Neural stem cell-derived exosome as a nano-sized carrier for BDNF delivery to a rat model of ischemic stroke

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Abstract
Our previous study demonstrated the potential therapeutic role of human neural stem cell-derived exosomes (hNSC-Exo) in ischemic stroke. Here, we loaded brain-derived neurotrophic factor (BDNF) into exosomes derived from NSCs to construct engineered exosomes (BDNF-hNSC-Exo) and compared their effects with those of hNSC-Exo on ischemic stroke both in vitro and in vivo. In a model of H2O2-induced oxidative stress in NSCs, BDNF-hNSC-Exo markedly enhanced cell survival. In a rat middle cerebral artery occlusion model, BDNF-hNSC-Exo not only inhibited the activation of microglia, but also promoted the differentiation of endogenous NSCs into neurons. These results suggest that BDNF can improve the function of NSC-derived exosomes in the treatment of ischemic stroke. Our research may support the clinical use of other neurotrophic factors for central nervous system diseases.

Key Words: brain-derived neurotrophic factor; exosome; inflammation; ischemic stroke; neural stem cell; neurogenesis; neurological recovery; transplantation

Introduction
Stroke has become a tremendous threat to human health and has placed an enormous burden on health care systems worldwide (Leira et al., 2020). A systematic analysis of the 2019 Global Burden of Disease Study showed a 70.0% increase in the absolute number of incident strokes and a 43.0% increase in stroke deaths worldwide from 1990 to 2019 (Ma et al., 2021). Ischemic stroke accounted for 62.4% of all strokes in 2019 and has remained the largest proportion of all incident strokes. Tissue-type plasminogen activator therapy (Hughes et al., 2020; Marko et al., 2020) remains the only effective drug for stroke and must be administered within 4.5 hours of the onset. However, because of the limited therapeutic window (Goldstein, 2007), only a small percentage of individuals can benefit from tissue-type plasminogen activator therapy (Hughes et al., 2020; Marko et al., 2020). Integrated neuroprotective techniques to minimize ischemic injury remain scarce (Zhang et al., 2016).

Neurotrophic factors are a group of proteins that are vital for the development, growth, survival, and differentiation of the nervous system (Muheremu et al., 2021). Brain-derived neurotrophic factor (BDNF) is the most abundant and widely distributed neurotrophic factor in the nervous system. BDNF promotes cell differentiation, cell growth, synaptogenesis, and neurogenesis in the nervous system by stimulating its tropomyosin receptor kinase B (Hao et al., 2021; Xiong et al., 2021). Importantly, BDNF showed to exhibit numerous neuroprotective properties in post-ischemic and traumatic brain injury (Li et al., 2021). Studies demonstrated that BDNF promotes the proliferation of endogenous neural stem cells (NSCs) and the regeneration of nerve axons in rats with spinal cord injury (Huang et al., 2021). However, exogenous administration of neurotrophic factors for stroke treatment is limited by their poor half-life, lack of blood-brain barrier permeability, and rapid degradation (Yuan et al., 2017; Naqvi et al., 2020).

Exosomes are small extracellular vesicles 30–200 nm in size that are secreted by various cell types and contain a variety of proteins, microRNAs, and other macromolecules (Zhang and Chopp, 2016b; Chen et al., 2020). Compared with conventional carriers (such as liposomes), exosomes offer a number of advantages, including minimal immunogenicity (Fang et al., 2020), minimal toxicity (Fu et al., 2019), biocompatibility (Wu et al., 2021), high circulation stability (Kim et al., 2020), and biological barrier permeability (Feng et al., 2021), making them ideal drug delivery vehicles (Xu et al., 2021). Recent studies have shown that NSCs exert therapeutic effects by releasing exosomes (Vogel et al., 2018). Zhong et al. (2020) found that NSC-derived exosomes (NSC-Exo) significantly promoted angiogenesis, constricted the spinal cavity, and improved motor function in spinal cord injury mice. Another study showed that miR-9, a key regulator of neurogenesis, was highly enriched in NSC-Exo exosomes, and miR-9-enriched NSC-Exo promoted the differentiation of NSCs into neurons and astrocytes (Yuan et al., 2021).

