# Study on the Therapeutic Effects and Mechanisms of Human Mesenchymal Stem Cell-Derived Exosomes Carrying *NGF* Gene in Treating Ischemic Stroke in Rats

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**Abstract:** *Objective:* To investigate the therapeutic effects and mechanisms of human mesenchymal stem cell-derived exosomes (hMSCs-Exo) carrying the *NGF* gene in treating ischemic stroke in rats, aiming to provide new insights and treatment methods for ischemic stroke therapy. *Methods:* After successful construction of the cerebral ischemia model in 40 male SPF-grade SD rats aged 6–8 weeks, the model rats were randomly divided into 4 groups: Sham group, PBS group, hMSCs-Exo group, and NGF-hMSCs-Exo group, with 10 rats in each group. The rat MCAO model was prepared using the classic filament method, and NGF-hMSCs-Exo were injected via the tail vein into the MCAO model rats. The expression of the *NGF* gene in brain ischemic tissues, neuronal regeneration, and rat neurological function recovery were observed using TTC staining, memory function evaluation, Western blot, qRT-PCR, and other methods. *Results:* Compared with the Sham group, neurological deficits were significant in the PBS group ($P < 0.01$). Compared with the PBS group, neurological scores improved in the hMSCs-Exo group and NGF-hMSCs-Exo group ($P < 0.05$). Compared with the hMSCs-Exo group, the improvement in neurological deficits was more significant in the NGF-hMSCs-Exo group ($P < 0.05$). The infarct area after NGF-hMSCs-Exo intervention was significantly reduced ($P < 0.05$) compared with the Sham group. Compared with the PBS group, relative expression levels of NGF mRNA and protein decreased, while Caspase-3 mRNA and protein expression significantly increased in the PBS group ($P < 0.01$). Compared with the PBS group and hMSCs-Exo group, there were differences in NGF and Caspase-3 mRNA and protein expression in the NGF-hMSCs-Exo group rat brain tissues ($P < 0.05$). *Conclusion:* Treatment with human mesenchymal stem cell-derived exosomes carrying the NGF gene improves cognitive function and exerts protective effects on SD rats while inhibiting apoptotic levels in cells.

**Keywords:** *NGF* gene; Human mesenchymal stem cell-derived exosomes; Ischemic stroke in rats; Mechanism of action

## 1. Introduction

Ischemic stroke leads to rapid necrosis of brain tissue, with limited clinical treatment efficacy and methods. An ideal treatment method aims to repair damaged brain tissue early and restore the neurovascular network [1]. Due

to their low immunogenicity and ability to penetrate the blood-brain barrier, exosomes are considered potential resources for repairing injured brain tissue [2,3]. In this study, focusing on the NGF gene, we developed an efficient and safe NGF-hMSCs-Exo nanodrug system and injected it into MCAO model rats. Through TTC staining, memory function tests, Western blot, qRT-PCR, and other techniques, we monitored NGF gene expression, neuronal regeneration, and neurological function recovery to assess the potential of NGF therapy for ischemic stroke and explore new approaches for treating ischemic stroke [2,3].

## 2. Materials and methods

### 2.1. Identification of exosomes from human-derived mesenchymal stem cell sources

Human bone marrow mesenchymal stem cells were purchased from the Shanghai Cell Bank. Exosomes were extracted and identified from these cells: the supernatant from hMSC cultures was collected and subjected to ultracentrifugation or an exosome extraction kit as per the manufacturer's instructions. After collection, the cell supernatant was centrifuged at 300 g and 2,000 g for 10 minutes each to remove cells and debris, then filtered through a 0.22 μm filter to eliminate any residual debris or impurities. For ultracentrifugation, the supernatant was centrifuged at 100,000 g (Beckman) at 4°C for 70 minutes, resuspended in 1 mL of pre-cooled PBS, and centrifuged again at 100,000g at 4°C for 70 minutes. The exosomes were finally collected in 100 μL of PBS. The exosome extraction and purification kit was used according to the operational steps, and the exosomes were collected in 100 μL of PBS.

