle, n=4; **Figure 6B**). These results indicate that AMSC-EXO promotes endothelial cell proliferation in the post-ischemic cortex, supporting its role in vascular remodeling after cerebral ischemia.

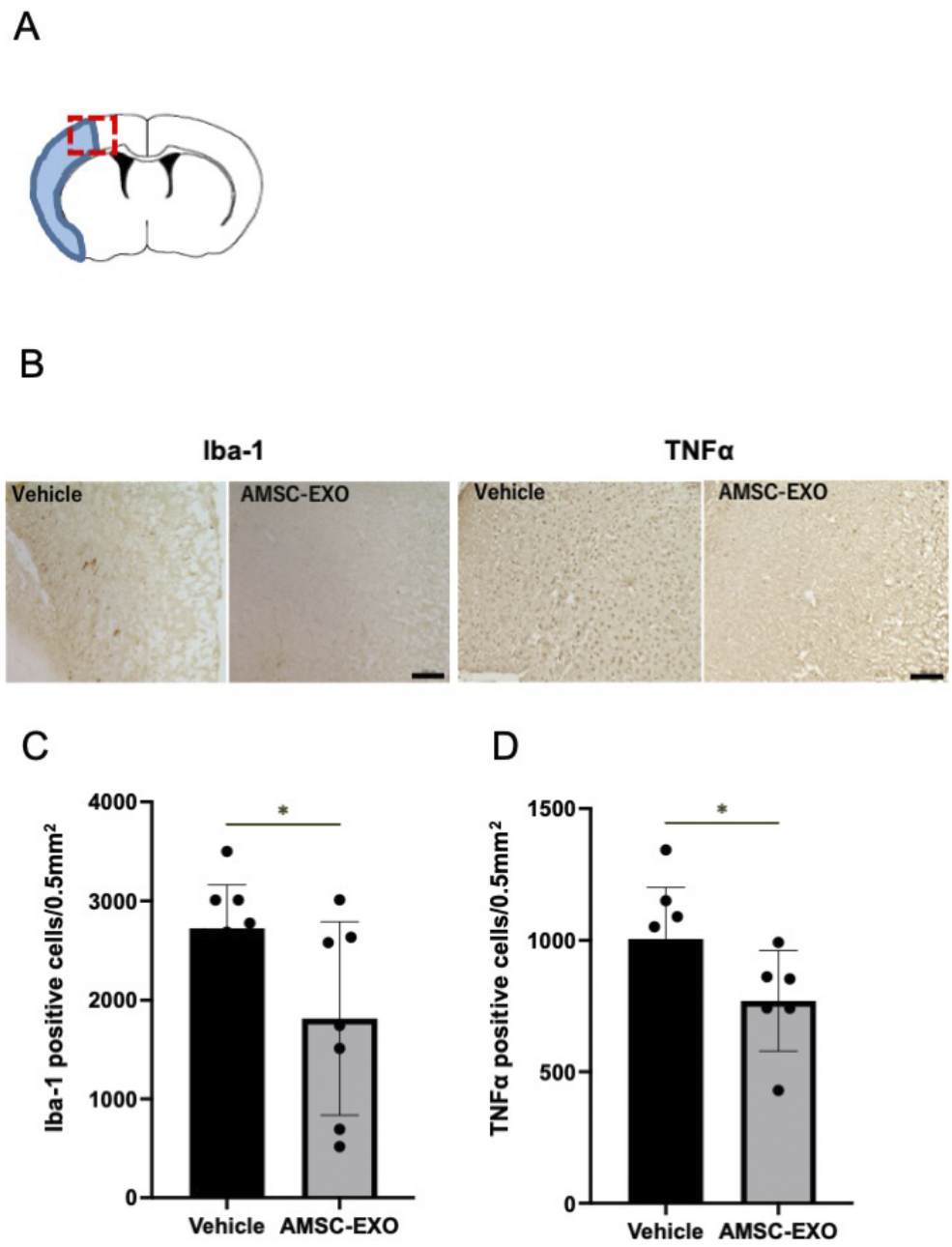

Figure 3 AMSC-EXO attenuates post-ischemic neuroinflammation
Microglial activation and inflammatory cytokine expression were evaluated at 3 days after permanent middle cerebral artery occlusion (pMCAO). (A) Schematic illustration of the cortical ischemic boundary zone. The red square on the brain map indicates the cortical ischemic boundary zone, and the black area represents the ischemic lesion. (B) Representative immunofluorescence images showing Iba-1–positive microglia (left) and TNF-α immunostaining (right) in the cortical infarct border zone (IBZ). Scale bar: 200 μm. (C) Quantification of Iba-1–positive cells in the IBZ revealed a significant reduction in the AMSC-EXO–treated group compared with the vehicle group (*$p<0.05$, $n = 7$). (D) Quantitative analysis demonstrated a significant decrease in the number of TNF-α–positive cells in the IBZ following AMSC-EXO treatment (*$p<0.05$, $n = 6$).