In this study, we loaded BDNF into human NSC (hNSC)-derived exosomes to construct BDNF-hNSC-Exo and investigated the potential application of BDNF-hNSC-Exo in ischemic stroke both in vitro and in vivo.
The differentiation ability of NSCs into neurons was detected by immunofluorescence staining. hNSCs were seeded in 24-well plates at a density of 3 × 10^5 cells/mL medium/well and treated with 500 μM H₂O₂ (MilliporeSigma, Cat# B8597) solution for 5 hours. Cells were treated with PBS, hNSC-Exo, or BDNF-hNSC-Exo (100 μg/mL) for 72 hours and then coated with anti-Tuj1, a marker for neurons; 1:500, Abcam, Cat# ab52623, RRID: AB_869991) dissolved in blocking solution. Following the addition of anti-BDNF monoclonal antibodies (1:400, Abcam, Cat# ab150078, RRID: AB_2722519) and DAPI (Boster, Cat# C1002), the specimens were observed under a fluorescence microscope.

**Cell Counting Kit-8 assay**

The cell viability of hNSCs was determined by Cell Counting Kit-8 (CCK-8) assay (Yasen, Shanghai, China, Cat# 402035650). hNSCs were seeded in 96-well culture plates at a density of 1 × 10^3 cells/100 μL medium/well and treated with a 500 μM solution of H₂O₂ for 5 hours to stimulate the process of cellular oxidative injury, prompting programmed cell death. The samples were then treated with 100 μg/mL PBS, hNSC-Exo, or BDNF-hNSC-Exo (100 μg/mL) at 37°C for 4 hours, 1 day or 2 days. Next, 10 μL of CCK-8 solution was added to each well, and the samples were incubated at 37°C for 4 hours. The absorbance of each well at 450 nm was measured using a multiplate reader (Thermo Fisher Scientific, Cat# M3K).

**Terminal deoxynucleotidyl transferase dUTP nick end labeling staining**

Apoptosis was measured using the CF488 TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) Kit (Servicebio, Cat# G15504). Briefly, hNSCs were fixed with 4% paraformaldehyde (Servicebio, Cat# G15101), washed three times with PBS, covered with 50 μL Equilibration Buffer and incubated at room temperature for 10 minutes, followed by incubation in TdT incubation buffer (Servicebio, Cat# G15014-1) at 37°C for 60 minutes. The hNSCs were then incubated with DAPI for 5 minutes for nuclear staining. Subsequently, the samples were washed with PBS, mounted with TUNEL-positive and TUNEL-negative cells were counted by two independent observers. The TUNEL-positive rate (%) was calculated as: TUNEL-positive cells/total number of cells × 100.

**Quantitative reverse transcription-polymerase chain reaction**

TRIZOL reagent (Thermo Fisher Scientific, Cat# 15599-026) was used to isolate total RNA from hNSCs following the company’s instructions. Total RNA (500 ng) was reverse transcribed to cDNA using the HiScript® 1st Strand cDNA Synthesis Kit (Vazyme, Cat# TM1000-03) in a 50 μL reaction volume using a Thermo Fisher Scientific Eppendorf mastercycler (Vazyme, Cat# Q341-02) in a Roche LightCycler 96 Sequence Detection System (Roche, Basel, Switzerland). The reaction conditions were: reverse transcription at 55°C for 15 minutes, followed by denaturation at 95°C for 30 seconds, followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 30 seconds. The relative expression of mRNA was calculated using the 2-ΔΔCT method and normalized using GAPDH mRNA as an internal control. The primer sequences used are listed in Table 1. The quantitative reverse transcription-polymerase chain reaction primers were designed using Primer Premier 5 software (Premier, Palo Alto, CA, USA). The primers were as follows: Tuj1: forward 5′-GGT CCA AGG TCC TGG TGG T-3′, reverse 5′-TGG TGC ATC GTG GAA GGA-3′, reverse 5′-GGT TGC ATG GTA GGA GGA-3′, reverse 5′-GGT TGC ATG GTA GGA GGA-3′.