### 2.2. Construction and characterization study of the NGF-hMSCs-Exo complex

To construct the NGF-hMSCs-Exo complex, 50–100 μg of hMSCs-Exo was co-incubated with 10 μg of NGF expression plasmid for 4 hours. The NGF expression plasmid, hMSCs-Exo, and electroporation buffer (250 μL) were then mixed in an electroporation cuvette. Electroporation was performed at 400 V and 350 μF. After electroporation, the exosomes were incubated at 37°C for 30 minutes. If necessary, sucrose density gradient centrifugation (15%–60%) was used to separate excess free NGF from the exosomes.

### 2.3. Construction of the rat MCAO model and grouping

The rat MCAO model was constructed using the classical suture method. Rats were first anesthetized with intraperitoneal injection of chloral hydrate, followed by a midline neck incision to expose and ligate the right common carotid artery and external carotid artery. The common carotid artery was ligated near its bifurcation, leaving a suture in place at the distal end. A miniature artery clamp was used to occlude the distal common carotid artery, and a small incision was made at the proximal end. A monofilament nylon suture was inserted through the incision into the origin of the middle cerebral artery, with an insertion depth of approximately 1.8 cm to block the blood supply to the middle cerebral artery for 90 minutes. All procedures were performed at room temperature, and rectal temperature was monitored. After model construction, neurological function was evaluated in awake rats using the Zea-Longa score, which ranges from 0 to 4. Scores of 1, 2, or 3 were considered successful model construction.

Forty male SPF-grade SD rats aged 6–8 weeks were successfully modeled for cerebral ischemia and then randomly divided into four groups: Sham, PBS, hMSCs-Exo, and NGF-hMSCs-Exo, with 10 rats in each group. Twenty-four hours later, approximately $10^{11}$ labeled exosomes were injected into the rats via the tail vein. An intraperitoneal injection regimen was implemented over three days before the rats' euthanasia. This included 5-bromo-2'-deoxyuridine (BrdU), administered at a standard dose of 50 mg/kg body weight, and given twice daily.

## 2.4. Neurological function deficit scoring

Neurological function deficits were assessed using the modified neurological severity score (mNSS) and rotarod test or grid walking test to evaluate the motor, sensory, reflex, and balance functions of ischemic rats. These tests were conducted at multiple time points: 1 day, 3 days, 7 days, 14 days, and 28 days post-modeling, to assess the improvement in neurological function following exosome transplantation treatment.

## 2.5. TTC staining for infarct volume percentage

TTC staining was used to determine changes in the infarct area, expressed as the infarct volume percentage. The infarct volume percentage was calculated as (infarct volume / total brain volume) × 100%.

## 2.6. Quantitative PCR (RT-PCR) for NGF and Caspase-3 mRNA expression

For the experiments, SD rats treated for one month were euthanized humanely using cervical dislocation, and brain tissue samples were quickly collected. Total RNA was extracted using Trizol reagent, and RT-PCR was performed according to the fluorescence quantitative PCR kit's guidelines. The relative expression levels of the target gene mRNA were quantified using the $2^{-\Delta\Delta CT}$ method.

## 2.7. Western blot for NGF and Caspase-3 expression

Fresh brain tissue from the rats was added to 1 mL RIPA cell lysis buffer and incubated on ice for 45 minutes. The lysate was then transferred to a cooled PE tube and centrifuged at 12,000 rpm for 15 minutes at 4°C using a pre-cooled high-speed centrifuge to obtain total protein. A 10% separation gel and 4% stacking gel were prepared, with 50 ng of protein loaded per lane. The gel was submerged in 1× SDS loading buffer and electrophoresed at 40 V for 2 hours. The proteins were then transferred to a nitrocellulose membrane. After washing with TBST, the membrane was incubated overnight with primary antibodies for NGF and apoptosis-related factor Caspase-3. The membrane was then washed with TBST and incubated at room temperature with a mouse anti-goat HRP secondary antibody for 30 minutes. β-actin served as a loading control, and DAB was used for detection. Protein bands were analyzed using ImageJ software.

## 2.8. Statistical analysis

Statistical analyses were performed using SPSS 26.0 software. Results are presented as the mean ± standard deviation (SD). Comparisons of measurement data were made using one-way analysis of variance (ANOVA) (F-test), and comparisons between groups were made using the *t*-test. A *P*-value of < 0.05 was considered statistically significant.

