Protective Effect of Mesenchymal Stromal Cell-Derived Exosomes on Traumatic Brain Injury via miR-216a-5p

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Background: Transplantation of exosomes derived from mesenchymal stem cells (MSCs-Exo) can improve the recovery of neurological function in rats after traumatic brain injury (TBI). We tested a new hypothesis that brain-derived neurotrophic factor (BDNF)-induced MSCs-Exo can effectively promote functional recovery and neurogenesis in rats after TBI.

Material/Methods: BM-MSCs of rats were extracted by whole bone marrow culture, BDNF was added to BM-MSCs as an intervention, supernatant was collected, and exosomes were separated and purified by ultracentrifugation. Exosomes were identified by Western blot (WB), transmission electron microscopy (TEM), and particle size analysis and were subsequently used in cell and animal experiments. The experimental animals were divided into a sham group, a PBS group, an MSCs-Exo group, and a BDNF-induced MSCs-Exo group (n=12). An electric cortical contusion impactor (eCCI) was used to cause TBI in all rats except the sham group. We investigated the recovery of sensorimotor function and spatial learning ability, inflammation inhibition, and neuron regeneration in rats after TBI.

Results: Compared with the MSCs-Exo group, the BDNF-induced MSCs-Exo group showed better effects in promoting the recovery of sensorimotor function and spatial learning ability. BDNF-induced MSCs-Exo successfully inhibited inflammation and promoted neuronal regeneration *in vivo* and *in vitro*. We further analyzed miRNAs in BDNF-induced MSCs-Exo and MSCs-Exo and found that the expression of miR-216a-5p in BDNF-induced MSCs-Exo was significantly higher than that in MSCs-Exo as determined by qRT-PCR. Rescue experiments indicated that miR-216a-5p had a similar function as BDNF-induced MSCs-Exo.

Conclusions: We found that BDNF-induced MSCs-Exo can improve cell migration and inhibit apoptosis better than MSCs-Exo in rats after TBI, and the mechanism may be related to the high expression of miR-216a-5p.

MeSH Keywords: Brain Injuries • Mesenchymal Stromal Cells • MicroRNAs

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Background

Due to increasing socio-economic development and motor vehicle injuries, as well as for other reasons, the incidence of traumatic brain injury (TBI) is increasing every year [1,2]. TBI is expected to be the third most expensive disease by 2020 [3]. TBI therapy has always been a medical puzzle due to the difficulty in central nervous system regeneration, the complex pathological mechanism, and severe secondary injury. Regeneration after neuronal injury is the most difficult problem in TBI therapy. To effectively treat TBI, problems such as nerve regeneration, inflammation, and oxidative stress must be solved.

In recent years, in-depth research on stem cells and exosomes has led to new perspectives on the treatment of TBI. MSCs are the focus of stem cell research, and many studies have confirmed that mesenchymal stromal cells (MSCs) and exosomes derived from mesenchymal stem cells (MSCs-Exo) can restore neuronal function after TBI [4–6]. Exosomes are extracellular vesicles formed by endocytosis, with a diameter of 30–100 nm. Exosomes are effective transmitters of intercellular communication, especially for the transmission of small molecules between cells. The cargos of exosomes are collections of proteins, lipids, mRNAs, microRNAs (miRNAs), and other substances that are transported to other cells to influence their functions and physiological states [7,8]. MSCs-Exo can improve functional recovery after TBI, rescue cognitive impairments, promote neurogenesis and angiogenesis, and reduce neuroinflammation in animal models of TBI [9–11]. Although MSCs-Exo cannot proliferate, they have some advantages over stem cells due to their low immunogenicity, easy storage and transportation, and free passage through blood vessels and the blood-brain barrier [9,12]. Brain-derived neurotrophic factor (BDNF) is one of the most widely distributed neurotrophic factors in the brain. BDNF can promote the growth of neurons and reduce the loss of neurons, as well as inducing the differentiation of neural stem cells and MSCs into neurons [13–15].

Studies have shown that the secretion and composition of exosomes are related to the intracellular environment or culture environment [16]. We speculated that MSCs-Exo can treat nerve injury after BDNF stimulation. In this study, we speculated that BDNF could induce more miRNAs with anti-inflammatory and neural regeneration characteristics in MSCs-Exo.

Material and Methods

Extraction and culture of rat bone marrow MSCs and exosome extraction

Rats were anesthetized with 4% chloral hydrate (7 ml/kg) and disinfected with 75% ethanol for 5 min. Femurs and tibias were isolated under aseptic conditions, the muscle attached to the bone was removed, and the bone marrow cavity was exposed at the metaphyseal ends of both ends of the bone. Bone marrow was flushed by low glucose-Dulbecco's modified Eagle's medium (L-DMEM) culture. The obtained cell suspension was centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The pellet was suspended in L-DMEM containing 10% fetal bovine serum (FBS) (Excel Scientific, USA) in a 37°C and 5% CO_2 incubator. The morphology of MSCs was observed under a microscope. After the adherent cells reached 80~90% confluence, the adherent cells were considered P0 cells. The cells were digested with 0.25% trypsin and passaged at a ratio of 1: 2 and stored. P3 MSCs that had been identified by immunofluorescence (Supplementary Figure 1) were used for the experiment, and the cell density was adjusted to $1×10^8$/L, which were divided into 2 groups. Exosome-free serum (Excel Scientific, USA) was used for culturing, BDNF (Bioss, Beijing, China) (30 ng/mL) was added to one group, and the supernatant was collected after culturing for 48 h.

The animal experiments were conducted at the animal laboratory of the Special Medical Center of PAP (Tianjin, China). The experimental protocol was approved by the Ethics Committee of the Armed Police Characteristic Medical Center (approval number 2017-0021.

Exosome extraction

Exosomes were extracted by ultracentrifugation [17]. The collected cell culture supernatant was centrifuged at 2 000 g at 4°C for 20 min, and the precipitate was discarded. The supernatant was centrifuged at 10 000 g at 4°C for 30 min, and the precipitate was discarded. The supernatant was centrifuged at 100 000×g for 70 min at 4°C, and the supernatant was discarded. The precipitate collected by resuspending in PBS was centrifuged again at 4°C for another 70 min at 120 000 g and resuspended in 50 µL PBS to obtain the exosome suspension, which was stored at −80°C for future use.