**Establishment of transient middle cerebral artery occlusion model rats and exosome treatment**

Healthy adult male Sprague-Dawley rats (n = 60, aged 8 weeks, weight 250–280 g, specific-pathogen-free grade) were purchased from Hangzhou Medical College (Hangzhou, Zhejiang, China, license No. SCXK (Zhe) 2019-007). Before the experiment, all animals were housed under standardized conditions at 20°C ± 2°C and a 12-hour light/dark cycle, with free access to water and food. All animal trials were approved by the Institutional of Animal Care and Use Committee at the Medical School of Southeast University (approval No. SYXK (Su) 2021-0022) on May 5, 2021 and were performed in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) (Pericic et al., 2020).

MIDDLE cerebral artery occlusion (MCAO) surgery was performed as previously described (Zhang et al., 2018). Briefly, rats were anesthetized with 1% pentobarbital sodium (MilliporeSigma, 25 mg/kg). The subcutaneous tissue and muscle were bluntly dissected with vascular forceps, and the common carotid, external, and internal carotid arteries were gently dissected with microtweezers without damaging the vagus nerve. A nylon thread (Cinon, Cat# A9036) was inserted into the right (supine) common carotid artery, and a nylon thread was inserted into the internal carotid artery. The nylon thread was removed after 1.5 hours of ischemia, and the wound was sutured closed. The body temperature of the rats was closely monitored during the operation until the rats were awake. The following 2–3 days were used as successful models, and a Longa score of 2 was used for subsequent experiments.

At 72 hours after MCAO, rats (n = 15/group) were randomly injected with PBS, hNSC-Exo, and BDNF-hNSC-Exo (5 × 10^6 granules in 10 μL PBS/rat) via stereotaxic intraventricular injection. The right striatum of the ischemic hemisphere was then perfused for 5 minutes after sacrifice. The brain was then sectioned and examined with haematoxylin and eosin staining.
Behavioral tests
To test the neuroprotective effects of BDNF-hNSC-Exo in vivo, the MCAO model rats were subjected to behavioral evaluation. Rats underwent three days of behavioral training before MCAO. Tests were performed before MCAO and on days 7, 14, and 28 after transplantation by two research assistants who were blinded to the groups.
For the rotator test, a motorized rotating rod (RWD, Shenzhen, Guangdong, China) was used to test the sensorimotor function of rats. Rats were placed on a rod that accelerated from 4 r/min to 40 r/min in 5 minutes, and the stick time of each rat was recorded.

The postural reflex examination assesses the vulnerability to brain and striatum damage on a scale of 0 to 10. For the postural reflex test, the rat was placed on a smooth surface or suspended in the air, and the rat’s behavior and muscle strength were evaluated. A higher score suggested that the animal had a more significant behavioral problem.

Neurological function was determined using the modified neurological severity score (mNSS) test. Scores ranged from 0 to 18 (normal score, 0; maximum score, 18). An mNSS score of 13–18 indicated severe damage, 7–12 indicated moderate damage, and 1–6 indicated mild damage. A higher score indicates more severe damage.

Immunostaining of brain slices
Rat brains were rapidly removed on the 28th day after transplantation; brain tissues were sliced into six equidistant slices (n = 6 each rat) and stained with a 2% 2,3,5-triphenyltetrazolium chloride (Servicbio, Cat# G0059) and cut into coronal sections of 10–20 μm thickness using a Leica VS1200 (Leica, Hesse, Germany). Sections were washed three times with PBS, incubated with Triton X-100 (MilliporeSigma, Cat# 93443) for 30 minutes and blocked in 10% donkey serum (MilliporeSigma, Cat# D9663) for 1 hour. The sections were incubated overnight at 4°C with diluted primary antibodies: rabbit polyclonal anti-BrdU (1:500, Abcam, Cat# ab152095, RRID: AB_2813002), mouse monoclonal anti-GFAP (1:50, Abcam, Cat# ab4648, RRID: AB_449329), mouse monoclonal anti-Iba-1 (1:500, Proteintech, Cat# 6375-T-1-lg, RRID: AB_2814998), and rabbit polyclonal anti-β-1 (1:300, Proteintech, Cat# 10904-1-AP, RRID: AB_2224573). The next day, the sections were washed three times with PBS and incubated with Alexa Fluor® 488-conjugated anti-mouse IgG (1:300, Abcam, Cat# ab150113, RRID: AB_2576208) or Alexa Fluor® 555-conjugated anti-rabbit IgG (1:400, Abcam, Cat# ab150578, RRID: AB_2722159) at room temperature for 1 hour. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (1:1000, Sigma). The slides were then washed in PBS and coverslipped with anti-fade reagent.