Discussion

In the present study, we demonstrated that systemic administration of exosomes derived from human amnion-derived mesenchymal stromal cells (AMSC-EXO) exerts significant therapeutic effects in the acute phase of cerebral ischemia. AMSC-EXO treatment markedly improved neurological outcomes, including both motor and cognitive functions, in a permanent middle cerebral artery occlusion model. These functional benefits were accompanied by robust anti-inflammatory effects, attenuation of

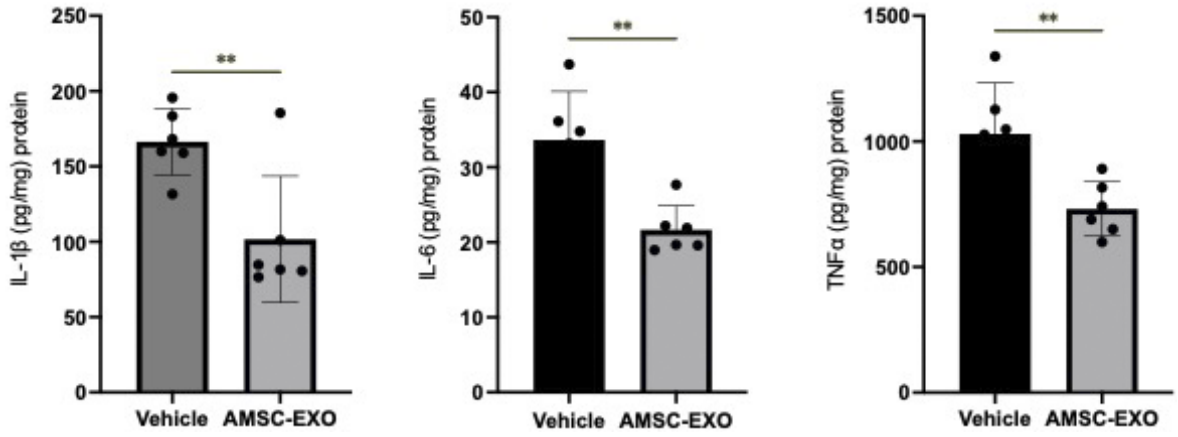

Figure 4 Quantification of pro-inflammatory cytokines expression in ischemic hemisphere extracts
Levels of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in the ischemic hemisphere were significantly reduced in the AMSC-EXO group compared with the vehicle group (**p<0.01, n = 6).

