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Original Article

Effect of erythropoietin-expressed neural stem cell transplantation on cerebral ischemic injury

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Abstract: The alleviation of neurological function and recovery after cerebral ischemia is of critical importance. Recent studies have identified certain roles of tissue repair by neural stem cell (NSC) transplantation. Erythropoietin (EPO) may exert tissue protective function. In this study, NSCs were genetically modified to express EPO, and were tested for neurological functions. Meanwhile, bcl-2 protein expression was measured along with the discovery of potential mechanisms, in an attempt to discover novel pathways for treating brain ischemia. A total of 45 SD rats were prepared for middle cerebral artery occlusion (MCAO) model, followed by the direct injection of cerebral cortical NSCs that were modified to express EPO genes *in vitro*. Neurological functions, along with bcl-2 protein expression levels, were quantified by functional scores, RT-PCR and Western blotting. EPO-modified NSCs, after transplantation, significantly elevated neurological function scores of model rat. In those animals, we also found remarkably increased expression of bcl-2 in the sense of both mRNA and proteins ($P < 0.05$). Our study illustrated the anti-apoptotic role of EPO-modified NSCs in cerebral ischemia rats via up-regulating bcl-2 protein expression, thus decreasing the focal ischemia damage and protecting neural functions.

Keywords: Neural stem cell transplantation, cerebral ischemia, Bcl-2 protein, genetic modification, erythropoietin

Introduction

Based on WHO survey, ischemia stroke is the primary reason causing morbidity and mortality in adults. Also called cerebral infarction, ischemia stroke is the focal tissue necrosis or softening caused by obstruction of brain tissue blood flow and tissue hypoxia. It is manifested as coma, paralysis, cognitive functions. Ischemia stroke brings heaving burdens for patients and their families. The study of alleviating neurological dysfunction and recovery of neural functions is thus of great importance. Current available treatment methods approved by FDA only include thrombolysis at super-acute stage, using urokinase or streptokinase. Such method, however, is only effective within narrow time windows (3~6 hours after onset), thus limiting the availability. Alternative method may include the thrombolytic enzyme introduction via carotid artery. This method, however, is still at immature stage and is currently not available for large-scale use.

Various *in vitro* and animal studies have found the potent pluripotency of neural stem cells (NSCs) in neural cell repair and regeneration in neurodegenerative diseases [1, 2]. Therefore NSC has been used as clinical trials for central nervous system diseases. As one glycoprotein from adult renal and fetal liver cells, EPO was originally recognized as one endocrine hormone stimulating red blood cells generation. Recent studies, however, suggested the potential tissue protection function of EPO in addition to neurotrophic functions in both peripheral and central nervous system [3-5]. We thus performed this study using previously established genetic manipulation technique [6-8] to express EP in NSCs *in vitro*, followed by the transplantation into rat ischemia stroke model for further observation of neurological functions. The expression of bcl-2 protein was then determined to reveal underlying mechanisms providing novel pathways for clinical treatment of cerebral infarction.

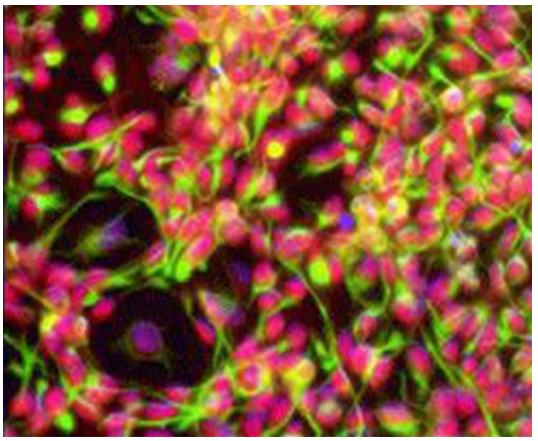

Figure 1. NSCs morphology in vitro.

Materials and methods

Animals and reagents

A total of 45 Sprague Dawley rats (body weight between 200 g and 300 g) were purchased from Laboratory Animal Center of Shanghai Jiaotong University. Protein extraction buffer, quantification reagents and developing kits in Western blotting were purchased from Shanghai Biotechnology Institute. All antibodies were products of Abcam (UK). PVDF membrane (0.45 μm thickness) was supplied by Roche (Swiss).

Experimentation protocols were submitted to and approved by the ethics committee of Shanghai Jiaotong University Affiliated 6th People's Hospital.