TUNEL staining
The rate of TUNEL-positive cells was evaluated in the normal contralateral hemisphere area, and then the entire infarction area of rats per superposition was computed (Arumugam et al., 2006).

Statistical analysis
All data are expressed as the mean with error bars representing standard deviation (SD). Multiple comparisons were performed using one-way analysis of variance (ANOVA) and Tukey’s post hoc test. Data were calculated by GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA, www.graphpad.com). P < 0.05 was considered statistically significant.

Results
Characterization of hNSCs and hNSC-Exo
hNSCs were in cell suspension and can form large neurospheres when cultured for 5–7 days. We used immunofluorescence to observe free single cells and neurospheres and confirmed that the NSC markers nestin and Sox2 were expressed (Figure 1A). Under induction with differentiation medium, NSCs were observed to differentiate into oligodendrocytes (identified by the marker MOG), neurons (Tuji3), and astrocytes (GFAP).
BDNF protein was encapsulated into neural stem cell-derived exosomes. Pure nanoparticles from the culture supernatant of hNSCs (hNSC-Exo and BDNF-hNSC-Exo) were evaluated by TEM, IFA, and western blotting. TEM revealed that the nanoparticles showed the typical cup-shaped exosome morphology (Figure 1B). NTA indicated that the diameter of the particles ranges from 50 to 150 nm (Figure 1C). Western blotting revealed the expression of typical exosome markers such as CD81, TSG101, and HSP70, whereas the calnexin negative control was not expressed (Figure 1D). Therefore, these isolated vesicles were considered to be hNSC-Exo. Notably, the diameter of exosomes did not change after encapsulating BDNF. These results suggest that BDNF loading does not affect the characteristics of exosomes derived from hNSCs. We also used exosomes for cell tracing in vitro. Panneled labeled exosomes accumulated around the nucleus of target cells, and the entrapment of BDNF did not affect the uptake into target cells (Figure 1E).

BDNF-hNSC-Exo reduces apoptosis in the H2O2 stress model
Oxidative stress is one of the key pathogenic mechanisms of nerve apoptosis and neurological dysfunction in ischemic stroke (Belavie et al., 2017; Lai et al., 2020). We used H2O2 to induce oxidative damage in NSCs. We transfected BDNF-hNSC-Exo into NSCs treated with H2O2, and western blotting confirmed the expression of BDNF in target cells transfected with BDKNF-Exo (Figure 2A).

BDNF-hNSC-Exo treatment improves neurological function after ischemic stroke and reduces infarct volume
We next evaluated the therapeutic effect of BDNF-hNSC-Exo after stroke using a MCAO rat model (Figure 3A). To establish the MCAO model, the common carotid artery and external carotid artery on one side of the rat were ligated, and the suture was inserted into the middle cerebral artery for 90 minutes. BDNF-hNSC-Exo, hNSC-Exo or PBS was injected into the striatal region of cerebral ischemic stroke rats 3 days after stroke (n = 15 animals/group). As expected, compared with the hNSC-Exo and PBS groups, the BDNF-hNSC-Exo injected rats showed increased BDNF expression in the brain tissue at 28 days after treatment (Figure 3B).

We used rotarod, mNSS, and postural reflex tests to evaluate the neurological function of rats with cerebral ischemia before modeling and 28 days after transplantation (Figure 3C–E). The neurological function of the BDNF-hNSC-Exo group and the hNSC-Exo group was markedly improved four weeks after transplantation compared with the PBS control group, and the BDNF-hNSC-Exo group performed better than hNSC-Exo group. In addition, the results of TTC staining showed that the infarct volumes of the BDNF-hNSC-Exo group and the hNSC-Exo group were significantly smaller than that of the PBS control group (Figure 3F).