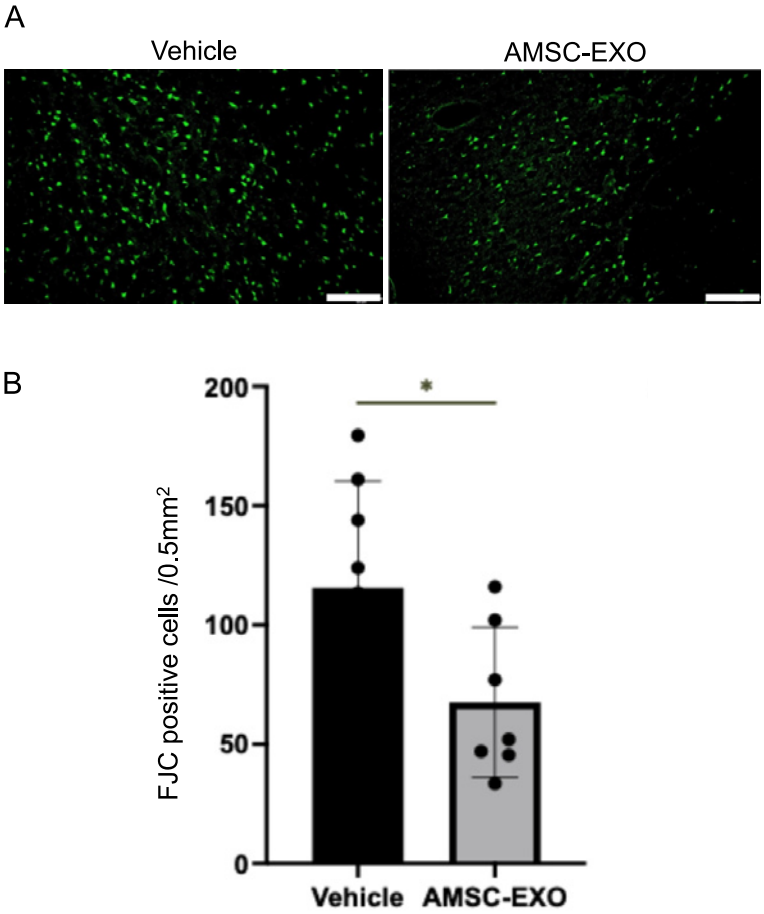

Figure 5 AMSC-EXO reduces neuronal cell death after ischemia
(A) Representative Fluoro-Jade C (FJC) staining and quantification of FJC-positive degenerating neurons in the cortical infarct border zone (IBZ) at 3 days after ischemia. Scale bar: 100 μm. (B) Quantification of FJC-positive cells showed a significant reduction in the AMSC-EXO–treated group compared with the vehicle group (*p<0.05, n = 7).

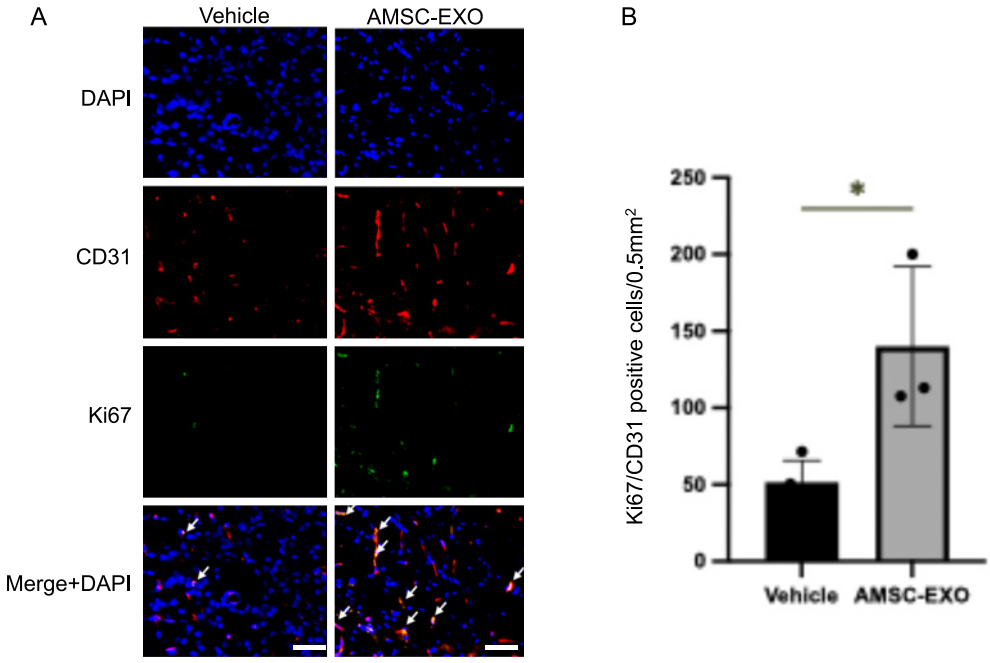

Figure 6 AMSC-EXO enhances endothelial cell proliferation in the ischemic cortex
(A) Representative double immunofluorescence images of Ki67 and CD31 in the cortical infarct border zone (IBZ) at 14 days after ischemia. Scale bar: 50 μm. (B) Quantification of Ki67/CD31 double-positive endothelial cells demonstrating a significant increase in the AMSC-EXO–treated group compared with the vehicle group ($p<0.05$; AMSC-EXO, n = 3; vehicle, n=4).

neuronal cell death, and enhanced endothelial cell proliferation in the ischemic brain, indicating a multifaceted neuroprotective and neurovascular restorative action of AMSC-EXO.

Post-ischemic neuroinflammation is a critical contributor to secondary brain injury and long-term neurological deficits following cerebral ischemia. Excessive activation of microglia and the subsequent release of pro-inflammatory cytokines amplify neuronal damage, disrupt neurovascular integrity, and impair functional recovery[18,19]. In this study, AMSC-EXO administration significantly suppressed microglial activation in the cortical infarct border zone and reduced the expression of key pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in the ischemic hemisphere. Importantly, these anti-inflammatory effects were closely associated with a reduction in Fluoro-Jade C–positive degenerating neurons, suggesting that AMSC-EXO confers neuroprotection, at least in part, by mitigating inflammation-mediated neuronal cell death.