Establishment of the cell damage model

PC12 (Bena Culture Collection, Beijing, China) cells in logarithmic growth phase were collected, and the cell density was adjusted to $1×10^8$/L. The cells were inoculated into 6-well plates in 4 groups. BDNF-induced MSCs-Exo was added to the first group, and MSCs-Exo was added to the second group. The total exosome protein concentration of both groups was 50 µg/ml, and an equal volume of PBS was added to the last 2 groups. The medium was changed with RPMI 1640 medium after 24 h, and the medium contained 0.521 mmol/L H_2O_2, with the exception of the last group. H_2O_2 was used to induce apoptosis, as described previously [18].

Cell proliferation assay

CCK-8 is a widely used method for detecting cell proliferation [19]. Ten hours after the cell damage, 10 µl Cell Counting Kit-8 (CCK-8) (Biosharp Life Sciences, Hefei, China) reagent was added to each well, and the optical density was measured at 450 nm. The results are expressed as a ratio to the control group values.

Determination of SOD and ROS

Superoxide dismutase (SOD) and reactive oxygen species (ROS) were tested according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). SOD is expressed in units of U/mg protein, and ROS are expressed as the ratio to the control group values.

TdT-mediated dUTP nick-end labeling (TUNEL) staining

TUNEL staining was performed 10 h after the cell damage, according to the protocol of the TUNEL kit (Keygen Biotech, Nanjing, China). Sections of the central part of the brain tissue injury in each group were obtained for TUNEL staining. Six high-magnification fields were randomly selected from the stained sections. The calculated stained cell count was used for statistical analysis.

Scratch wound-healing assay

PC12 cells were plated into 6-well plates, and 2 horizontal and 2 vertical lines were scratched on the bottom of the plates with a sterile 10-µl micropipette tip after the cells were confluent. After the cells were washed with phosphate-buffered saline (PBS), RPMI 1640 medium without FBS was added to the 6-well plate. MSCs-Exo and BDNF-induced MSCs-Exo were added to the culture medium in the first group and the second group, and the concentration of exosomes was 50 µg/ml. An equal volume of PBS was added to the third group. Images of the same view were obtained under a microscope at 0, 6, 12, and 24 h after the scratch was made. The obtained images were analyzed using ImageJ software.

Establishment of animal models

The TBI model was established by the electric cortical contusion impactor (eCCI) (CDF, USA). The specific methods were as in previous studies [20,21]. Adult male Sprague-Dawley (SD) rats (weight 220–250 g) were anesthetized with 4% chloral hydrate (7 ml/kg) by intraperitoneal injection. The skull was exposed, a bone flap with a diameter of 8 mm was removed from the parietal bone, the bone flap was 3 mm from the sagittal suture and herringbone suture, and the dura mater was protected during the operation. The eCCI strike parameters were set as follows: 4 m/s strike speed, 2.5 mm strike depth, and 200 ms strike dwell time. The bone flap was removed without striking in the sham group (n=12), and the other 3 groups (n=12) were struck with the above parameters. Twenty-four hours after surgery, 0.5 ml PBS was injected into each rat through the tail vein, and the PBS of the MSCs-Exo group and the BDNF-induced MSCs-Exo group contained 100 µg exosomes (total protein) [6,11]. Rats were sacrificed on days 3 (n=3), 7 (n=3), and 35 (n=6) for the corresponding experiments.

Neurobehavioral assessment

Modified neurological severity score (mNSS) mNSS tests were performed on all rats preinjury and at 1, 7, 14, and 28 days after TBI. This test included a motion test, sensation test, and reflection test. The possible test scores were 0–18, and the higher the score, the more severe the functional deficiency.

Morris water maze (MWM)

This test was performed using a previously described protocol [22]. The MWM test was performed 31–35 days after TBI, 4 times a day. The water in a swimming pool with a diameter of 1.8 meters inside the maze was dyed black with ink. A hidden platform with a diameter of 12 cm was placed under the water. During the test, the experimental conditions were strictly controlled, including the starting time each day and the temperature of the water in the pool (25±1°C). A dim light and quiet environment were ensured, and all tests were carried out by the same experimenter. The pool was divided into 4 equal quadrants, and the rats were placed in the pool from the midpoint of each quadrant near the arc. The rats swam for 90 s or until they found the platform. If the rat could find the platform within 90 s, it was allowed to remain on it for 10 s. If the rat could not find the platform within 90 s, it was placed on the platform for 10 s. On the 5th day of the MWM test, the underwater platform was removed, and the swimming track of the rats in the pool was recorded. The track of rats in the swimming pool was recorded by camera and analyzed using a professional software system (Ethovision 2.0, Noldus, Wageningen, the Netherlands).

Brain tissue preparation

The rats were anesthetized with 4% chloral hydrate, and the heart was perfused with normal saline, followed by perfusion with 4% paraformaldehyde. The skin and skull were cut layer by layer to expose the brain tissue and carefully remove it. The brain tissue was soaked in 4% paraformaldehyde for 24–48 h. After dehydration, the brain tissue was embedded with paraffin, and then brain slices (5 µm thick) were cut from the damaged area and the hippocampus.

Immunofluorescent staining

To identify new immature neurons in rats, we collected brain tissue sections of rats 7 days after TBI and labeled them with doublecortin (DCX) (Bioss, Beijing, China) for immunofluorescence staining. We also identified immature and mature neurons in the injured peripheral areas and the dentate gyrus of the hippocampus. After dewaxing and rehydration, the sections were boiled in citric acid buffer (pH 6) for 10 min. Slices were processed with 0.1% Triton X-100 for 15 min. Sections were incubated with 5% BSA in PBS. Sections were incubated overnight with rabbit anti-DCX (1: 200) (Bioss, Beijing, China) at 4°C. After washing with PBS, goat anti-rabbit antibody (Alex Fluor 488, 1: 500) (Bioss, Beijing, China) was added, followed by incubation for 2 h. After washing with PBS, sections were incubated with DAPI (Bioss, Beijing, China) for 10 min at room temperature. The sections were then sealed with glycerin and observed under a fluorescence microscope.

MiRNA analysis

To determine microRNA (miRNA) expression, total RNA was extracted from MSCs-Exo and BDNF-induced MSCs-Exo. RNA was converted to cDNA using the QuantiMir RT system. miRNA expression levels were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) using the QuantiMir System (TransGen Biotech, Beijing). cDNAs were mixed with SYBR Green Mastermix (TransGen Biotech) and a universal reverse primer. Specific primers (1 μl) were added to each qPCR plate. The expression levels of each mature miRNA were evaluated using the comparative threshold cycle (Ct) method ($2^{-\Delta Ct}$). Glyceraldehyde-3-phosphate dehydrogenase 9 (GAPDH) was used as the internal control.

