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Neural stem cell transplantation improves neurological function in focal cerebral ischemia through GDNF/PI3K/AKT axis

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ABSTRACT

The aim of this experiment was to analyze the ameliorating effect of neural stem cells (NSCs) on focal cerebral ischemia (FCI) through GDNF/PI3K/AKT axis, so as to provide evidence for future clinical application of NSCs. In this study, the 15 Sprague-Dawley (SD) male rats were modeled for middle cerebral artery occlusion (MCAO)-induced FCI and then grouped: NSCs group was treated with NSC transplantation, GDNF/NSCs group was transplanted with recombinant adenovirus pAdEasy-1-pAdTrackCMV-GDNF-transfedcted NSCs, and the blank group was treated with normal saline transplantation. Rats were tested by rotarod and corner turn tests at 1 week and 4 weeks after NSC transplantation, and the levels of tumor necrosis factor-α (TNF-α), interleukin-6/8 (IL-6/8), superoxide dismutase (SOD) and malondialdehyde (MDA) were quantified. Then all rats were killed and their brain tissues were HE stained for the determination of and GDNF/PI3K/AKT axis-associated protein expression. The results of the experiment showed that: at the 1st and 4th week after transplantation, the time on the rod, number of turnings and SOD were the lowest in the blank group among the three groups, while IL-6, IL-8, TNF-α and MDA were the highest (P<0.05). Increased time on the rod, number of turnings and SOD, as well as decreased IL-6, IL-8, TNF-α and MDA were observed in NSCs and GDNF/NSCs groups after transplantation, with better performance in GDNF/NSCs group (P<0.05). Based on HE staining of brain tissue, GDNF/NSCs group had the most significant improvement in tissue injury and the highest GDNF, PI3K, AKT and p-AKT protein expression among the three groups (P<0.05). In conclusions, NSC transplantation can ameliorate neurological function in MCAO-induced FCI rats through the GDNF/PI3K/AKT axis.

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Introduction

As the incidence of cerebral ischemia (CI) constantly increases, it has become one of the major diseases of human mortality and disability, with more than 600,000 new cases worldwide every year (1). According to statistics, CI diseases have become the third cause of unnatural death of human beings, second only to malignancies and cardiovascular diseases (2). FCI results from a series of complex pathophysiological events, including excitotoxicity, oxidative stress, inflammation, and apoptosis (2). However, there is currently no optimal clinical treatment for FCI, as the cellular and molecular changes in such a disease are not fully understood, which is also one of the reasons leading to neuronal apoptosis. Currently, thrombolytic therapy remains the mainstay of clinical treatment for CI. Although timely treatment can effectively relieve patients' symptoms and ensure their life safety, it still cannot reverse the neuronal damage and apoptosis caused by CI, resulting in a greater possibility of disability in most patients (3, 4). Thus, finding a more valid treatment plan for cerebral ischemic diseases is of great significance to the life and prognosis of patients.

As the technology of cultivating neural stem cells (NSCs) in vitro gradually matures, accumulating evidence has found that exogenous NSCs transplanted into rat brains can save dying neurons and integrate with host cells to form a good neural network, which provides hope for the treatment of cerebral ischemic diseases (5). The endogenous nervous system consists of intestinal neurons and intestinal glial cells, of which the latter can maintain the integrity of the enteric nervous system and secrete glial cell-derived neurotrophic factor (GDNF), a nutritional factor that promotes neuronal growth, differentiation, and repair (6). The PI3K/AKT axis involved in GDNF has also been shown to work in the maintenance and differentiation of NSCs in the developing brain and contributes to the recovery of neurological function after CI injury (7, 8). However, it remains to characterize whether NSC transplantation plays a role in treating ischemic brain injury through the GDNF/PI3K/AKT axis.

Therefore, it is of great significance to study the molecular mechanism of FCI in order to formulate effective treatment plans for improving patient outcomes. In this study, by establishing a rat model of middle cerebral artery occlusion (MCAO)-induced focal cerebral ischemia (FCI) and conducting NSC transplantation, this study explores whether NSC transplantation is neuroprotective in CI rats

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and observes changes in GDNF/PI3K/AKT axis expression, in the hope of rendering more reliable theoretical guidance for the future application of stem cell transplantation therapy.