# 3. Results

## 3.1. Comparison of neurological function deficits in each group of rats

Rats in the Sham group showed no abnormal symptoms. Compared to the Sham group, the PBS group exhibited motor impairments such as the inability to fully extend the contralateral forepaw or turn to the opposite side, with significant neurological deficits ($P < 0.01$). Compared to the PBS group, the hMSCs-Exo group and NGF-hMSCs-Exo group showed improvements in neurological function scores ($P < 0.05$), with the NGF-hMSCs-Exo group showing more significant improvements compared to the hMSCs-Exo group ($P < 0.05$). See **Figure 1**.
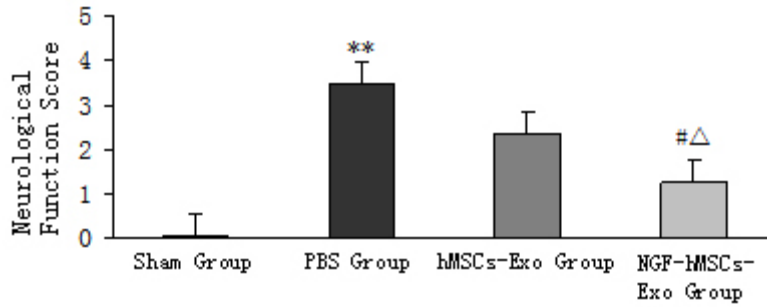
**Figure 1.** Effect of NGF-hMSCs-Exo injection on neurological function deficits. Compared to the Sham group, \*\*$P < 0.01$; compared to the PBS group, [#]$P < 0.05$; compared to the hMSCs-Exo group, [Δ]$P <0.05$

## 3.2. Comparison of infarct area changes in each group of rats

MCAO modeling resulted in a 43.3% infarct area in the ipsilateral hemisphere of the rats, indicating successful preparation of the cerebral ischemia model. Compared to the PBS group, the hMSCs-Exo group and NGF-hMSCs-Exo group had significantly reduced infarct areas ($P < 0.01$), with the NGF-hMSCs-Exo group showing a more significant reduction in infarct area compared to the hMSCs-Exo group ($P < 0.05$). See **Figure 2**.
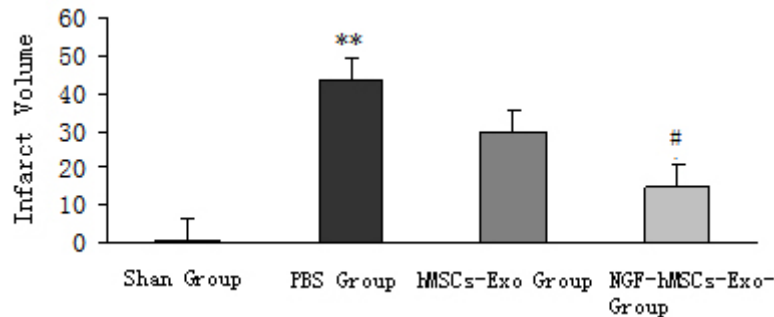


**Figure 2.** Effect of NGF-hMSCs-Exo injection on infarct area in rats. Compared to the Sham group, \*\*$P < 0.01$; compared to the PBS group, [#]$P < 0.05$; compared to the hMSCs-Exo group, [Δ]$P <0.05$

## 3.3. Expression of NGF and apoptosis-related factor Caspase-3 mRNA in each group of rats

Using RT-PCR, it was observed that compared to the Sham group, the PBS group had decreased relative expression levels of NGF mRNA and significantly increased relative expression levels of Caspase-3 mRNA ($P < 0.01$). Compared to the PBS group, the NGF-hMSCs-Exo group had significantly increased NGF mRNA relative expression levels and significantly decreased Caspase-3 mRNA relative expression levels ($P < 0.05$). See **Table 1**.

**Table 1.** Comparison of relative expression levels of NGF and apoptosis-related factor Caspase-3 mRNA in each group of rats

| Group | n | NGF mRNA expression | Caspase-3 mRNA expression |
|---|---|---|---|
| Sham group | 10 | 1.00 ± 0.01 | 1.00 ± 0.01 |
| PBS group | 10 | 0.82 ± 0.02 | 2.14 ± 0.03 |
| hMSCs-Exo group | 10 | 2.07 ± 0.03 | 0.78 ± 0.02 |
| NGF-hMSCs-Exo group | 10 | 2.95 ± 0.04 | 0.43 ± 0.01 |
| F-value | | 22.185 | 14.756 |
| P-value | | 0.000 | 0.000 |