NSC isolation

SD rats with pregnancy day 14 were anesthetized and decapitated. Cerebral cortex was isolated from fetal rats for preparing NSC single cell suspension. NSCs were incubated in DMEM/F12 medium containing bFGF and EGF. Cells were passed at day 7, followed by 5-bromodexyuridine incubation for 72 hours. The purity of NSCs was also determined by nestin immunohistochemical (IHC) staining.

rAAV-EPO vector packing

rAAV-EPO viral vector was packaged, purified and condensed (Zhengyang Gene Tech., China). The viral titer was determined as 2×10^{11} vg/mL. The viral vector was used to infection NSCs, which were firstly prepared for single-cell colony and were seeded into 6-well plate (1×10^5 per well) in complement culture medium (containing DMEM/F12, N2, 10 μg/L bFGF, 20 μg/L EGF, 4×10^4 U/L, 1×10^5 U/L penicillin, and 1×10^6 U/L streptomycin) in a humidified chamber with 5% CO_2.

Rat stroke model

Silk lines (0.2 mm diameter, 8 cm length) were coated with 0.1% polylysine overnight, and were sterilized. 45 adult SD rats were anesthetized by 1% pentobarbital sodium (30 mg/kg) via intraperitoneal injection. Right common carotid artery, external and internal carotid arteries were exposed and ligated by silk lines. An incision was then made at the bifurcation of common carotid artery, for inserting silk line until the bifurcation of internal carotid artery for ligation. The incision was then sutured to fix the inserted line. 2 hours later, the obstruction line was retracted to the extracranial segment of internal carotid artery. After recovery, neurological functions of animals were evaluated. Those animals with > 7 scores were enrolled to NSC transplantation experiment.

NSC transplantation

Rats were transfected with saline (control group, N = 15), NSCs (N = 15), or EPO/NSCs (N = 15). In brief, 20 μL cell suspensions (4~5 × 10^5 cells) were injected into the right lateral ventricle at 5 μL/min velocity. After retracting the needle, the skull was repaired by bone wax.

Neurological function score

Modified neurologic severity scores (MNSS) scale [2-4] was used to evaluate the neurological function of 5 animals from each group at D7, D14 and D21 after surgery. The scale system mainly reflects the motor function, sensory and balance ability, in addition to neural reflexes, all of which are quantified by a total score of 18.

RT-PCR

Total RNA was extracted from cerebral cortex tissues of experimental rats. RT-PCR was performed to using specific primers for bcl-2 (Forward, 5'-GUG CAC GAC UCA AUA TA-3'; reverse, 5'-TTC ACG UGC UGA CGC AG -3') and β-actin as internal reference (Forward, 5'-GGT

Table 1. NSS scores

	7 days	14 days	21 days
Saline	7.65 ± 1.21	6.85 ± 0.76	6.15 ± 0.81
NSCs	7.60 ± 1.14*	6.80 ± 0.84*	6.20 ± 0.84*
EPO-NSCs	7.20 ± 0.84*	5.60 ± 0.55*	4.40 ± 0.55*

*P < 0.05 compared to saline group.

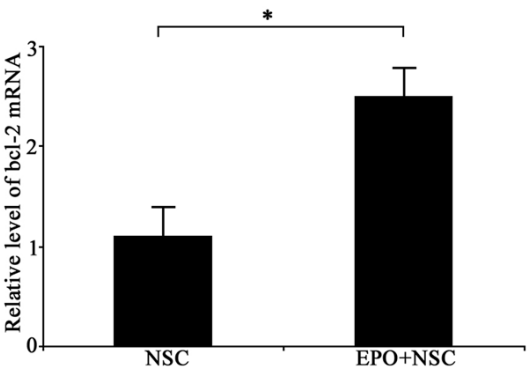

Figure 2. Bcl-2 mRNA relative levels. *P < 0.05 compared to NSC group.

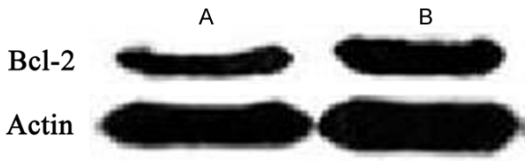

Figure 3. Representative Western blotting bands of Bcl-2 protein. A. NSC group; B. EPO/NSC group.

GTG ATGGTG GGT ATG GGT-3'; reverse, 5'-CTG GGT CATCTT TTC ACG GTC-3'). The PCR conditions were: 94°C pre-denature for 5 min, followed by 30 cycles each containing 94°C denature for 1 min, 60°C annealing for 1 min and 72°C elongation for 5 min. The relative mRNA expression level of all samples was determined by $2^{-\Delta\Delta Ct}$ method.