RNA transfection

RNA transfection was performed by lentivirus transfection [23]. Lentivirus containing X rat pre-miR-216a-5p was purchased from GenePharma (Shanghai, China). When the cell density reached 40%, the cells were transfected according to the manufacturer's instructions, and the cells were harvested at 48 h for qRT-PCR analysis and cell function testing.

Western blot analysis

This assay was performed using a previously described protocol [24]. The bicinchoninic acid (BCA) protein assay kit was used to quantify the protein concentration after total protein was extracted. The proteins were denatured by boiling and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was soaked in 10% fat milk for 1 h at room temperature.

The antibody (Bioss, Beijing, China) (1: 1000) was dissolved in 5% fat milk and incubated overnight with the PVDF membrane at 4°C. The next day, the corresponding secondary antibody (Bioss, Beijing, China) (1: 5000) was incubated at room temperature for 1 h. The PVDF membrane was visualized by enhanced chemiluminescence reagent.

Statistical analysis

The results are all expressed as the mean±standard error (SEM). SPSS version 19.0 software (IBM, Chicago, IL, USA) was used for data analysis. One-way analysis of variance (ANOVA) was used for statistical comparison, and Fisher's post hoc multiple comparison test was used. $P < 0.05$ was regarded as statistically significant.

Results

Characterizations of MSCs-Exo and BDNF-induced MSCs-Exo

To understand the characteristics of exosomes, we evaluated the exosomes by transmission electron microscopy (TEM), Western blot (WB), and nanoparticle tracking analysis (NTA). TEM showed that exosomes were flat vesicular structures with a diameter of 30–150 nm, and there was no significant difference in morphology between MSCs-Exo and BDNF-induced MSCs-Exo (Figurte 1A, 1B). WB analysis showed that the exosome markers CD63 and CD9 were positively expressed in MSCs-Exo and BDNF-induced MSCs-Exo (Figure 1C). NTA detection showed that the concentration of MSCs-Exo was 8.31×10^8 particles/frame after being diluted 10 times, with a peak value of 110 nm (Figure 1D). The concentration of BDNF-induced MSCs-Exo was 9.0×10^8 particles/frame after being diluted 10 times, with a peak value of 118 nm (Figure 1E). There were no significant differences in the concentration and diameter distribution between the MSCs-Exo and BDNF-induced MSCs-Exo.

BDNF-induced MSCs-Exo significantly enhanced migration and inhibited oxidative stress injury in PC12 cells

PC12 cells can be used as neuronal model cells in neurobiology experiments. To verify the effect of BDNF-induced MSCs-Exo on the proliferation and migration of neurons, we conducted a series of experiments with PC12 cells. Compared with MSCs-Exo, BDNF-induced MSCs-Exo significantly promoted the migration of PC12 cells at 12 and 24 h (Figure 2A). After H_2O_2 treatment, the viability of PC12 cells decreased in each group, and the viability of PC12 cells pretreated with BDNF-induced MSCs-Exo was significantly higher than that of other groups (Figure 2B). Compared with those of the control group, the SOD values of PC12 cells treated with H_2O_2 decreased significantly. Compared
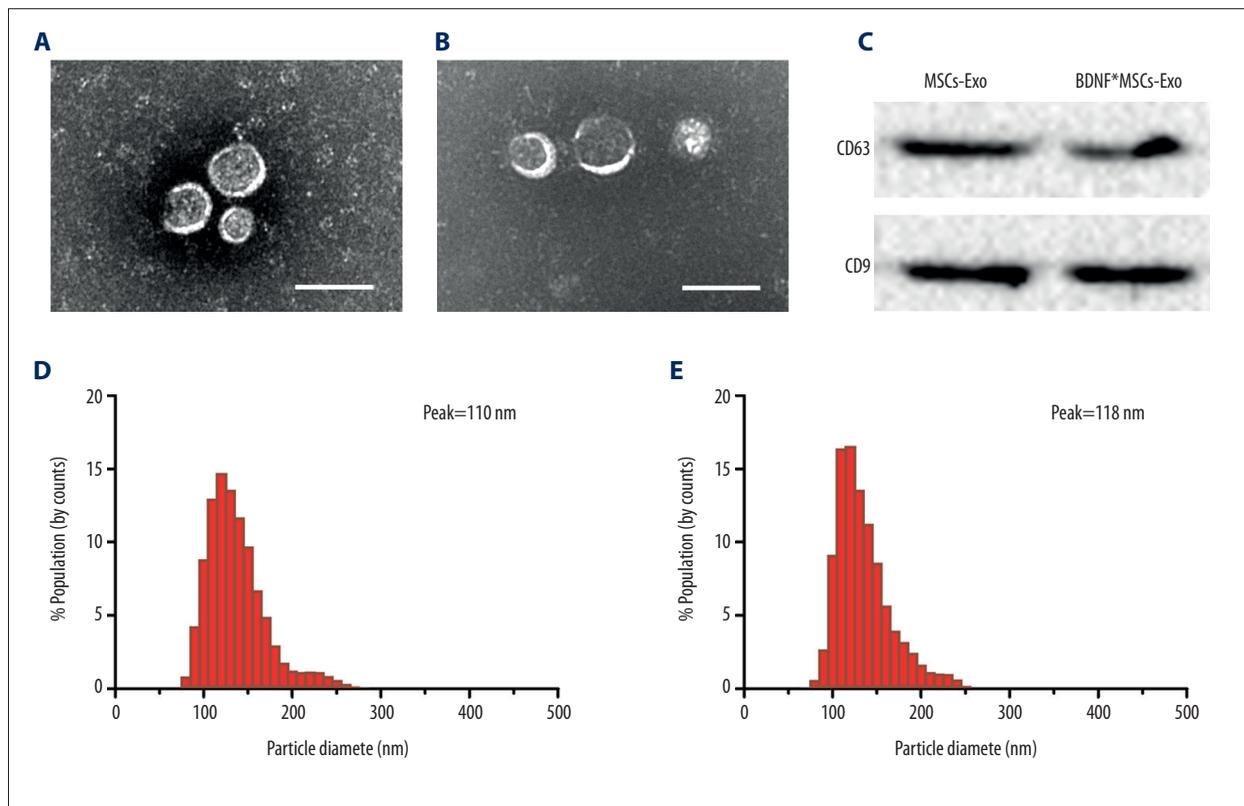
Figure 1. Characterizations of exosomes. (**A, B**) Transmission electron microscopy (TEM) image of MSCs-Exo and BDNF-induced MSCs-Exo (BDNF*MSCs-Exo) (scale bar: 100 nm). (**C**) Both CD9 and CD63 were positively expressed in MSCs-Exo and BDNF-induced MSCs-Exo as determined by Western blot assay. (**D, E**) The peak of MSCs-Exo diameter was 110 nm, and the peak of BDNF-induced MSCs-Exo diameter was 118 nm as detected by NTA. There were no significant differences in the concentration and diameter distribution between MSCs-Exo and BDNF-induced MSCs-Exo.