Materials and Methods

Animal data

Fifteen clean-grade healthy male Sprague-Dawley (SD) rats (weight: 260-300 g), ordered from Suzhou Truway Biotechnology Inc. with the animal license number SYXK (Su) 2022-0032, were fed in the environment of 23-26°C, 50-60% humidity and 12h-12h day-night alternating cycle with feed and water freely available.

Model building

Referring to the study of Yu et al. (9), a model of left MCAO-induced FCI was established: Rats who had been adaptively fed for one week were given an intraabdominal injection of 10% chloral hydrate (3 mL/kg) for anesthesia and then fixed supine to create a longitudinal incision in the middle of the posterior neck. The muscular nerves were then bluntly separated layer by layer, exposing the left common (CCA), internal (ICA), and external carotid arteries (ECA). After that, a diagonal incision was made in the ECA, through which a suitable thread plug was inserted and passed through the ICA to the middle cerebral artery. The thread plug was inserted 1.8~2.0cm away from the CCA bifurcation and pulled out after 2 hours of ischemia to restore the filling of the ICA. Animals were scored for their neurological function (10) after recovery from anesthesia: 0: normal with no obvious behavior changes; 1: inability to fully extend the right forelimb; 2 points: turn slightly to the right side when walking; 3 points: difficulty in walking and tipping to the right side; 4 points: no spontaneous activity and loss of consciousness. Rats with a score of 0 or 4 were considered modeling failures and were removed from the study.

Isolation and culture of NSCs

Neonatal SD rats were killed by cervical dislocation under anesthesia. The collected whole brain tissue was rinsed in D-Hank's solution, the meninges were peeled off, and the brain tissues were cut into pieces for culture with a small amount of DMEM/F12 basal medium. The cell suspension was formed after mechanical pipetting. Cells were then collected by centrifugation and dripped into DMEM/F12 culture medium containing B27, bFGF, EGF and streptomycin. After filtration, they were inoculated in a 25cm^2 culture flask at 1.0×10^9/L and incubated at a constant temperature. The medium was added once every 2 ~ 3 days, and the passage was carried out once every 5 ~ 6 days by mechanical pipetting or Accutase digestion.

Grouping and treatment

Rats were randomized into the following three groups for corresponding treatment: (1) NSCs group was treated with NSC transplantation. NSCs were labeled continuously with 10 μmol/L BrdU for 3 days, and the cell concentration was adjusted to 2.5×10^7 cells/ml. 20 μL cell suspension (number of transplanted cells: 5×10^5) was used for transplantation. (2) GDNF/NSCs group was subjected to transplantation of NSCs transfected with recombinant adenovirus pAdEasy-1-pAdTrackCMV-GDNF. According to the rat GDNF cDNA sequence, a pair of specific primers were designed and synthesized as follows: sense: 5'-TTTGGTACCATGAAGTTATGGGATGTCGT-3', anti-sense: 5'-TTTAAGCTTTCAGATACATCCACATCCACACCGTT-3'. Before transplantation, 2 wells of cells were collected and the supernatant was discarded by centrifugation, followed by the addition of 20 μL culture medium. The number of transplanted cells was $4\sim5\times10^5$. (3) Blank group was treated with normal saline transplantation. Intracerebral transplantation was performed using a rat brain stereotaxic instrument. The transplantation time was 3 days after reperfusion, and the transplantation site was the right ventricle.

Behavior testing

Rat behavior tests were performed at 1 and 4 weeks after NSC transplantation, including the rotarod test (the initial speed was 4 RPM/min, which was accelerated to the maximum rotation speed of 40 RPM/min within 5 minutes. The test ended when the rat fell from the rotating rod, clung to the rotating rod for 2 consecutive rotations without any movement, and/or the movement time of the rat on the rotating rod reached 300s. The number of rod turns was recorded) and the corner turn test (two boards, 30cm×20cm×1cm, was used to form a 30° angle, and the rats were placed in the corner of the edge. When the rats started to move, the board was turned, and the turning times were recorded 10 consecutive times. The turning times of the rats were recorded).

ELISA

Carotid artery blood samples were collected at 1 and 4 weeks after NSC transplantation to quantify inflammatory factors (IFs) such as tumor necrosis factor-α (TNF-α) and interleukin-6/8 (IL-6/8) and stress injury markers like superoxide dismutase (SOD) and malondialdehyde (MDA), using kits all ordered from Wuhan Fine Biotech.

