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Stroke

ORIGINAL CONTRIBUTION

Circulating Extracellular Vesicles in Stroke Patients Treated With Mesenchymal Stem Cells: A Biomarker Analysis of a Randomized Trial

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BACKGROUND: Mesenchymal stem cells (MSCs) secrete trophic factors and extracellular vesicles (EVs). However, the level and role of EVs after MSC therapy in patients with stroke are unknown. We investigated whether circulating EVs and trophic factors are increased after MSCs and are related to the therapeutic benefits in the STARTING-2 trial (Stem Cell Application Researches and Trials in Neurology-2) participants.

METHODS: In this prospective randomized controlled trial, patients with chronic major stroke were assigned, in a 2:1 ratio, to receive autologous MSC intravenous injection (MSC group, n=39) or standard treatment (control group, n=15) and followed for 3 months. Detailed clinical assessment and neuroplasticity on diffusion tensor image and resting-state functional magnetic resonance imaging were evaluated. Serial samples were collected, before/after MSCs therapy. The primary outcome measure was circulating factors that are associated with the clinical improvement in the Fugl-Meyer Assessment (secondary end point of the trial) and neuroplasticity on diffusion tensor image and resting-state functional magnetic resonance imaging. Additional outcome measures were microRNAs and trophic factors enriched in the plasma EVs, obtained using quantitative polymerase chain reaction and ELISA, respectively.

RESULTS: Circulating EV levels were increased ≈5-fold (mean±SD, from $2.7\times10^{9}\pm2.2\times10^{9}$ to $1.3\times10^{10}\pm1.7\times10^{10}$ EVs/mL) within 24 hours after injection of MSCs (P=0.001). After adjustment of age, sex, baseline stroke severity, and the time interval from stroke onset to treatment, only the EV number was independently associated with improvement in motor function (odds ratio, 5.718 for EV number^{Log} [95% CI, 1.144–28.589]; P=0.034). Diffusion tensor image and resting-state functional magnetic resonance imaging showed that integrity of the ipsilesional corticospinal tract and intrahemispheric motor network were significantly correlated with circulating EV levels, respectively (P<0.05). MicroRNAs related to neurogenesis/neuroplasticity (eg, microRNA-18a-5p) were significantly increased in circulating EVs after MSC therapy (P=0.0479). In contrast, trophic factor levels were not changed after MSC therapy.

CONCLUSIONS: This trial is the first to show that treatment of ischemic stroke patients with MSCs significantly increases circulating EVs, which were significantly correlated with improvement in motor function and magnetic resonance imaging indices of plasticity.

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Key Words: biomarkers ■ clinical trial ■ mesenchymal stem cells ■ stem cells ■ stroke

Stroke is the leading cause of physical disability among adults. Stem cell therapy is a potential regenerative strategy for patients with neurological deficits. Several clinical trials of mesenchymal stem cell (MSC) therapy have been conducted in patients with stroke.[1–7] Although most MSC therapies appeared to be

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ORIGINAL CONTRIBUTION

Nonstandard Abbreviations and Acronyms

Ang-1	angiotensin 1
BDNF	brain-derived neurotrophic factor
CST	corticospinal tract
DTI	diffusion tensor image
EV	extracellular vesicle
FMA	Fugl-Meyer Assessment
GO	gene ontology
HRP	horseradish peroxidase
KEGG	Kyoto Encyclopedia of Genes and Genomes
L1CAM	L1 cell adhesion molecule
miRNA	microRNA
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
NSE	neuron-specific enolase
rs-fMRI	resting state functional magnetic resonance imaging
SDF-1	stromal cell-derived factor-1
STARTING-2	Stem Cell Application Researches and Trials in Neurology-2
VEGF	vascular endothelial growth factor

of some benefit, their effects were modest and heterogeneous among patients.

We have recently reported the results of the STARTING-2 trial (Stem Cell Application Researches and Trials in Neurology-2), a randomized controlled trial of intravenous application of autologous MSCs, expanded with autologous serum.[7] In this trial, changes in detailed motor function and functional outcome were measured over 90 days after randomization to either the MSC group or the standard care group. Multimodal brain magnetic resonance imaging (MRI), including resting-state functional MRI (rs-fMRI) and diffusion tensor imaging (DTI), was performed. The secondary analyses showed significant improvements in detailed motor function in the MSC group compared with the control group, but there was a relatively large variation in the changes of motor function among patients of the MSC therapy. In this trial, a prespecified biomarker study was performed to investigate possible mechanisms of action of MSCs.

MSCs secrete a variety of bioactive substances, including trophic factors and extracellular vesicles (EVs), into the injured brain, which may be associated with enhanced neurogenesis, angiogenesis, neurite outgrowth and remodeling, and neuroprotection. However, the level and role of EVs after MSC therapy in patients with stroke are unknown. We hypothesized that the effects of MSC therapy are mediated via EVs. Thus, we serially measured the circulating trophic factors and EVs, before and after intravenous application of MSCs. We investigated whether these circulating factors are independently associated with clinical improvement in the Fugl-Meyer Assessment (FMA; the secondary end point of the trial) and neuroplasticity measured using DTI and rs-fMRI. In addition, we investigated the EV cargo, that is, microRNAs (miRNAs) and proteins, to identify the possible mechanisms of action of MSC therapy. This was a preplanned secondary analysis.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Trial Design

The STARTING-2 trial (NCT01716481) was an investigator-initiated, prospective, randomized, open-label, controlled trial with blinded outcome evaluation (PROBE design [prospective, randomized, open-label, controlled trial with blinded outcome evaluation]). The details of trial protocol, bone marrow aspiration, MSC isolation, cell preparation, and intravenous infusion are described elsewhere.[7,8] Chronic cases of stroke with fixed neurological deficits were included and followed for 3 months. Eligible participants were adult, aged 30 to 75 years, who had moderate-to-severe persistent neurological deficits (National Institutes of Health Stroke Scale score of 6–21 points), stroke observed within 90 days of the onset of symptoms, and nonlacunar infarcts within the middle cerebral artery territory, but sparing more than a half of ipsilateral subventricular zone. We excluded patients who had a significant disability before the current stroke, whom we defined as those having a prestroke modified Rankin Scale score of ≥2. We also excluded patients with lacunar stroke, hemorrhagic stroke, and recurrent stroke within 7 days of screening. Detailed information on inclusion and exclusion criteria is reported elsewhere.[7,8] Participants were enrolled from 4 University Medical Centers and transferred to the Samsung Medical Center to receive comprehensive rehabilitation and undergo serial dedicated MRI and biomarker study.