with that of the PBS group, the SOD activity of the MSCs-Exo and BDNF-induced MSCs-Exo groups increased significantly, and the increase in the BDNF-induced MSCs-Exo group was more significant, close to that in the control group (Figure 2C). Compared with the levels in the control group, the ROS levels increased significantly in each group after treatment with H_2O_2. Compared with those in the PBS group, the ROS levels in the MSCs-Exo and BDNF-induced MSCs-Exo groups decreased significantly, and the decline was more obvious in the BDNF-induced MSCs-Exo group (Figure 2D). To verify the effect of BDNF-induced MSCs-Exo on neuronal apoptosis, TUNEL staining was performed on PC12 cells and brain tissue. In the sham group without H_2O_2, only a few cells were apoptotic, while cells in the 3 groups treated with H_2O_2 were significantly more apoptotic than those in the sham group. There were significantly fewer TUNEL-positive cells in the BDNF-induced MSCs-Exo group than in the PBS group and MSCs-Exo group, indicating that BDNF-induced MSCs-Exo had an obvious anti-apoptotic effect (Figure 2E, 2F).

BDNF-induced MSCs-Exo significantly promoted the recovery of sensorimotor function and spatial learning function in rats after TBI

After training and learning to find the platform in the MWM test, the shorter the time the rats spent finding the platform, the better their spatial learning ability. On the third and fourth days of training, all rats spent significantly less time finding the hidden platform. The time needed for the BDNF-induced MSCs-Exo and MSCs-Exo rats to find the hidden platform was significantly shorter than in the PBS group, and the BDNF-induced MSCs-Exo group took the shortest time (Figure 3A, 3B). On day 5 of the test (35 days after TBI), after the platform was removed, rats in the BDNF-induced MSCs-Exo group spent significantly more