HE staining

Rats were sacrificed with the neck broken under anesthesia to obtain complete brain tissue, which was preserved in 4% paraformaldehyde and prepared into paraffin sections after dehydration with gradient ethanol. After baking at 60°C for 2 h, the paraffin slices were subjected to xylene dewaxing, HE dyeing, dehydration with ethanol gradient, and xylene permeation. Following air drying and mounting, the slices were observed and photographed under a light microscope.

Western blotting

RIPA was added to the rat brain tissue for lysis, and then the protein was quantified by BCA. After SDS-PVDF, the protein was sealed with 5% skim milk. GDNF, PI3K, AKT and p-AKT primary antibodies (1: 500) were then added to incubate at 4°C overnight. Following three TBST washes the next day, the membrane was immersed in a secondary antibody (1: 3000) to incubate for 1 hour. After ECL development, the relative expression was calculated with GAPDH as the reference protein.

Statistical analyses

Data were input into SPSS19.0 for statistical analysis. All the tests were run in triplicate, and the results were described as ($\chi\pm s$). One-way analysis of variance (ANO-

VA) was used for comparisons. LSD-t tests were used for data that met the normality and variance homogeneity, and nonparametric tests were used otherwise. $P<0.05$ was the significance level.

Results

Modeling results

All the rats were successfully modeled without developing peritonitis. In the blank group, the nerve defect score of one rat was 4 points and was therefore excluded. Before NSC transplantation, the nerve defect scores of blank, NSCs, and GDNF/NSCs groups were (2.75±0.50), (2.80±0.45) and (2.80±0.45) respectively, showing no statistical difference among them ($P>0.05$). Figure 1

Rat behavior changes

One week after transplantation, the time on the rod and number of turnings of rats in the blank group was the lowest in the three groups ($P<0.05$), but no difference was identified between NSCs and GDNF/NSCs groups ($P>0.05$). In the fourth week, the time on the rod in NSCs and GDNF/NSCs groups increased ($P<0.05$), but the number of turnings remained unchanged ($P>0.05$); while in the blank group, the time on the rod and number of turnings were further reduced ($P<0.05$). The comparison among the three groups also identified that the time on the rod and number of turnings were the lowest in the blank group, while those in the NSCs group were lower compared with GDNF/NSCs group ($P<0.05$). Figure 2

Inflammatory reaction changes

According to the detection results of IFs, IL-6, IL-8 and TNF-α were also the lowest in the blank group among the three groups one week after transplantation ($P<0.05$), while no difference was determined between NSCs and GDNF/NSCs groups ($P>0.05$). In the 4th week after transplantation, elevated IFs were observed in the blank group, higher than those in the other two groups ($P<0.05$); while IL-6, IL-8 and TNF-α in NSCs and GDNF/NSCs groups were reduced compared with the levels at the 1st week after transplantation, especially in GDNF/NSCs group ($P<0.05$). Figure 3

Stress damage changes

Subsequently, detection of stress damage indexes showed that SOD was the lowest and MDA was the highest in the blank group at 1 and 4 weeks after transplantation; while compared with GDNF/NSCs group, SOD in the NSCs group was lower and MDA was higher ($P<0.05$). Compared with the value at 1 week after transplantation, the SOD was reduced in the blank group at week 4 after transplantation, while it was elevated in the other two groups ($P<0.05$); MDA was increased in the blank group at the 4th post-transplantation and was decreased in the other two groups ($P<0.05$). Figure 4

Comparison of brain tissue injury

HE staining showed that the ischemic side of rat brain tissue in the blank group was loose, with interstitial edema, blurred cell contour, invisible nucleoli, increased cy-

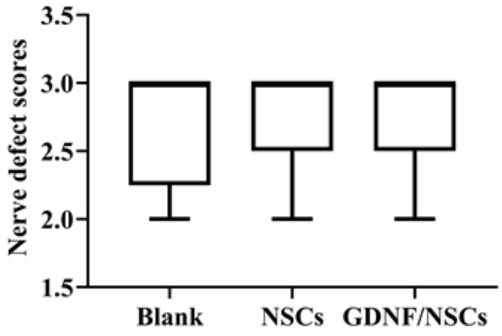
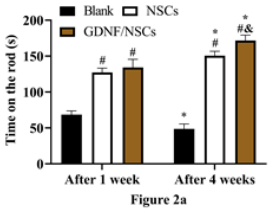


Figure 1. Neural deficit scores after modeling.