Participants were assigned, in a 2:1 ratio, to receive intravenous MSC injection (MSC group) or standard care alone (control group), using computer-generated random permuted blocks with blocks of 6 subjects. After randomization, all participants received conventional rehabilitation therapy (physical, occupational, speech/language, or cognitive rehabilitation therapy as needed) during the inpatient rehabilitation period. Participants in the MSC group underwent MSC treatment. Expanded autologous MSCs at 1×106 cells/kg (maximum, 1.2×108) were infused via the antecubital vein with 5×106 cells/mL of normal saline over 10 minutes at 55.9±19.1 days after the onset of stroke. Comprehensive and objective measurements using multimodal MRI and detailed functional assessments were performed. The clinical trial protocol and consent form were approved by the Korean Food and Drug Administration (No. 12218) and the Institutional Review Board of the Samsung Medical Center (IRB-2011-10-047). The trial was registered and reported according to the Consolidated Standards of Reporting Trials statement. Written informed consent was obtained from all patients or their first-degree relatives.

Clinical and Imaging Assessments

Changes in detailed motor function and the functional outcome were evaluated by the modified Rankin Scale score and FMA. Changes of each score were serially measured over a period of 90 days after randomization. We designated each stroke patient as either a good or poor responder according to the minimal clinically important difference in the FMA.[9,10] Good responders for motor recovery were defined as those having a total score of >15 (FMA-T), an upper limb score of >9 (FMA-UL), and a lower limb score of >6 (FMA-LL) from baseline to 90 days.

Multimodal brain MRI, including rs-fMRI and DTI, was performed as described elsewhere.[11] In brief, to measure tract-wise DTI-derived parameters, a template corticospinal tract (CST) generated from the healthy controls was used instead of tracking the CST in every patient. The use of a template CST has been suggested as a standardized approach to measure the integrity of the CST in patients with stroke using DTI data.[12] In every patient, DTI-derived parameters in the native space were transformed to the same standard space on which the template CST was placed. The tract-wise fractional anisotropy value was calculated as the average of the parameters read over the extent of the template CST on the ipsilesional side. To extract FA values of the CST, individual DTI data were preprocessed using the FMRIB Diffusion Toolbox implemented in the FSL software package 5.0.9 (FMRIB Software Library, FMRIB, Oxford, United Kingdom; http://www.fmrib.ox.ac.uk/fsl). The DTIfit algorithm was used to fit a tensor model and reconstruct FA maps. The FA maps were registered to the MNI standard space (FMRIB58_FA standard-space image) using the nonlinear registration algorithms of the tract-based spatial statistics technique. Lesioned voxels were masked out, and stroke lesion was not considered during the spatial registration. The spatially normalized FA maps were visually checked. To obtain the FA value of the CST, the CST template descending from the primary motor cortex (M1) obtained from probabilistic tractography in the 9 healthy DTI data was used.[2] The CST was binarized and masked on the spatially normalized FA maps. The FA values of the CST were obtained by averaging the FA values within each region. The proportional FA values (affected/unaffected hemisphere) were used as the integrity measure for each region. Because we included MCA infarction patients with moderate-to-severe motor involvement, all participants had infarct involvement of the CST. The preprocessing of the rs-fMRI data included slice timing correction, spatial realignment, lesion-masked spatial normalization, and spatial smoothing with a 6-mm full-width half-maximum gaussian Kernel. All processes were performed with the SPM12 package (Welcome Trust Centre for Neuroimaging, University College London, London, United Kingdom; http://www.fil.ion.ucl.ac.uk/spm). Nuisance signals were removed with a linear regression of 9 parameters, including 6 head motion parameters and 3 temporal parameters for each of the white matter, ventricle, and global signals. Band-pass filtering between 0.009 and 0.08 Hz and linear detrending were performed to remove constant offsets and linear trends. These processes were performed with MATLAB R2014b (Mathworks, Natick, MA). The functional network was constructed with 24 predefined (motor related) regions obtained from a previous meta-analysis[13] from 36 neuroimaging studies for upper extremities of patients with stroke. Lesioned voxels were masked out. The network was constructed by calculating the Pearson correlation for the mean time course of each region defined as the 10-mm-diameter sphere around the predefined MNI coordinates.

Measurement of Circulating EVs and Trophic Factors

We serially measured the circulating EVs and trophic factors, before and after intravenous application of MSCs. Sampling was performed before MSC treatment (the day before and immediately before MSC injection) and after treatment (within 24 hours of treatment [postTxD0], posttreatment day 3 [postTxD3], day 7 [postTxD7], day 14 [postTxD14], and day 90 [postTxD90]) for the MSC group and for the control group at 30 days (preTx), 44 days (postTx14), and 120 days (postTx90) of enrollment. Plasma samples were prepared from citrated whole blood following immediate centrifugation for 15 minutes at 2000*g* and stored at −80 °C until further analyses. To isolate and measure EVs, citrate plasma was diluted 1:1 in 0.22 µm filtered PBS and centrifuged at 10 000*g* for 15 minutes at 4 °C. EVs from the precleared supernatant were then pelleted at 100 000*g* for 1 hour. Centrifugation steps were performed at 4 °C using an Optima TLX ultracentrifuge (Beckman Coulter, Brea, CA) and a TLA120.2 rotor. Pellets were resuspended in filtered PBS and centrifuged again at 100 000*g* for 60 minutes at 4 °C using the same tubes. The final pellet containing EVs was resuspended in 100 µL PBS.

For optimal analysis, EVs were prediluted in vesicle-free water, and the concentration and size distribution of EVs were characterized using a NanoSight NS300 instrument (Malvern, Worcestershire, United Kingdom). The mean size and concentration (particles/mL) were calculated by integrating the data from 3 individual measurements.

The direct visualization of EVs was examined by Cryo-transmission electron microscopy. Carbon grids (Quantifoil, R1.2/1.3, 200 Mesh, Electron Microscopy Solutions) were made hydrophilic surface with glow-discharged in Pelco EasiGlow system. An aliquot (4 µL) of samples was applied on to the carbon side of EM grid and blotted for 1.5 s with humidity and temperature of 100% and 4 °C. Then the sample was plunge frozen into the precooled liquid ethane with Vitrobot Mark IV (FEI). The samples were analyzed by cryo-electron microscope Talos L120C (FEI) at 120 kv.

Twenty micrograms of EV protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was incubated with primary antibodies against CD63, flotillin-1, and HSP70 ([heat shock protein 70]; 1:1000; Cell Signaling Technology, Beverly, MA) or calnexin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C and then was incubated with HRP (horseradish peroxidase)-conjugated secondary antibodies (1:1000; anti-rabbit; Cell Signaling Technology) for 2 hours. Proteins were detected using a chemiluminescence substrate from ThermoFisher Scientific, Inc (Waltham, MA), and were visualized on x-ray film (Agfa, Mortsel, Belgium).