Western blotting

Total protein was firstly extracted from cerebral tissues, followed by separation in 15% SDS-PAGE. Proteins were transferred to PVDF membrane, which was added with anti-bcl-2 antibody (1:500) or anti-β-actin monoclonal antibody (1:2,000). After incubation overnight, the membrane was washed in TBST for 3 times, followed by the addition of horseradish peroxidase (HRP)-conjugated anti-IgG (1:1,000) for 1-hour incubation at room temperature. ECL method was then used to develop the membrane. Using β-actin as the reference, integrated optical density (OD) values of each band was measured by an imaging system for semi-quantitative analysis. Each experiment was conducted in triplicates.

Statistical analysis

SPSS 17.0 software was used to process all collected data, which were presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used to compare means across all groups. A statistical significance was defined when $P < 0.05$.

Results

Morphology of NSCs and immunohistochemical (IHC) staining

Neurons separated from fetal brain tissues had near-round shape and similar size. After incubation for 4~5 days, dozens of round cell colonies were formed with dark color, sharp edge and small size. Those "balls" with small burrs at the edge were names as neurospheres. Those neurospheres were again separated into single cell suspensions for continuous culture. Cell division occurred after 2~3 days incubation for the-reforming of large neurospheres with clear edges and strong light refraction. Those features suggest the embryonic origin of those neurosopheres cultured (**Figure 1**).

NSS scores

As shown in **Table 1**, NSS score of rats was significantly improved after transfecting with EPO-expressed NSCs.

mRNA level of bcl-2

RT-PCR showed significantly elevated mRNA expression of Bcl-2 gene after EPO/NSC transplantation, as compared to NSC group ($P < 0.05$, **Figure 2**).

Bcl-2 protein level

Those rats with EPO/NSCs transfection had significantly elevated bcl-2 protein levels compared to NSC group, as shown in **Figures 3** and **4**.

Discussion

Having potent division ability and self-renewal possibility, NSCs may differentiate into all types of neural cells of CNS, thus providing the cell

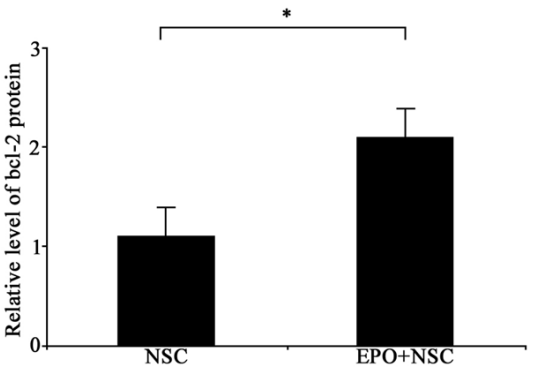

Figure 4. Relative protein level of bcl-2. *P < 0.05 compared to NSC group.

population for regenerating brain tissues [1-3]. More importantly, NSCs exist in almost all tissue types of CNS [9-12].

Medicines, surgery and habitation have made great progresses in treating central nervous system diseases in recent years. There are still huge amounts of patients, which, however, had severely compromised life quality. Recent progress in cells and animals indicated the ability of certain NSCs inside adult brain tissues to regenerate new neural cells. Those NSCs mostly existed in the sub-trophic ventricular zone (SVZ) and dentate gyrus of hippocampus. In 1992, mouse NSCs were firstly isolated to demolish the classical view of non-regenerating of neural cells [1]. In 1997, the theory of NSCs was established as to differentiate into neural cells with self-renewal potency [3]. The pluripotency of NSCs, plus other features such as low immunogenic property, make the clinical transplantation of NSCs possible [13-15]. The long-term survival and self-renewal ability of transplanted NSCs may help *de novo* regeneration of neural cells under certain conditions [16-18].

As one endocrine hormone, EPO mainly stimulate the generation of blood cells. Recent studies also revealed the potent tissue protective role of EPO in both central and peripheral nervous systems [3-5]. In this study, we for the first time expressed EPO in NSCs, which were then infused into cerebral ventricles of ischemia stroke rats. The neurotropic role of EPO further accelerated the proliferation of NSCs Our results showed significantly improved motor function scores in ischemia stroke rats after EPO/NSCs transplantation, thus providing a novel potential method in clinical treating ischemia stroke.

Our results also showed the decreased neural cell apoptosis after EPO introduction, along with elevated bcl-2 protein expression. Although the detailed mechanism underlying EPO for decreasing neural cell apoptosis is still unclear, some scholars found the blockade of inflammatory necrosis and consequence cell apoptosis by EPO, which thus exerts a neural protective effect. Other opinions such as cellular hypoxia and neurotoxicity have been proposed but without conclusion [19, 20]. Further studies thus should focus on the exploration of molecular mechanisms, thus providing in-depth evidences for clinical treatment.

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Disclosure of conflict of interest

None.

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