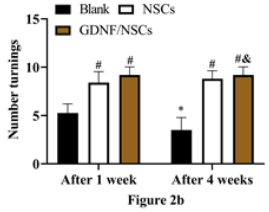


Figure 2. Results of behavioral tests. a) Results of rotarod test. b) Results of corner turn test. Note: # indicates a statistically significant difference from the blank group, & indicates a statistically significant difference from the NSCs group, and * indicates a statistically significant difference from the results of the same group after 1 week (i.e., $P<0.05$).

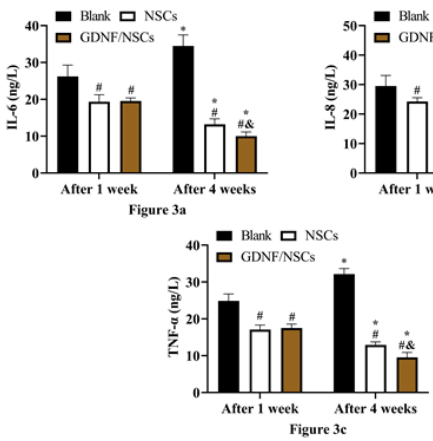
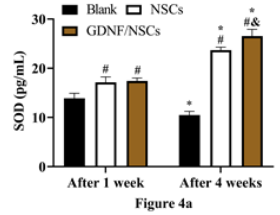


Figure 3. Inflammatory reaction changes. a) Comparison of IL-6 assay results. b) Comparison of IL-8 assay results. c) Comparison of TNF-α assay results. Note: # indicates a statistically significant difference from the blank group, & indicates a statistically significant difference from the NSCs group, and * indicates a statistically significant difference from the results of the same group after 1 week (i.e., $P<0.05$).



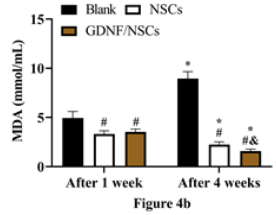


Figure 4. Stress damage changes. a) Comparison of SOD assay results. b) Comparison of MDA assay results. Note: # indicates a statistically significant difference from the blank group, & indicates a statistically significant difference from the NSCs group, and * indicates a statistically significant difference from the results of the same group after 1 week (i.e., $P<0.05$).

toplasmatic eosinophilia and obvious vacuole formation. In contrast, the pathological damage of rat brain tissue was ameliorated in both the NSCs group and the GDNF/NSCs group, and the neurons in GDNF/NSCs group were arranged regularly and tightly, with almost normal morphology and clear nucleoli. Figure 5

Comparison of GDNF/PI3K/AKT axis-related protein expression

Finally, the detection of GDNF/PI3K/AKT axis-related protein expression in rat brain tissue showed that GDNF, PI3K, AKT and p-AKT protein levels were lower in a blank group than in the other two groups, while those in GDNF/NSCs group were higher compared with NSCs group (P<0.05). Figure 6

Discussion

NSCs complete self-renewal and differentiation by means of symmetric or asymmetric division of metrocytes. There is increasing evidence that NSC transplantation is an effective method to treat ischemic brain injury and that NSCs can rebuild neural circuits by replacing and repairing damaged brain cells (11). Besides, NSCs can secrete a variety of neurotrophic factors, improve the local microenvironment and reduce the expression of genes related to microglia activation and inflammation (12). In this study, we transplanted NSCs into rats with FCI. The results showed that compared with rats in the blank group, the behavior, inflammatory reaction and stress injury of animals in NSCs and GDNF/NSCs groups were significantly improved; moreover, with the increase of transplantation time, the injury of NSCs and GDNF/NSCs groups ameliorated more obviously, while the injury of the blank group was getting worse. Subsequently, by observing the brain tissue injury of the three groups of rats, it can be seen that the injury of NSCs and GDNF/NSCs groups were also more significantly relieved. Therefore, NSC transplantation has an excellent ameliorating effect on FCI. In previous studies, NSC transplantation has been repeatedly confirmed to exert excellent repairing effects on nerve injuries such as cerebral infarction and stroke (13-15), which undoubtedly further supports our findings. The protective mechanism of NSCs on brain tissue is mainly divided into direct action (differentiation into neurons) and indirect action (paracrine active secretions) (16). We believe that NSCs may secrete brain-derived neurotrophic factor, GDNF, microvesicles and other active secretions through paracrine action to inhibit inflammatory responses of nerve cells and protect neurons. On the other hand, inflammation is known to aggravate blood-brain barrier injury, microvascular failure, brain edema and oxidative stress, and directly induce neuronal death. NSCs may also play a neuroprotective role by reducing inflammatory reactions and inhibiting neuronal apoptosis (17), which has also been confirmed in the research of Luciani et al. (18). Furthermore, the death of nerve cells in FCI has been suggested to be a unique process different from apoptosis and necrosis and is related to mitochondrial dysfunction (19), but whether this is related to NSCs remains to be further studied and confirmed.