We quantified levels of trophic factors in plasma using ELISAs. ELISAs were performed using commercial kits according to the manufacturer's instructions. The following ELISA kits were used: human VEGF (vascular endothelial growth factor; R&D Systems, Minneapolis, MN), human CXCL12 (C-X-C motif chemokine ligand 12)/SDF-1 (stromal cell-derived factor-1;

R&D Systems), human BDNF (brain-derived neurotrophic factor; R&D Systems), and human NSE (neuron-specific enolase; R&D Systems).

Measurement of miRNAs and Proteins Within Circulating EVs

We measured the levels of miRNA within EVs sequentially (before treatment, on the day of treatment, 14 days of treatment, and 90 days of treatment) in both control and MSC groups. The levels of miRNAs within EVs related to therapeutic efficacy of stem cells and potential recovery after stroke were measured in individual patients using the quantitative polymerase chain reaction technique and analyzed by 2−ΔCt quantitative method. The total RNA of EVs was extracted using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) and XENOPURE miRNA extraction Kit (Xenohelix, Incheon, Korea; CAT: 93667873-EV) in accordance with the manufacturer's instructions. The concentration of RNA was quantified by NanoDrop 1000 (NanoDrop, Wilmington, DE). The primers for the miRNAs were performed by TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA). We selected a total of 16 miRNAs to be analyzed; miR-17-92 (miR-17-3p, 18a-5p, miR-20a-5p, and miR-92-1) and miR-133b, which are associated with neuronal plasticity, neurogenesis, and oligodendrogenesis[14,15]; miR-126-5p, miR-132-3p, miR-181b-5p, and miR-494-3p, which are involved in angiogenesis[16–19]; miR-19a-3p, miR-146a-5p, miR-210-3p, and miR-223-3p, which are related to cytoprotection/anti-inflammation[20–23]; and miR-21-5p and miR-196a-5p, which are involved in recovery after stroke.[24,25] The absolute copy numbers of 7 miRNAs within EVs were determined by performing the XENO-Q miRNA detection assay (Xenohelix, Incheon, Korea; CAT: 93661000), following the manufacturer's instructions. This method relies on the sequence-specific hybridization between a fixed amount of Xeno nucleic acid–based sensors and their target miRNAs that precisely calculates the copy number of multiple target miRNAs. We detected the function of expressed miRNAs in target genes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and gene ontology (GO) pathway analysis. GO analysis and KEGG pathway enrichment analysis were performed by the miRWalk2.0 web-based tool (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/).

Circulating brain-derived EVs were analyzed using immune capture for neuronal surface antigen LICAM (L1 cell adhesion molecule; also known as CD171) to selectively isolate EVs enriched for neuronal origin.[26,27] The level of trophic factors related to neuronal recovery in brain-derived EVs was serially measured to evaluate the changes of these levels in the brain after MSC treatment. We defibrinated plasma samples using thrombin (System Biosciences, Inc, Mountainview, CA), precipitated total EVs using particle precipitation by Exoquick (System Biosciences, Inc), and immunoprecipitated brain-derived EVs expressing L1CAM. Proteins of L1CAM-positive brain-derived EVs were quantified by electrochemiluminescence using the Mesoscale Discovery platform and kits, including human VEGF-a, BDNF, and b-NGF ([beta-nerve growth factor]; catalog No. K151ACL-1, MESO SECTOR S 600, Gaithersburg, MD).

Statistical Analysis

Differences in discrete variables between the groups were examined using the χ^2, Fisher exact, or Mann-Whitney *U* test as appropriate. Differences in continuous variables were examined using 1-way ANOVA, Kruskal-Wallis test, or *t* test. In addition, independent factors for the clinical improvement were evaluated using multivariate logistic regression. Adjustment variables in the multivariable regression models were selected from the literature and selected as age, sex, the National Institutes of Health Stroke Scale at the time of MSC injection, MSC injection, and circulating EV levels after MSC injection for the MSC group and the level at the corresponding time in the control group. Significance levels were set at 2-tailed *P* of <0.05. All statistical analyses were performed using commercially available software (STATA, version 13.1; Stata Corp, College Station, TX).

RESULTS

Baseline Characteristics

All the 54 patients (women, n=27; mean age, 63.4±13.9 years old; control group of 15 patients and MSC group of 39 patients) enrolled in the trial participated in this study. Baseline characteristics are shown in Table S1. Nine of 39 patients of the MSC group and 2 of 15 patients of the control group were classified as good responders according to the FMA-T score after 90 days of intervention. Although good responders were numerically higher in the MSC group than in the control group, there was no significant difference (*P*>0.05).

Plasma Trophic Factor Levels Were Not Changed After MSC Therapy

The levels of circulating cytokines, chemokines, and trophic factors were not different between the control and MSC groups (Figure 1). In addition, there were no differences in the levels between patients who showed clinical improvement in motor function and those who did not (Figure S1A through S1C).

Plasma EV Levels Are Increased in Patients With MSC Treatment and Correlated With Improvement of Motor Function

Most EVs had a round shape with an electron dense structure observed by Cryo-transmission electron microscopy analysis, and their mean (SD) diameter was 156.98 (17.5) nm, respectively (Figure 2A and 2B). The circulating EVs were positive for EV markers including CD63, flotillin-1, and HSP70, whereas calnexin, an EV-negative marker, was not detected (Figure 2C).

Pretreatment EV levels varied among patients (mean±SD, 4.5×109±7.9×109 EVs/mL) and were not different between the control and MSC groups (*P*>0.05). Before injection of MSCs, circulating EV levels were serially measured (n=21), which showed that the levels of EV did not change with time (Figure S2).

The number of EVs in the blood were measured immediately after MSC therapy. The level of EVs