time in the area around the platform (Figure 3C). From the first day after TBI, we used the mNSS to evaluate the neurological function of rats, and the higher the score, the more serious the functional injury. On the first day after TBI, the mNSS of the rats was approximately 12 points, which gradually decreased in subsequent tests. From day 7, the mNSS of the BDNF-induced MSCs-Exo group was the lowest among the injury groups (Figure 3D). The results showed that both
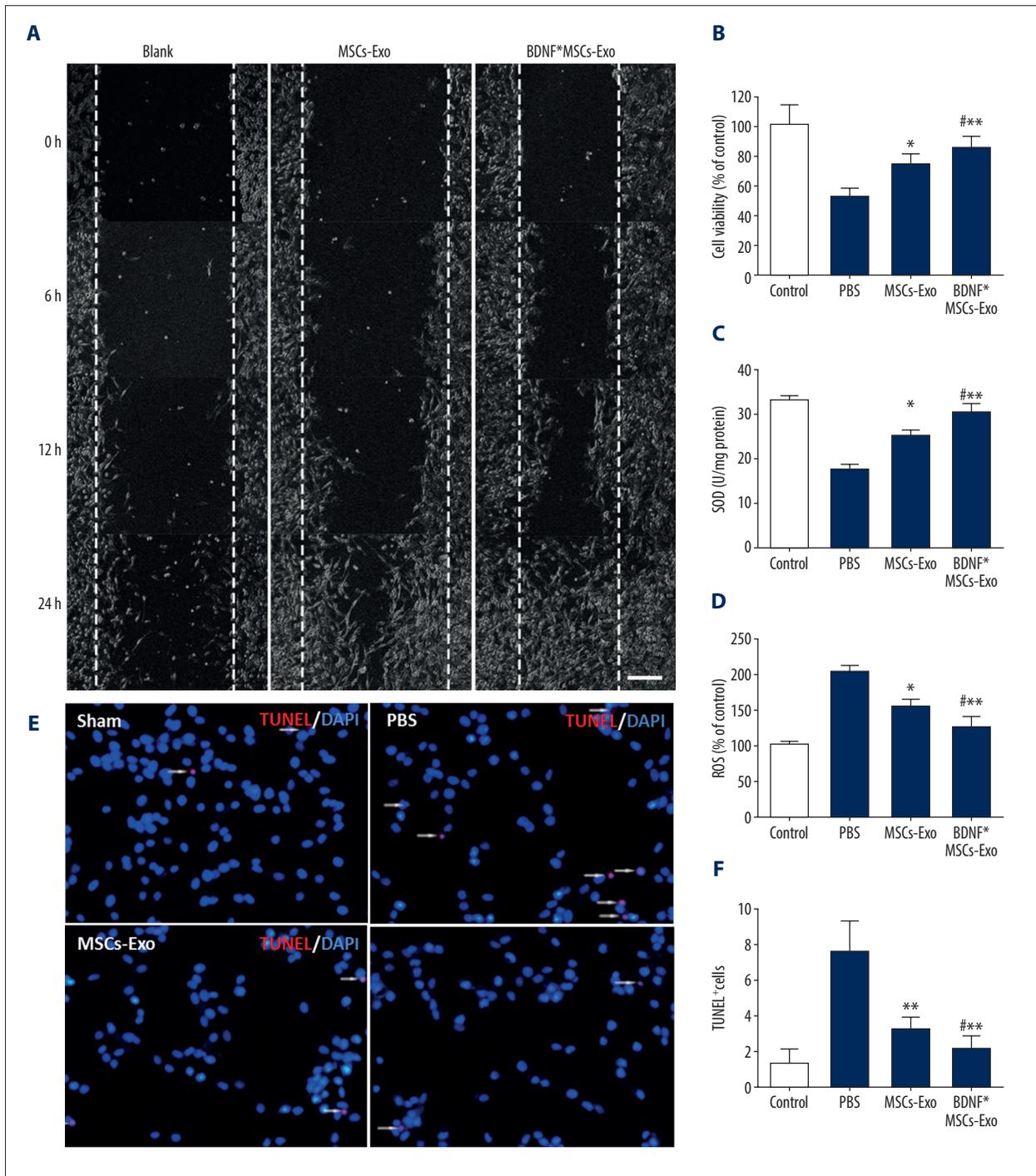
Figure 2. BDNF-mediated MSCs-Exo significantly enhanced migration and inhibited oxidative stress injury and apoptosis in PC12 cells. (**A**) Both MSCs-Exo and BDNF*MSCs-Exo promoted cell migration after cell scratch (scale bar: 100 μm). (**B**) BDNF*MSCs-Exo significantly increased the viability of PC12 cells after oxidative stress injury (n=5). (**C, D**) BDNF*MSCs-Exo significantly increased SOD activity and decreased ROS levels after oxidative stress injury (n=5). (**E**) Apoptosis of PC12 cells was detected by TUNEL staining after H_2O_2 injury (scale bar: 100 μm). (**F**) The average TUNEL+cell number at high magnification (n=6). * Mean significant difference *vs.* the PBS group (* P<0.05, ** P<0.01). # Mean significant difference *vs.* the MSCs-Exo group (# P<0.05).
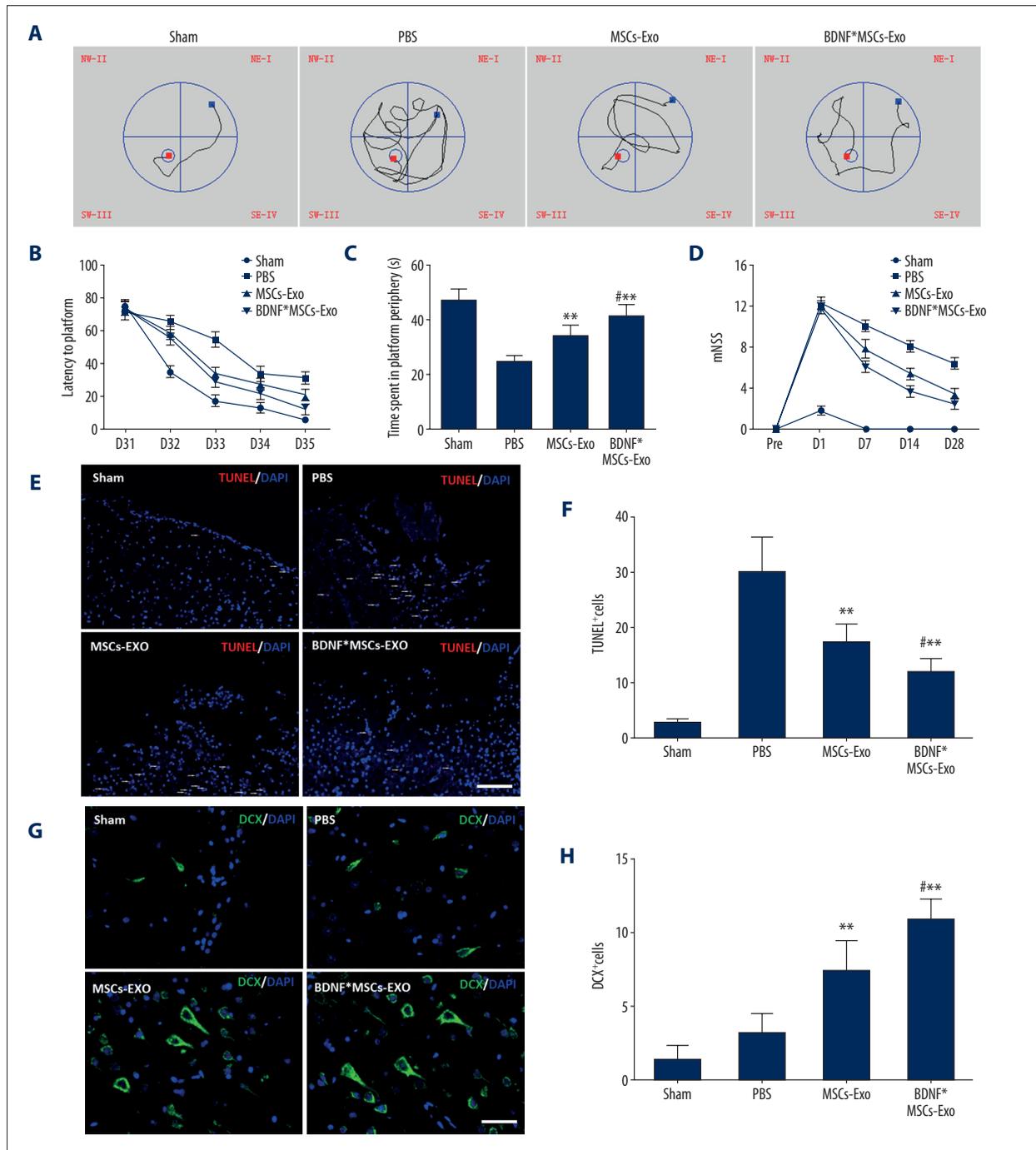
Figure 3. BDNF-induced MSCs-Exo significantly promoted the recovery of sensorimotor function and spatial learning function, and BDNF-induced MSCs-Exo inhibited cell apoptosis and promoted neuron regeneration in rats after TBI. (**A**) The movement path diagram of the rats in the water maze was analyzed by the software after 4 days of training in the water maze. (**B**) BDNF-induced MSCs-Exo significantly improved the spatial learning ability of TBI rats (n=6). (**C**) The time rats swam around the platform after removing the