BDNF-hNSC-Exo treatment promotes tissue repair in the ischemic model rats
The rate of BrdU/Tuj1 and BrdU/GFAP double-positive cells was evaluated in the peri-infarct zone. Immunofluorescence staining showed that there were more BrdU/Tuj1 double-positive cells around the infarct area in the BDNF-hNSC-Exo and hNSC-Exo groups compared with the PBS group, indicating that more functional neurons were produced (Figure 4A). The percentage of BrdU/GFAP double positive cells around the ischemic area was lower in the hNSC-Exo group and the BDNF-hNSC-Exo group than in the PBS group (Figure 4B). To evaluate the inflammatory response of BDNF-hNSC-Exo to the brain after cerebral ischemia, the rate of Iba-1-positive cells near the injury site was evaluated using immunofluorescence. The results confirmed that BDNF-hNSC-Exo significantly inhibited the activation of microglia (Figure 4C).
The features of hNSCs and engineered exosomes (Exo).

BDNF-hNSC-Exo promotes the survival and differentiation of neural stem cells

Discussion

In this study, we found that BDNF-hNSC-Exo attenuated NSCs stress injury and promoted the differentiation of NSCs into neurons. BDNF-hNSC-Exo also inhibited inflammation, thereby creating a suitable immune microenvironment for nerve regeneration.

Although BDNF has emerged as a promising contender for new strategies for stroke treatment, the vast majority of small molecule drugs and biomacromolecules have poor brain bioavailability (Ahmad et al., 2022). Recent studies have shown that exosomes have advantages in their ability to cross biological barriers and their high biocompatibility. Exosomes fuse with the recipient cell membrane to deliver their contents to the recipient cells (Kalluri and LeBleu, 2020). In this study, we used NSC-derived exosomes as delivery vehicles to improve the bioavailability of BDNF. Our results confirmed that BDNF-hNSC-Exo showed standard vesicle shape and diameter, and NTA revealed no difference in the shape and density between hNSC-Exo and TEM revealed no difference in the shape and density between hNSC-Exo and BDNF-hNSC-Exo. In addition, there was no significant difference between BDNF-hNSC-Exo and BDNF-hNSC-Exo uptake by hNSCs. These results confirmed that BDNF loading did not affect the native properties of exosomes and that BDNF could be efficiently internalized into NSCs. The survival rate of NSCs is reduced due to oxidative stress, but if exosomes are added, the survival rate of NSCs increases.

In addition, the expression levels of BDNF, NF-κB, and Tuj1 in hNSC-Exo were lower than in PBS. There was no significant difference in cell viability between cells treated with PBS and hNSC-Exo or BDNF-hNSC-Exo. However, at 4 h, there was a significant difference between hNSC-Exo and BDNF-hNSC-Exo. Therefore, our results confirmed that NSCs showed similar effects in NSC-Exo and BDNF-NHS-Exo.
apoptosis of NSCs and inhibited the expression of apoptosis-related markers caspase-3 and Bax. Most endogenous NSCs undergo apoptosis in response to hypoxia, oxidative stress, and inflammation (Song et al., 2013; Zhang and Chopp, 2016a). We speculate that BDNF-hNSC-Exo promotes the recovery of neurological function after cerebral ischemia, at least in part, by inhibiting the apoptosis of NSCs.

NSCs have been shown to be helpful in the treatment of various neuropathological diseases (Lv et al., 2021; Peruzzotti-Jametti et al., 2021), including stroke (Hamblin et al., 2022). Previous studies have revealed the proliferation and differentiation of NSCs in the ventricle of MCAO model rats (Li and Clevers, 2010). However, the effects of hypoxia on endogenous NSCs leads to inhibition of neuronal differentiation in the process of self-repair, which in turn affects neuronal network remodeling after ischemia (Kriegstein and Alvarez-Buylla, 2009). Our study revealed that more functional neurons in the peri-infarct area were generated in the BDNF-hNSC-Exo and hNSC-Exo groups than in the PBS group. BrdU (red) co-localized with TuJ1 (green). Nuclei were stained by DAPI (blue). Scale bar: 20 μm. n = 5 rats/group.