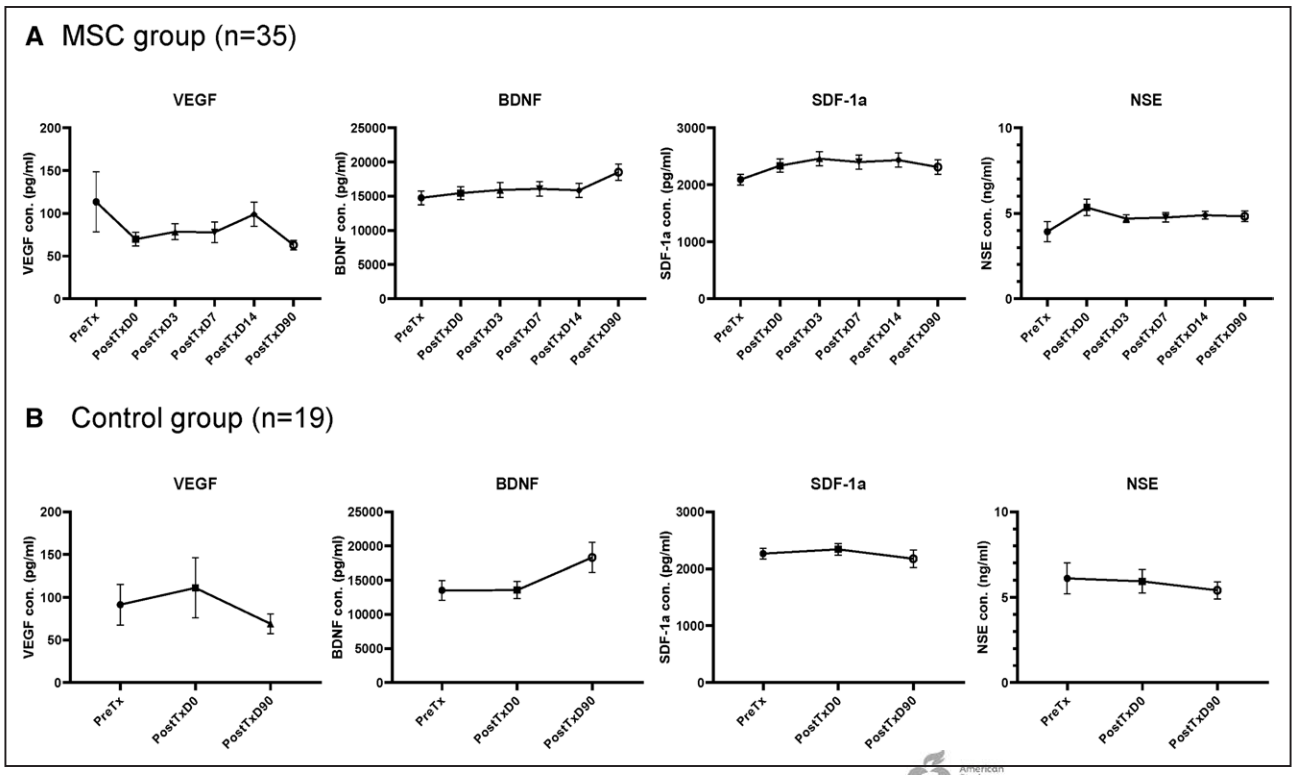

Figure 1. The temporal profiles in the levels of circulating trophic factors and chemokines after mesenchymal stem cell (MSC) therapy.

A, The temporal profiles in the levels of circulating trophic factors and chemokines on MSC group. **B**, The temporal profiles in the levels of circulating trophic factors and chemokines on control group. Mean±SEM, $P>0.05$ in all cases. BDNF indicates brain-derived neurotrophic factor; NSE, neuron-specific enolase; SDF-1, stromal cell-derived factor-1; and VEGF, vascular endothelial growth factor.

was significantly increased ≈5-fold (mean±SD, from 2.7×109±2.2×109 EVs/mL to 1.3×1010±1.7×1010 EVs/mL) within 24 hours after injection of MSCs ($P=0.001$) The percentage increase in EV levels compared with the pretreatment levels is shown in Figure 3A and 3B. In the MSC group, the level of circulating EVs after MSC injection varied greatly among patients, although the same body weight–adjusted number of MSCs were injected. This level was marked increased in patients who showed clinical improvement in motor function than in those who did not show clinical improvement (EV number^{Log}, $P=0.039$ for FMA-total, $P=0.023$ for FMA-upper, $P=0.291$ for FMA-lower; Figure 3C and 3D). Correlation analysis showed that circulating EV number^{Log} after MSC injection was significantly correlated with improvement in motor function (r=0.444, $P=0.001$) but not in patients of the control group (r=0.359, $P=0.207$; Figure S3). The time interval between stroke onset and time of MSC injection was not different between the good and poor responders (51.0±11.7 versus 57.9±20.9 days, $P>0.05$). The time interval between stroke onset to placebo injection was 53.4±11.0 days. After adjustment of age, sex, baseline National Institutes of Health Stroke Scale, and the time interval from stroke onset to treatment of MSCs or placebo, only the number of circulating EVs was independently associated with clinical improvement (good responders; odds ratio, 5.718 for EV number^{Log} [95% CI, 1.144–28.589]; $P=0.034$). In addition, the association between circulating EV levels and DTI and rs-fMRI indices of neuroplasticity was measured on day 90 (Figure 4). Integrity of ipsilesional CST and intrahemispheric motor network were significantly correlated with the circulating EV levels ($P<0.05$ in both cases), suggesting the influence of increased EV levels on the ipsilesional motor network, and on both anatomic and functional recovery, in MSC-treated patients with stroke. There was a good correlation of circulating EV numbers measured by NanoSights with CD9 (a tetraspanines) expressing EV levels on nano-flow cytometry and EV protein levels measured by the microBCA method (Figure S4).

MiRNAs Related to Neurogenesis Are Increased in Circulating EVs After MSC Therapy

In addition to the increased number of EVs, the relative expression of miRNAs within EVs related to neuroplasticity was increased in patients with the MSC group. Specifically, miRNA-18a-5p was significantly increased in the MSC group ($P=0.0479$; Figure 5A). The GO and KEGG analysis showed that enriched miR-18a-5p was linked to signal pathways and biological processes related to the nervous system development, the nerve growth factor receptor