platform (n=6). (**D**) The mNSS test indicated that rats in the BDNF-induced MSCs-Exo group recovered better than those in the MSC-Exo group (n=6). (**E**) The apoptosis of brain cells in the BDNF-induced MSCs-Exo group was significantly less than that in the MSC-Exo group, as detected by TUNEL staining after TBI (scale bar: 200 μm). (**F**) The average TUNEL+cell number at high magnification (n=6). (**G**) The expression of DCX+cells in brain tissues was observed 7 days after TBI in rats (scale bar: 100 μm). (**H**) The average number of DCX+cells at high magnification (n=6). * Mean significant difference *vs.* the PBS group (* P<0.05, ** P<0.01). # Mean significant difference *vs.* the MSCs-Exo group (# P<0.05).
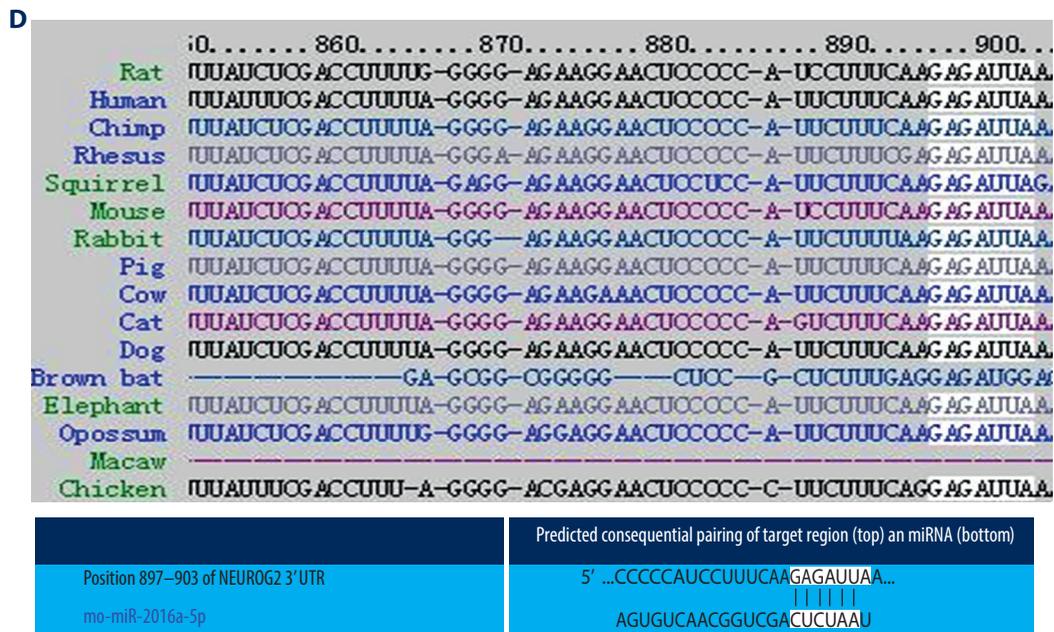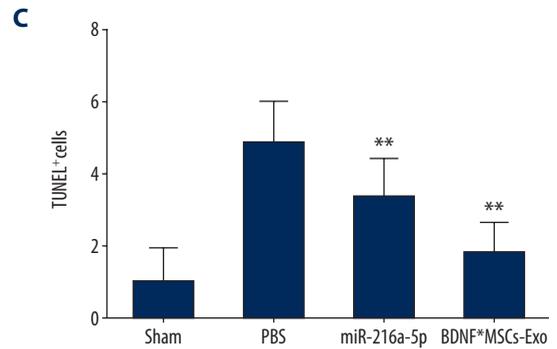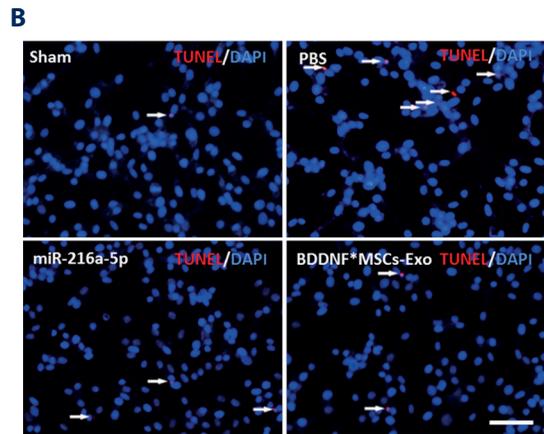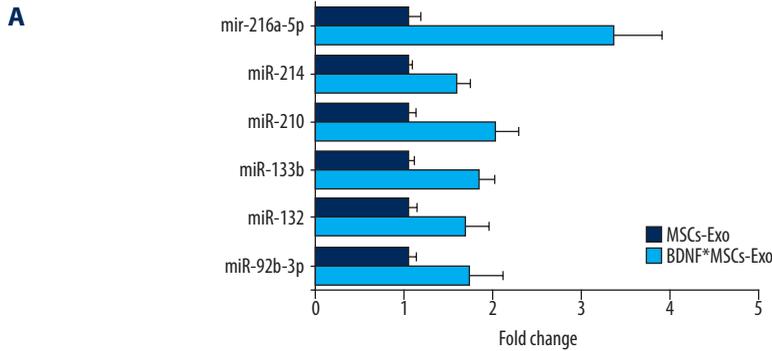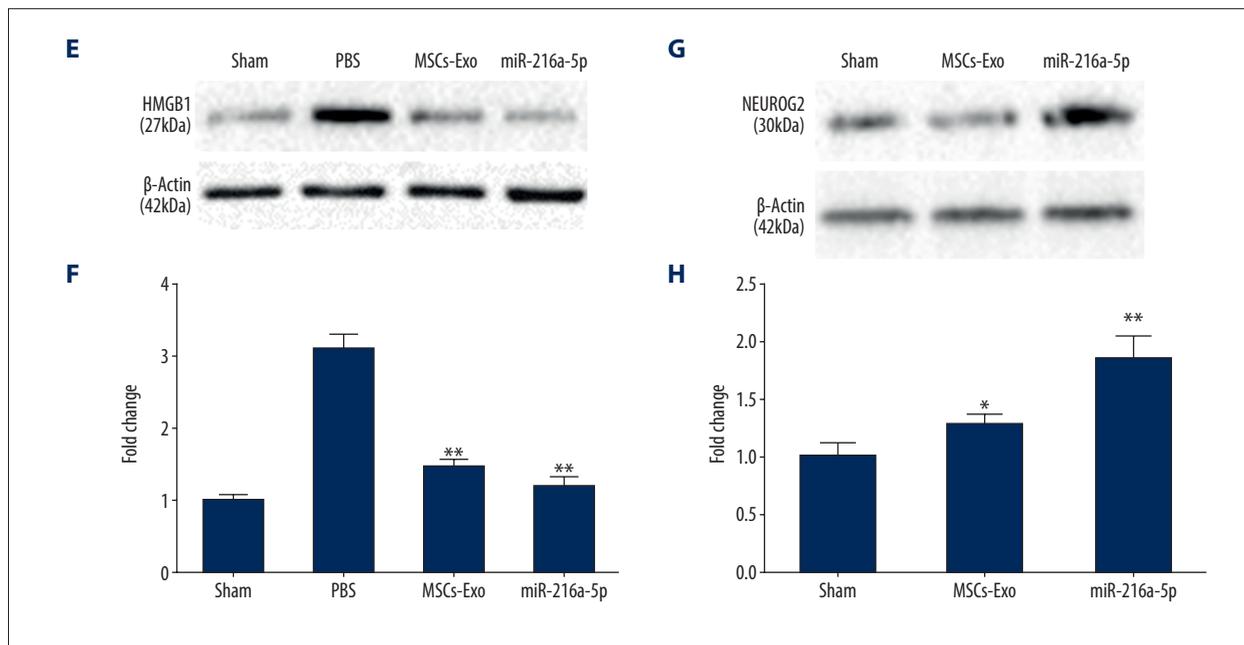
Figure 4. The high expression of miR-216a-5p in BDNF-induced MSCs-Exo inhibited inflammation and promoted neurogenesis. (**A**) Fold changes in miRNA expression within EVs between MSCs-Exo and BDNF-induced MSCs-Exo (n=5 per group). (**B**) Apoptosis of PC12 cells was detected by TUNEL staining after H_2O_2 injury (scale bar: 200 μm). (**C**) The average TUNEL+cell number at high magnification (n=6). (**D**) According to miRBase, NEUROG2 is the downstream target protein of miR-216a-5p. (**E**) Western blot analysis of HMGB1 in PC12 cells. (**F**) Quantitative data of HMGB1 levels were normalized to the relevant β-actin levels (n=4). (**G**) Western blot analysis of NEUROG2 in PC12 neural stem cells. (**H**) Quantitative data of NEUROG2 levels were normalized to the relevant β-actin levels (n=4). * Mean significant difference *vs.* the PBS group (* P<0.05, ** P<0.01). # Mean significant difference *vs.* the MSCs-Exo group (# P<0.05).