Several comprehensive reviews have summarized the therapeutic potential of MSC-derived exosomes in ischemic brain injury; however, these reviews predominantly focus on exosomes derived from bone marrow, adipose tissue, or umbilical cord MSCs, with relatively little emphasis on AMSC-EXO[10,20]. This relative scarcity of AMSC-EXO–specific evidence underscores the importance of the present study and highlights the need for further focused investigations of this unique exosomal source in cerebral ischemia.

Previous studies have demonstrated that MSC-derived exosomes promote macrophage polarization toward the anti-inflammatory M2 phenotype[21], induce the conversion of activated T cells into regulatory T cells[22], suppress the production of pro-inflammatory mediators such as TNF-α, COX-2, IL-1β, and MCP-1, and enhance the expression of anti-inflammatory cytokines including IL-10[23]. Consistent with these findings, our results suggest that AMSC-EXO effectively modulates the post-ischemic inflammatory microenvironment, thereby contributing to neuroprotection and functional recovery. In addition to their anti-inflammatory properties, MSC-derived exosomes have been reported to promote angiogenesis and vascular remodeling in various pathological conditions[24]. In experimental stroke models, enhanced angiogenesis is closely associated with improved tissue perfusion, neuronal survival, and long-term neurological recovery[25]. In line with these reports, we observed a significant increase in Ki67/CD31 double-positive endothelial cells in the cortical infarct border zone following AMSC-EXO administration, indicating enhanced endothelial cell proliferation during the subacute phase of ischemia. However,

it should be emphasized that increased endothelial cell proliferation alone does not directly demonstrate angiogenesis. While our findings suggest a potential role of AMSC-EXO in promoting neurovascular remodeling, direct evaluation of angiogenesis will require additional analyses, such as measurements of vascular density, vessel length, or cerebral perfusion. These issues should be addressed in future studies to further clarify the angiogenic and functional vascular effects of AMSC-EXO. Angiogenesis and subsequent neurovascular remodeling are essential components of brain repair after cerebral ischemia and likely contribute to the sustained functional improvements observed in this study.

From a translational perspective, exosome-based therapies offer several advantages over conventional cell-based approaches. Due to their small size and reduced biological complexity, exosomes present a lower risk of vascular occlusion, uncontrolled differentiation, tumor formation, and immune rejection[10]. Moreover, exosomes can be more readily standardized, stored, and transported than living cells, enabling off-the-shelf therapeutic use. Importantly, exosome therapy circumvents key challenges associated with MSC transplantation, including limited engraftment, poor survival in the hostile ischemic environment, and functional deterioration caused by repeated in vitro passaging.

AMSC-EXO is particularly attractive as a therapeutic candidate because AMSCs can be obtained non-invasively and in large quantities from the amniotic membrane, a medical byproduct of childbirth, with minimal ethical concerns. AMSCs exhibit high proliferative capacity, low immunogenicity, and secrete abundant bioactive exosomes, enabling efficient and scalable large-scale production[26]. These features position AMSC-EXO as a promising and practical cell-free therapeutic modality for ischemic stroke.

Nevertheless, several limitations of the present study should be acknowledged. Given the still limited number of studies investigating AMSC-EXO in cerebral ischemia, further validation across different ischemia models, dosing regimens, routes of administration, and time windows will be required. In addition, although the present findings demonstrate clear anti-inflammatory, neuroprotective, and angiogenic effects, the specific molecular pathways and exosomal cargos responsible for these effects were not fully elucidated. Future studies focusing on exosome-associated microRNAs, proteins, and lipids will be essential to clarify the mechanisms underlying AMSC-EXO–mediated neurovascular protection and to optimize therapeutic efficacy[9,10].

In summary, this study provides evidence that systemic administration of human AMSC-derived exosomes confers significant neuroprotective and neurorestorative effects in the acute phase of cerebral ischemia. By suppressing post-ischemic neuroinflammation, reducing neuronal cell death, and promoting endothelial cell proliferation, AMSC-EXO significantly improves neurological outcomes. These findings not only expand the currently limited evidence base for AMSC-EXO in ischemic stroke but also support its potential as a safe, scalable, and effective cell-free therapeutic strategy.

Author Contributions: S.T., C.N., and Y.M. conceived and designed the research. S.T., C.N., H.K., and M. Saito performed the experiments. S.T., C.N., and M. Saito analyzed the data. S.T. and C.N. wrote the manuscript. Y.S., T.K., M. Sakai, and S.S. supervised the research. All authors reviewed and edited the manuscript.

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Conflict of Interest: C.N. and Y.M. are co-inventors of intellectual property licensed to Kaneka Corporation.

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process: ChatGPT (OpenAI, San Francisco, CA, USA) was used to assist with English language editing and to improve clarity. All content was critically reviewed and edited by the authors, who take full responsibility for the manuscript.

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