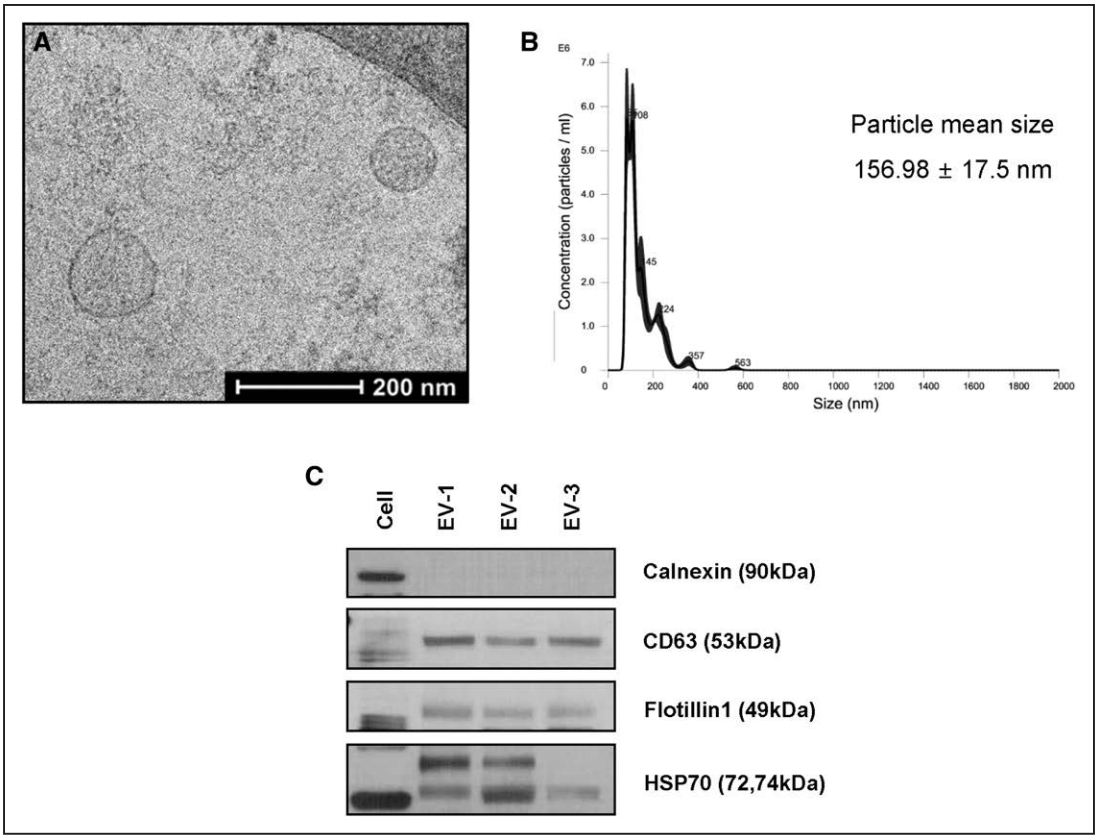

Figure 2. Characterization of circulating extracellular vesicles (EVs).
A, EVs imaged using Cryo-transmission electron microscopy. **B**, Histogram representing the size distributions and concentrations of the EVs using NanoSight. **C**, EV-positive markers, including CD63 (cluster of differentiation 63), flotillin-1, and HSP70 (heat shock protein 70), and EV-negative markers, including calnexin, using Western blot (n=3).

signaling pathway, and axonal guidance (Figure 5B). The KEGG pathways were linked to axon guidance, neurotrophin signaling, and Wnt signaling pathway. GO analysis showed that transcription DNA dependent, nervous system development, nerve growth factor receptor signaling pathway, and axon guidance in the biological process were significantly associated. Other selected miRNAs measured were not different between the groups (data not shown).

Trophic Factor Levels Are Increased in Brain-Derived EVs After MSC Therapy

To evaluate the possible mechanisms of action of MSC or MSC-derived EVs in the brain, circulating brain-derived EVs were collected, and the levels of trophic factors in these EVs were measured in all the patients (n=15 of the control group and n=39 of the MSC group). Compared with the pretreatment, the trophic factor levels in the circulating brain-derived EVs were increased at posttreatment day 3 (Figure 6). VEGF levels were significantly increased after MSC therapy, and the levels of BDNF and NGF (nerve growth factor) were increased but did not reach significant level, due to the small sample size (P=0.3132 for BDNF, P=0.0407 for VEGF, and P=0.0932 for NGF).

DISCUSSION

The major finding of this study is that EV levels were significantly increased in the blood after injection of MSCs, especially in patients who showed improvement in motor function outcome and neuroimaging after MSC therapy. MiRNAs related to neurogenesis were significantly increased in circulating EVs after MSC therapy. To the best of our knowledge, this is the first study performed in a randomized controlled trial of stroke, which suggests the importance of EVs in mediating the stem cell therapy.

EVs are released from various cells, including blood cells, organs, and endothelial cells, and distribute to organs and rapidly cleared by macrophages from blood circulation.[28] In patients with stroke, EVs in the blood can be derived from many sources, including infarcted brain, cerebrovasculature, platelets, and MSCs from patients' bone marrow. However, our serial analysis of plasma EVs showed no increase in EV levels before MSC treatment. A pharmacokinetic analysis of mouse plasma-derived EVs showed a balanced homeostasis by rapid secretion and clearance of endogenous EVs.[28] Moreover, patients with chronic stroke were included in this trial precluding the possible influence of acute stroke in EV levels.

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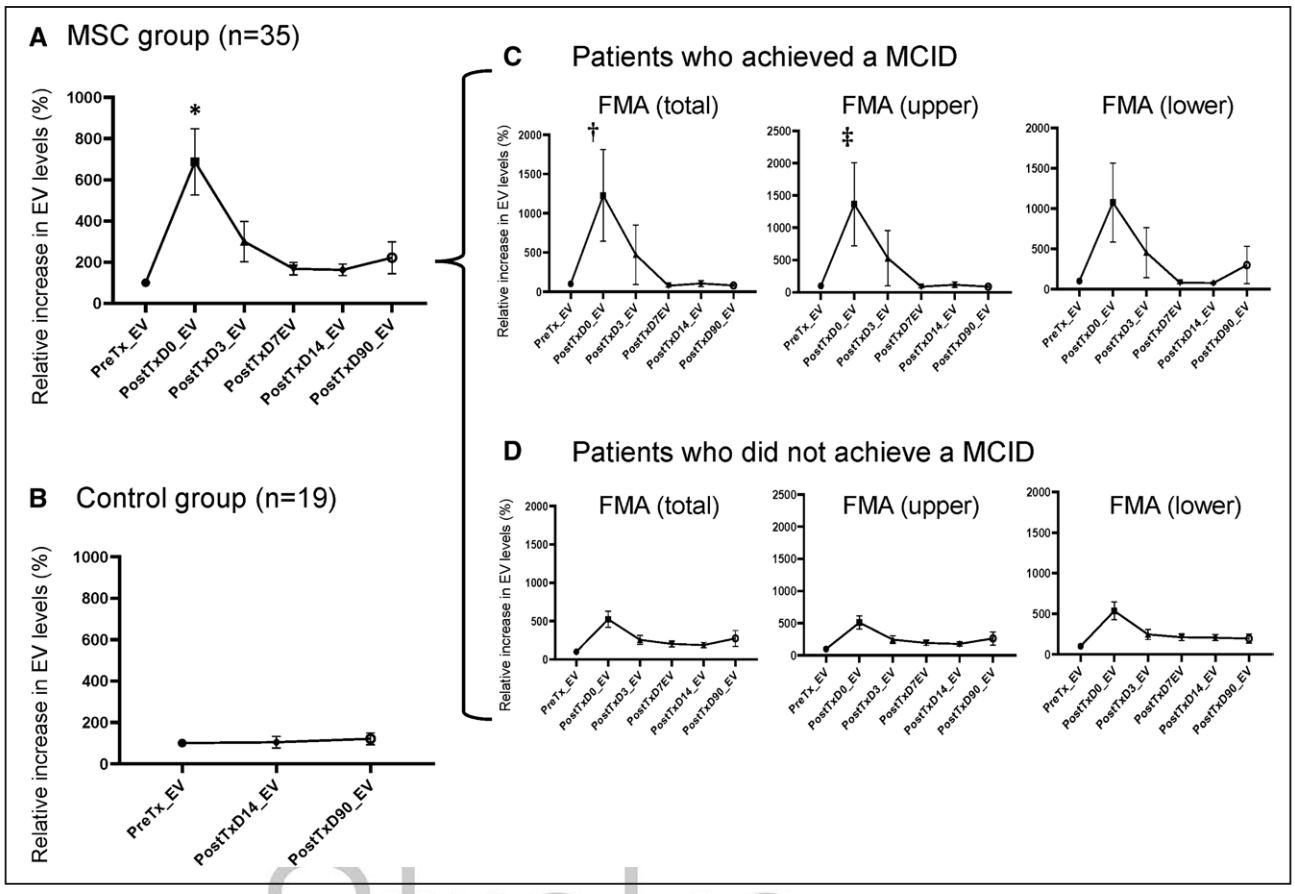