MSCs-Exo and BDNF-induced MSCs-Exo could improve spatial learning ability and motor ability in rats after TBI. The BDNF-induced MSCs-Exo were more beneficial to the recovery of spatial learning ability and motor function than MSCs-Exo.

BDNF-induced MSCs-Exo inhibited cell apoptosis and promoted neuron regeneration after TBI in rats

Only a few cells in the sham group were TUNEL-positive in brain tissue sections, while the other 3 groups had a large number of TUNEL-positive cells (Figure 3E). The number of TUNEL-positive cells was calculated by observing 5 random fields under 200× magnification (Figure 3F). DCX is a protein expressed in immature neurons and is often used as a marker of immature neurons. We collected images of DCX-positive cells from the subcortex of the injured side of the brain. The number of DCX-positive cells in the BDNF-mediated MSCs-Exo group was significantly higher than that in the other groups 7 days after TBI (Figure 3G). Similarly, the number of DCX-positive cells was calculated by observing 5 random fields under 200× magnification (Figure 3H). The results showed that BDNF-induced MSCs-Exo were better than MSCs-Exo at inhibiting apoptosis and increasing immature neurons.

The high expression of miR-216a-5p in BDNF-induced MSCs-Exo inhibited apoptosis and increased immature neurons

To verify whether the role of BDNF-induced MSCs-Exo in inhibiting apoptosis and increasing immature neurons is related to miRNA, both MSCs-Exo and BDNF-induced MSCs-Exo microRNAs were assayed for the levels of 22 selected miRNAs scientifically demonstrated to play a role in neuroprotective effects (Supplementary Table 1). According to the detection results, the expression levels of miR-92b-3p, miR-132, miR-133b, miR-210, miR-214, and miR-216a-5p in BDNF-induced MSCs-Exo were higher than those in MSCs-Exo (Figure 4A). The expression of miR-216a-5p in the BDNF-induced MSCs-Exo group was significantly increased (3.32 times that of the MSC-Exo group). Due to the high expression of miR-216a-5p in BDNF-induced MSCs-Exo, we designed experiments to verify whether the role of BDNF-induced MSCs-Exo neurogenesis is related to miR-216a-5p. We transfected pre-miR-216a-5p into PC12 cells to test the repair ability after oxidative stress injury.

HMGB1 is a protein that promotes inflammation, and it is a downstream target protein of miR-216a-5p [25]. miR-216a-5p can protect cells from oxidative stress induced by H_2O_2 by

targeting HMGB1/NF-κB pathways [25]. mir-216a-5p can inhibit apoptosis after oxidative stress induced by H_2O_2 in PC12 cells (Figure 4B, 4C). PC12 cells in each group were extracted for the WB test, and HMGB1 expression in the miR-216a-5p and BDNF-induced MSCs-Exo groups was significantly lower than that in the PBS group (Figure 4E, 4F).

The expression of endogenous neurogenin-2 (NEUROG2) was investigated as a downstream target of miR-216a-5p (Figure 4D). NEUROG2 is a protein that inhibits apoptosis and promotes neurogenesis [26]. The WB test showed that the expression of NEUROG2 in NSCs significantly increased after miR-216a-5p and BDNF-induced MSCs-Exo intervention compared to the control group (Figure 4G, 4H).

Discussion

MSCs-Exo have been shown to have therapeutic benefits in stroke, TBI, and other neurodegenerative diseases [6,27–29]. As stem cells, MSCs are capable of producing large numbers of exosomes. The quality and quantity of exosome production is not compromised by immortalization to create a permanent MSC cell line [30]. Because lipid membranes protect their biological activity, MSCs-Exo reduce the difficulty of keeping cells alive and functioning for storing and delivering them to patients. Exosomes carry proteins and genetic material with biochemical potential to participate in a variety of biochemical and cellular processes, so MSCs-Exo are an ideal and promising drug carrier.