Figure 3 | BDNF-hNSC-Exo improves neurological deficits and brain damage in rats following cerebral ischemia.

(A) Experimental design. (B) BDNF-hNSC-Exo increases the expression of BDNF in the infarct areas. Data were normalized to GAPDH expression. (C–E) Evaluation of the behavioral function of MCAO rats at 1, 3, 7, 14, and 28 days after BDNF-hNSC-Exo treatment by rotarod, postural reflex, and mNSS tests. (A) Experimental design. (B) BDNF-hNSC-Exo increases the expression of BDNF in the infarct areas. Data were normalized to GAPDH expression. (C–E) Evaluation of the behavioral function of MCAO rats at 1, 3, 7, 14, and 28 days after BDNF-hNSC-Exo treatment by rotarod, postural reflex, and mNSS tests. (A) Experimental design. (B) BDNF-hNSC-Exo increases the expression of BDNF in the infarct areas. Data were normalized to GAPDH expression. (C–E) Evaluation of the behavioral function of MCAO rats at 1, 3, 7, 14, and 28 days after BDNF-hNSC-Exo treatment by rotarod, postural reflex, and mNSS tests.

Figure 4 | BDNF-hNSC-Exo inhibit neuroinflammation and promote neurogenesis in the peri-infarct zone of rats.

(A) Immunofluorescence double-labeling demonstrated that more functional neurons in the peri-infarct area were generated in the BDNF-hNSC-Exo and hNSC-Exo groups than in the PBS group. BrdU (red) co-localized with TuJ1 (green). Nuclei were stained by DAPI (blue). Scale bar: 20 μm. n = 5 rats/group. (B) Double immunofluorescence staining showed that in the BDNF-hNSC-Exo group, the proportion of BrdU/GFAP double-positive cells in the peri-infarct area was lower than that in the hNSC-Exo group and the PBS group on day 28 after treatment. BrdU (red) co-localized with GFAP (green). Nuclei were stained by DAPI (blue). Scale bar: 20 μm. n = 5 rats/group. (C) Immunofluorescence showed that BDNF-hNSC-Exo significantly reduced the expression of Iba1 (red), indicating reduced neuroinflammation. Nuclei were stained by DAPI (blue). Scale bar: 20 μm. n = 5 rats/group. All data are shown as the mean ± SEM. *P < 0.05 (one-way analysis of variance followed by Tukey’s post hoc test). BDNF: Brain-derived neurotrophic factor; BrdU: bromodeoxyuridine; Exo: exosomes; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; hNSC: human neural stem cell; Iba1: induction of brown adipocytes 1; MCAO: middle cerebral artery occlusion; mNSS: modified neurological severity score; PBS: phosphate-buffered saline; TTC: 2,3,5-triphenyltetrazolium chloride.

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(A) Immunofluorescence double-labeling demonstrated that more functional neurons in the peri-infarct area were generated in the BDNF-hNSC-Exo and hNSC-Exo groups than in the PBS group. BrdU (red) co-localized with TuJ1 (green). Nuclei were stained by DAPI (blue). Scale bar: 20 μm. n = 5 rats/group. (B) Double immunofluorescence staining showed that in the BDNF-hNSC-Exo group, the proportion of BrdU/GFAP double-positive cells in the peri-infarct area was lower than that in the hNSC-Exo group and the PBS group on day 28 after treatment. BrdU (red) co-localized with GFAP (green). Nuclei were stained by DAPI (blue). Scale bar: 20 μm. n = 5 rats/group. (C) Immunofluorescence showed that BDNF-hNSC-Exo significantly reduced the expression of Iba1 (red), indicating reduced neuroinflammation. Nuclei were stained by DAPI (blue). Scale bar: 20 μm. n = 5 rats/group. All data are shown as the mean ± SEM. *P < 0.05 (one-way analysis of variance followed by Tukey’s post hoc test). BDNF: Brain-derived neurotrophic factor; BrdU: bromodeoxyuridine; Exo: exosomes; GFAP: glial fibrillary acidic protein; hNSC: human neural stem cell; Iba1: induction of brown adipocytes 1; MCAO: middle cerebral artery occlusion; PBS: phosphate-buffered saline; TuJ1: β-tubulin.

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