Figure 3. The temporal profile of plasma levels of circulating extracellular vesicles (EVs) after mesenchymal stem cell (MSC) therapy.

The level of circulating EVs was significantly increased in the MSC group (**A**) but not increased in the control group (**B**). In the MSC group, the level of EV was significantly increased in patients who achieved a minimal clinically important difference (MCID) of the Fugl-Meyer Assessment (FMA; **C**) but not in patients who achieved no MCID (**D**). FMA-LL indicates lower limb score of the Fugl-Meyer Assessment; FMA-T, a total score of the Fugl-Meyer Assessment; and FMA-UL, upper limb score of Fugl-Meyer Assessment. Mean±SEM, **P*=0.001, †*P*=0.039, ‡*P*=0.023.

The robust increase of plasma EV levels after systemic administration of MSC was an unexpected and interesting finding. The number of EVs increased from 2.7×109 to 1.3×1010 EVs/mL within 24 hours after injection of MSCs. The value appears to be larger compared with the estimated number of EVs secreted from MSCs in the body after MSC injection. The number of EVs increased in the blood was calculated as ≈7×107 EVs/mL, given that 1×106 cells/kg were injected, one MSC secreted ≈5×103 EVs in our ex vivo experiment, and blood volume is 70 to 80 mL/kg. However, it should be noted that the secretion rate of EVs from MSCs in the blood is much higher than that of cell cultured EVs.[28] In addition, MSCs release EVs in response to the ischemic brain microenvironment.[29] We have previously shown that treatment of bone marrow MSCs with ischemic brain extract increased the number of EVs released from MSCs, >100-fold.[29] Further studies on the biodistribution of EVs after systemic administration of MSCs are needed. A biodistribution study of EVs revealed the rapid disappearance of EVs in the blood with a very short half-life after a single intravenous injected labeled Evs,[28] while an MSC biodistribution study in patients with liver cirrhosis showed that labeled MSCs were accumulated in the organs up to day 10 after systemic administration.[30]

Stem cell therapies exert their actions through paracrine secretion effects of stem cells, such as trophic factors, cytokines, chemokines, and EVs. MSC-derived EVs act as key messengers between MSCs and injured cells, which is one of the major paracrine actions of MSCs.[31–33] However, it is unknown whether EVs are indeed formed by systemically administered MSCs. Our present results show that EV levels increased in patients after injection of MSCs. Importantly, the levels of EVs differed among patients although the same dose-adjusted numbers of MSCs were administered in the MSC group. Furthermore, the number of EVs was the only independent factor for the favorable clinical and imaging outcomes. Such interindividual variation in EV levels may, in part, explain the heterogeneity of the effects of MSCs among patients in clinical trials of stem cells for stroke. Our present results of a significant correlation of anatomic and functional improvement in motor function with numbers of plasma EVs are consistent with previous preclinical studies that showed that MSC-derived EVs