Exosomes transfer proteins and RNA to target cells and then alter the function of recipient cells. When studying the mechanism of exosomes, some scholars have focused on the changes in miRNA in exosomes. miR-124-3p was increased in microglial exosomes after TBI, which inhibited neuronal inflammation and promoted the outward growth of neurites [3]. The exosomes of astrocytes pretreated with oxy-glucose deprivation (OGD) showed high expression of miR-92b-3p and attenuated OGD-induced neuronal death and apoptosis [31]. Treatment of stroke with customized exosomes rich in miR-17-92 clusters can increase neuroplasticity and functional recovery after stroke [32]. MSCs-Exo can regulate the outward growth of neurites by transferring miR-133b to neurons [33]. The mechanism of exosomes is closely related to the miRNA contained in exosomes.

In this study, the data showed that BDNF-induced MSCs-Exo are superior to MSCs-Exo in promoting migration and inhibiting apoptosis. Since exosome secretion is related to the cell environment, we hypothesized that the contents of MSCs-Exo undergo neurogenesis-beneficial changes due to the intervention with BDNF. Because the mechanism of exosomes may be related to miRNAs, we assessed miRNAs contained in BDNF-induced MSCs-Exo and MSCs-Exo by qRT-PCR, showing that the levels of miR-216a-5p were significantly increased in BDNF-induced MSCs-Exo, as determined by qRT-PCR. Previous studies have shown that miR-216a-5p inhibits inflammation in various ways [34,35]. Moreover, we found that pre-miR-216a-5p could inhibit the inflammation of PC12 cells. Pre-miR-216a-5p partially replicated the effects of BDNF-induced MSCs-Exo in promoting migration and inhibiting apoptosis. Therefore, we speculate that the mechanism by which BDNF-mediated MSCs-Exo promotes migration and inhibits apoptosis involves miR-216a-5p.

BDNF is a neuroprotective protein, but it cannot effectively cross the blood-brain barrier due to its large molecular weight, which limits its clinical application. Since the double lipid membrane of exosomes is derived from cells, they can freely cross the blood-brain barrier and can be used as an effective carrier of nervous system drugs. In this study, we first included the substances that BDNF may be beneficial to for the nervous system in exosomes and then used the exosomes in TBI rats to obtain therapeutic effects. At present, no studies have been conducted on the role of exosomes after BDNF intervention in MSCs. Our study provides a novel approach for the clinical application of BDNF and other large molecules that have difficulty crossing the blood-brain barrier.

Although we speculated that the mechanism by which BDNF-induced MSCs-Exo improve cell migration and inhibit inflammation might be related to miR-216a-5p and its downstream target proteins, miR-216a-5p could not completely recapitulate the effects of BDNF-induced MSCs-Exo from the data. Exosomes contain components that are too complex for us to fully analyze their composition and bioactivity. Some other components in exosomes could also have neurogenic and anti-inflammatory effects, and, due to their relatively weak effects and complex types, we did not focus on them.

Conclusions

Our study demonstrated that BDNF-induced MSCs-Exo can improve cell migration and inhibit apoptosis, which was confirmed by *in vivo* and *in vitro* experiments. Although there are no clinical trials to verify this result, it also provides a new direction for the treatment of TBI. This is an alternative to stem cell therapy because exosome therapy avoids the ethical issues, as well as tumorigenic, thrombotic, and other issues associated with cell therapy.

Conflict of interest

None.

Supplementary Data
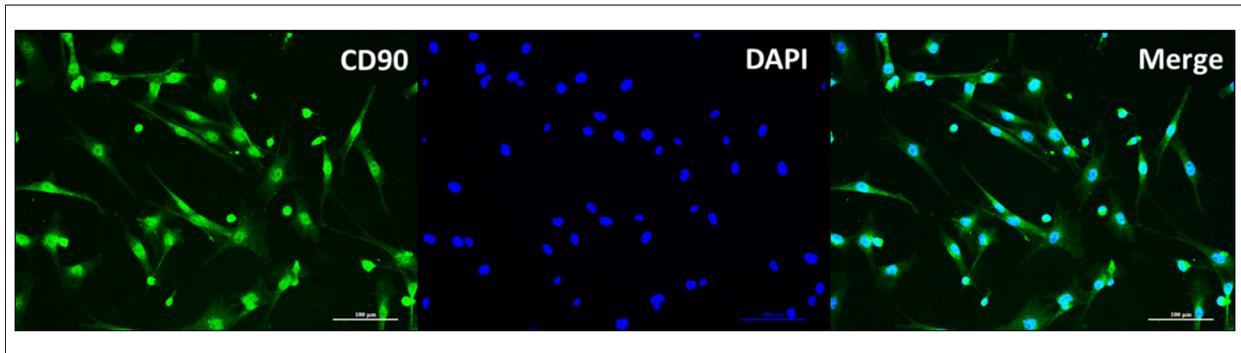
Supplementary Figure 1. Immunofluorescence identification of MSCs. MSCs expressed CD90 positively.

Supplementary Table 1. The selected 22 miRNAs have been scientifically proven to play a role in neuroprotective effects.

miRNA	References
1.miR-137-5p	(DOI: 10.26355/eurrev_201906_18030)
2.miR-184	(DOI: 10.1002/jcp.27158)
3-4.miR-199, miR-214	(DOI: 10.1038/mp.2017.86)
5.miR- 485-3p	(DOI: 10.1111/jcmm.14743)
6.miR-126	(DOI: 10.1016/j.neuroscience.2019.10.043)
7.miR -26a	(DOI: 10.1111/jcmm.14774)
8. miR- 129-5p	(DOI: 10.1096/fj.201801094RR)
9.miR- 24-3p	(DOI: 10.1007/s00018-019-03290-3)
10.miR-133b	(DOI: 10.1007/s12035-018-1005-0)
11.miR- 106b	(DOI: 10.1186/s13287-019-1387-6)
12.miR- 21a	(DOI: 10.1186/s12964-019-0418-3)
13.miR- 153	(DOI: 10.1038/s41418-019-0388-4)
14.miR -135a-5p	(DOI: 10.1016/j.stemcr.2019.04.020)
15.miR- 3099	(DOI: 10.1016/j.gene.2019.02.014)
16.miR-92b-3p	(DOI: 10.1016/j.brainres.2019.04.009)
17.miR- 27b	(DOI: 10.1002/2211-5463.12614)
18.miR-132	(DOI: 10.1002/term.2759)
19.miR-195	(DOI: 10.1016/j.omtm.2018.11.011)
20.miR-210	(DOI: 10.1523/JNEUROSCI.1777-16.2017)
21.miR-17-92	(DOI: 10.1096/fj.201801019R)
22.miR-216a-5p	(DOI: 10.1016/j.lfs.2019.116948)

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