## 3.4. Western blot analysis of NGF and apoptosis-related factor Caspase-3 protein expression levels

Western blot analysis showed that compared to the Sham group, the PBS group had decreased NGF protein expression levels and significantly increased Caspase-3 protein expression levels ($P < 0.01$). Compared to the PBS group, the NGF-hMSCs-Exo group had significantly increased NGF protein expression levels and significantly decreased Caspase-3 protein expression levels ($P < 0.05$). See **Figures 3** and **4**.
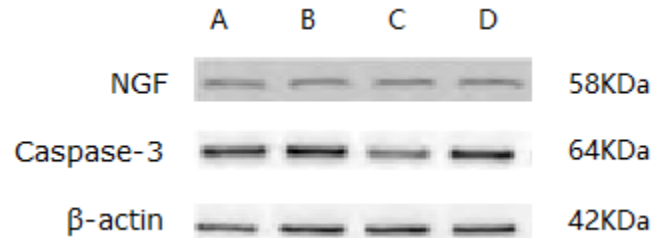


**Figure 3.** Western blot analysis of NGF and Caspase-3 protein expression. Note: A, Sham group; B, PBS group; C, hMSCs-Exo group; D, NGF-hMSCs-Exo group
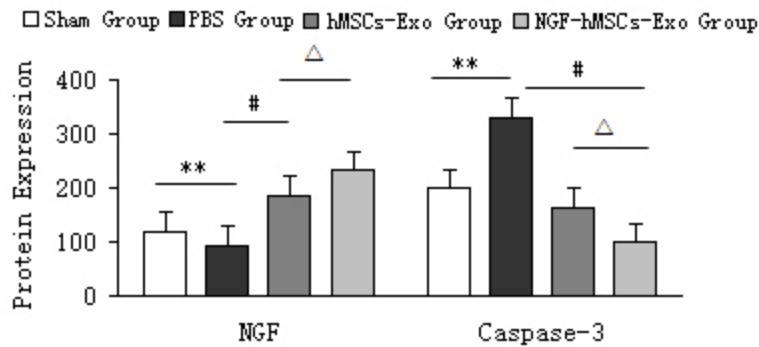


**Figure 4.** Protein expression levels of NGF and Caspase-3 in rat brain tissue for each group. Compared to the Sham group, **$P < 0.01$; compared to the PBS group, #$P < 0.05$; compared to the hMSCs-Exo group, Δ$P < 0.05$

## 4. Discussion

Ischemic stroke, commonly known as a stroke, along with coronary heart disease and cancer, ranks among the three major diseases affecting human health. Mesenchymal stem cells (MSCs), important members of the stem cell family, are multipotent stem cells that have garnered increasing attention due to their multi-lineage differentiation potential, immune modulation, and self-renewal capabilities [4,5]. Recent studies confirm that paracrine effects are the primary mechanism through which stem cells exert their effects after transplantation [6,7]. It is currently known that the survival efficiency of stem cells in vivo post-transplantation is very low, possibly less than 5% [8]. However, extracellular vesicles secreted by the cells, which mediate paracrine effects, are considered to be the main functional molecules, carrying the effective components and functional attributes of their source cells, and can be developed into a novel therapeutic method for clinical regenerative medicine [9]. Nerve growth factor (NGF) is a neurocellular growth regulator with dual biological functions of neuronal nourishment and axonal growth promotion. It plays a crucial neuroprotective role post-stroke by promoting nerve regeneration, axonal reconstruction, and long-lasting neurotrophic effects, thereby facilitating neurological recovery [10].

In the experimental results of this study, we observed that rats in the Sham group did not exhibit any abnormal behaviors or physiological symptoms. In stark contrast, rats in the PBS group exhibited significant