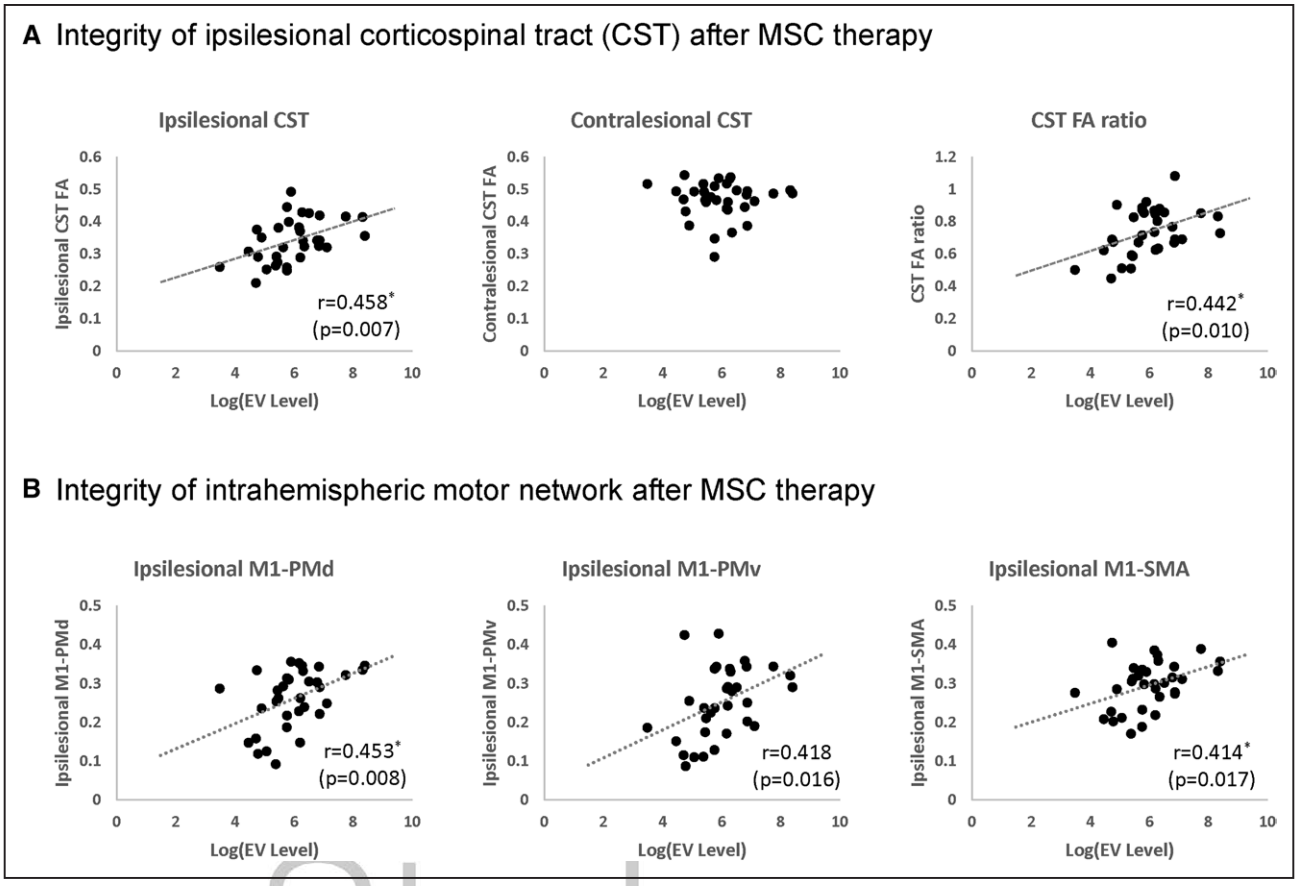

Figure 4. The association between circulating extracellular vesicle (EV) levels within 24 h after mesenchymal stem cell (MSC) therapy and diffusion tensor imaging and resting-state functional magnetic resonance imaging indices of neuroplasticity measured on day 90 (n=54 including both control and MSC groups).
Integrity of ipsilesional corticospinal tract (CST; **A**) and intrahemispheric motor network (**B**) was significantly correlated with the circulating EV levels. *P*<0.05 in all cases except contralesional CST. FA indicates fractional anisotropy; M1, primary motor cortex; PMd, dorsal premotor area; PMv, ventral premotor area; and SMA, supplementary motor area.

promote neural plasticity in animal models of stroke.[34,35] Recently, Barzegar et al[36] showed that the protective effects of MSCs in a stroke mouse model were decreased by blocking the formation/release of EVs from MSCs. It is possible that trophic factors that modify the cerebral microenvironment contribute to the efficacy of cell therapy. A recent multicenter study of patients with nonlacunar stroke within 24 hours from symptom onset showed that high serum levels of VEGF, Ang-1 (angiotensin 1), G-CSF (granulocyte colony stimulating factor), and SDF-1α at day 7 and 3 months after ischemic stroke are associated with good functional outcome and smaller residual lesion at 1 year of follow-up.[37] Therefore, we serially measured the levels of growth factors and SDF-1α after MSC therapy. The present study showed that levels of trophic factors and chemokines were not changed and apparently were not related to clinical outcome after MSC therapy. However, we only measured a selected number of these factors, and further studies are needed to preclude the possibility of a contribution of other factors.

In this study, we analyzed the EV-miRNA profile and brain-derived EV-trophic factor levels to investigate the possible mechanisms of action of MSC therapy. Biomarker studies in clinical trials of stem cell therapy may provide insights of action mechanisms of stem cells in patients with stroke. In a secondary analysis of the Exenatide-PD randomized clinical trial, the investigators measured the levels and target pathways of circulating EVs after treatment of patients with Parkinson disease with exenatide (a glucagon-like peptide 1 agonist).[27] Our results showed that circulating EVs contained miRNA-18a-5p, one of miRNA-17-92 cluster, which significantly increased after MSC therapy, and that the enriched miR-18a-5p was linked to signal pathways and biological processes related to neuroplasticity. Our GO and KEGG analyses are in line with previous studies that showed bone marrow MSC-derived EVs enriched with the miRNA-17-92 cluster mediate the proliferation/survival of neuronal progenitor cells and neuroplasticity after stroke.[14,38,39] Further studies are needed to confirm our results because other individual components of the miR-17-92 cluster did not show a similar change. However, our quantitative analysis of the absolute copy numbers of individual miRNA-17-92 cluster using the XENO-Q miRNA detection assay showed