To confirm the repair mechanism of NSCs on brain injury, we further set up NSCs transfected with GDNF for intervention (GDNF/NSCs group). The results showed that

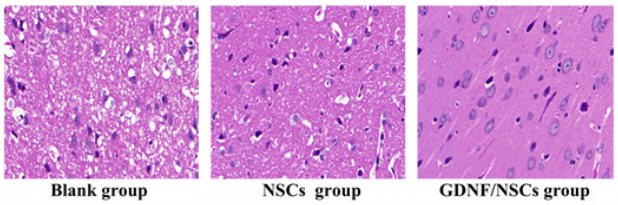


Figure 5. Comparison of brain tissue injury (200×). The ischemic side of rat brain tissue in the blank group was loose, the neurons in the NSCs and GDNF/NSCs groups were regularly and closely arranged.

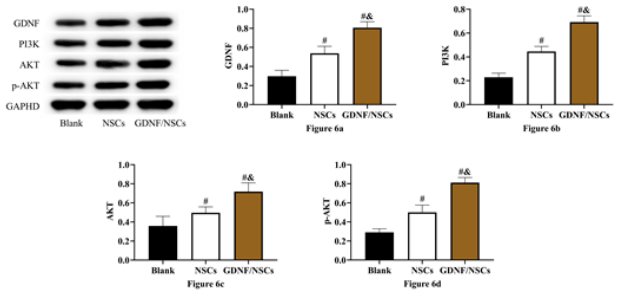


Figure 6. Comparison of GDNF/PI3K/AKT axis-related protein expression. a) Comparison of GDNF protein. b) Comparison of PI3K protein. c) Comparison of AKT protein. d) Comparison of p-AKT protein. Note: # indicates a statistically significant difference from the blank group, & indicates a statistically significant difference from the NSCs group (i.e., P<0.05).

compared with conventional NSC transplantation (NSCs group), the behavior, inflammatory reaction, stress injury, and brain histopathology of rats in the GDNF/NSCs group were more significantly alleviated. Moreover, further detection of GDNF/PI3K/AKT axis-related proteins in each group identified that compared with the blank group, GDNF, PI3K and AKT protein levels in NSCs and GDNF/NSCs groups were activated, especially in GDNF/NSCs group. This indicates that the repair of FCI-induced nerve injury by NSC transplantation may be carried out through the GDNF/PI3K/AKT axis, consistent with the results of a number of previous studies (20-22). Undoubtedly, this fully reveals the action pathway of NSC transplantation and can provide a more reliable reference for the future clinical application of NSCs.

However, this study still has many limitations that need to be addressed. For example, more experiments are needed to confirm the repair mechanism of NSC transplantation on nerve injury, and the role played by the GDNF/PI3K/AKT axis requires more precise experimental validation. In the future, we will also conduct more in-depth research on the above limitations to provide a more comprehensive reference for clinical practice.

NSC transplantation can improve neurological function and relieve inflammatory responses and stress injury in MCAO-induced FCI rats via the GDNF/PI3K/AKT axis, which is expected to be a treatment scheme for MCAO-induced FCI in the future, providing a reliable guarantee for the prognosis and health of patients.

Ethics Approval and Consent to Participate

Not applicable.

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Not applicable.

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Conflict of interest
The authors declare no conflict of interest.

Authors contributions
QL—Conceptualization, review and editing;HBX—Writing and original draft; FZ—Data curation and Methodology;HSW—Formal analysis and Supervision. All authors gave final approval of the submitted and published versions.

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