motor dysfunction, manifested as an inability to fully extend the contralateral forepaw and an unstable state of the body turning to the opposite side, indicating notable neurological deficits. Compared to the PBS group, the NGF-hMSCs-Exo group showed improved neurological function scores. MCAO modeling resulted in a 43.3% infarct area in the ipsilateral hemisphere of the rats, indicating successful preparation of the cerebral ischemia model. Compared to the PBS group, the hMSCs-Exo and NGF-hMSCs-Exo groups had significantly reduced infarct areas, with the NGF-hMSCs-Exo group showing a more substantial reduction in infarct area compared to the hMSCs-Exo group. This indicates that treatment with NGF gene-carrying human mesenchymal stem cell-derived exosomes can improve neurological function and reduce infarct area. Compared to the Sham group, the PBS group had decreased relative expression levels of NGF mRNA and protein, with significantly increased expression levels of Caspase-3 mRNA and protein. Compared to the PBS and hMSCs-Exo groups, the NGF-hMSCs-Exo group showed statistically significant differences in the expression of NGF and Caspase-3 mRNA and protein in rat brain tissue. This suggests that treatment with NGF gene-carrying human mesenchymal stem cell-derived exosomes has a protective effect on cognitive function in SD rats while inhibiting the level of apoptosis.

Therefore, by analyzing the effects and molecular mechanisms of NGF-hMSCs-Exo in treating ischemic stroke through neuroprotection and neurovascular regeneration, as well as assessing the potential of exosomes as endogenous carriers for treating ischemic stroke, this study provides a solid scientific basis for exploring clinical treatments for ischemic stroke.

## Funding

## Disclosure statement

The authors declare no conflict of interest.

## References

[1]  You T, Tan W, 2020, The Effect of Tanreqing Injection on Motor Function and Nerve Growth Factor Content in Acute Stroke Complicated with Pulmonary Infection. Chinese Journal of Traditional Chinese Medicine, 38(8): 187–190.

[2]  Chen N, Fan F, Li S, et al., 2022, The Role and Mechanism of Traditional Chinese Medicine in Regulating Mesenchymal Stem Cell-Derived Exosomes for the Treatment of Ischemic Stroke. Chinese Journal of Tissue Engineering Research, 26(13): 2081–2086.

[3]  Zhou G, Bai W, Shao J, 2023, Research Progress on the Effects of Exosomes and Their Carried microRNAs on Brain Ischemia-Reperfusion Injury. Journal of Chongqing Medical University, 48(7): 840–847.

[4]  Wang Y, Lai X, Wu D, et al., 2021, Umbilical Mesenchymal Stem Cell-Derived Exosomes Facilitate Spinal Cord Functional Recovery Through the miR-199a-3p/145-5p-Mediated NGF/TrkA Signaling Pathway in Rats. Stem Cell Res Ther, 12(1): 117. https://doi.org/10.1186/s13287-021-02148-5

[5]  Hao L, Lu L, Li Q, et al., 2024, The Effect of Bone Marrow Mesenchymal Stem Cell-Derived Exosomes Overexpressing miR-124-1 Gene on the M2 Polarization of Microglia in Stroke. Journal of Army Medical University, 46(5): 458–466.

[6]  Liu A, Chen Y, Zhang J, et al., 2021, Correlation Between Prognosis of Acute Ischemic Stroke and Serum Fibroblast Growth Factor 23 and Chemokine CXCL12. Chinese Journal of Gerontology, 41(20): 4350–4352.

[7]    He J, Chen L, Tang F, et al., 2023, Multidisciplinary Team Collaboration Impact on NGF, BDNF, Serum IGF-1, and Life Quality in Patients with Hemiplegia after Stroke. Cell Mol Biol (Noisy-Le-Grand), 69(12): 57–64. https://doi.org/10.14715/cmb/2023.69.12.10

[8]    Chen S, Zhu J, Zhao X, et al., 2023, Effects of Neural Stem Cell-Derived Exosomes on Astrocyte TGF-β1 Signaling and Inflammatory Factors in Rats with Cerebral Ischemia-Reperfusion Injury. Practical Journal of Medicine, 39(13): 1600–1605.

[9]    Tani A, Sakakima H, Otsuka S, et al., 2023, Stimulation of Functional Recovery Via Neurorepair Mechanisms by the Traditional Japanese Kampo Medicine, Ninjin'yoeito, and Physical Exercise in A Rat Ischemic Stroke Model. J Ethnopharmacol, 302(Pt B): 115927. https://doi.org/10.1016/j.jep.2022.115927

[10]   Povarnina PY, Antipova TA, Gudasheva TA, et al., 2022, Neuroprotective and Neuroregenerative Properties of Dimeric Dipeptide Mimetics of Individual NGF and BDNF Loops Under Conditions of an Experimental Ischemic Stroke Model. Pharm Chem J, 56: 1019–1023. https://doi.org/10.1007/s11094-022-02745-5