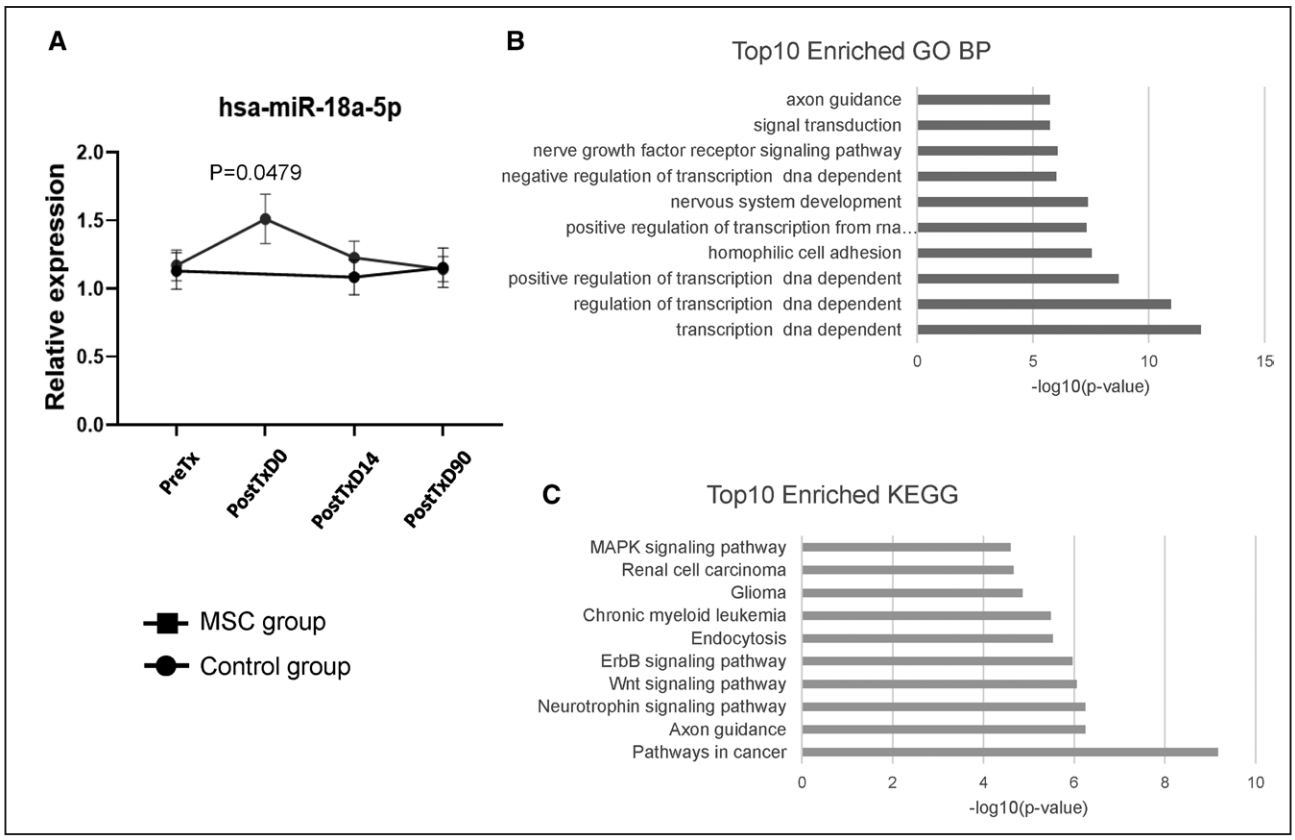

Figure 5. Relative expression of microRNAs related to neuroplasticity in the control and mesenchymal stem cell (MSC) group.
A, Significantly increased miRNA-18a-5p expression in the circulating extracellular vesicles (EVs) of the MSC group (*P*=0.0479). **B**, Gene ontology (GO) of biological processes (BP) and (**C**) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways are presented using only the 10 most relevant terms on target genes of miR-18a-5p enriched in the EVs.

the most tested miRNA levels were numerically increased in almost 2-fold after treatment of MSC therapy (Table S2; Figure S5). In addition, there were similarities in the miRNA profile between conditioned media EVs obtained during ex vivo cultivation and plasma EVs obtained within 24 hours after injection of MSCs, suggesting that the administered EVs have a direct impact on the plasma EVs (Figure S6). Moreover, trophic factors related to neuronal recovery after stroke were increased in the plasma brain-derived EVs, suggesting that MSCs or MSC-EVs exert their action via the regulation of trophic factor levels within the recipient brain cells.

This study had some limitations. This is a preliminary study, limited in patient populations investigated and

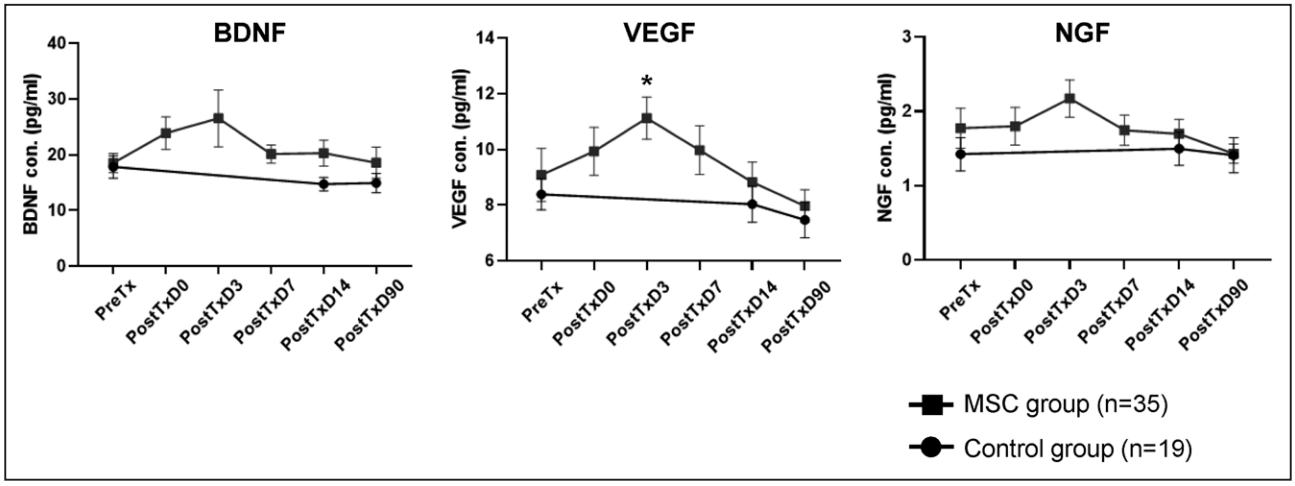

Figure 6. Trophic factors enriched in the brain-derived *L1CAM*-positive extracellular vesicles were increased after 3 d of mesenchymal stem cell (MSC) therapy.
BDNF indicates brain-derived neurotrophic factor; con., concentration; NGF, nerve growth factor; and VEGF, vascular endothelial growth factor. Mean±SEM, **P*=0.0407.

molecular markers measured. In this study, owing to the experimental nature of the treatment, a relatively small number of patients were included, and only selected miRNAs were tested because of the limitation in the amount of plasma of trial participants. In addition, this study included only patients with MCA infarction patients, so limiting generalizability to most stroke patients. Second, multiple correlation analyses were performed in this study based on the limited data set, the possibility of type II error cannot be excluded. Characteristics of patients, including age, stroke severity, and comorbidity, may impact on the results of this study, but the findings of robust increase in EVs after MSC treatment and their correlation with recovery warrants further studies. Third, beside MSCs, the source of EVs could be blood, endothelium, and other tissues. Further studies are needed evaluating the pharmacokinetics and biodistribution of MSC-derived EVs using various tagging strategies. Fourth, the change in the clinical outcomes would be influenced based on when the cells were injected after stroke. Our substudy of responder analysis showed that time from stroke onset to MSC therapy was related to the patients' response to MSC therapy.[40] However, in the present study, the number of circulating EV levels was independently associated with clinical improvement after adjustment of the time of MSC injection after stroke. Fifth, plasma EV levels were measured at 24 h posttreatment in the MSC group, but EV levels were not measured at a comparable time point in the control group after stroke. The EV levels could be influenced by acute stroke or other factors unrelated to MSCs. However, in our opinion, it is unlikely that plasma EV levels change with time in patients with chronic ischemic stroke (Figure S2). Lastly, the methods for quantifying EVs are limited by the technologies available, and experimental manipulation would alter the recovery or loss of EVs.

CONCLUSIONS

Our results showed that treatment of ischemic stroke patients with MSCs significantly increased circulating EVs, but the level of circulating EVs varied greatly among patients. The finding of nonresponders in the MSC-treated group (ie, patients who do not show a significant increase in circulating EVs and no significant effects of MSC therapy) needs to be investigated further to refine this treatment approach.

In addition, our results are suggestive of a therapeutic role of MSC-derived EVs and provide a mechanistic context for clinical findings of the trial. This study did not test the therapeutic role of EVs by injecting the patients' circulating EVs into an animal model of stroke. Further studies are needed examining the effects and safety of MSC-derived EV therapeutics in patients with stroke. An increasing body of literature has demonstrated that MSC therapeutic efficacy for treatment of stroke is mediated by the release of their EVs suggesting that stem cell–derived EVs can be used for stroke therapy as an alternative approach to stem cell infusion methods.[31,32]

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Disclosures

None.

Supplemental Material

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