Functional Recovery after Transplantation of Neural Stem Cells Modified by Brain-derived Neurotrophic Factor in Rats with Cerebral Ischaemia

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Functional recovery after transplantation of brain-derived neurotrophic factor (BDNF)-modified neural stem cells (NSCs) was evaluated in a rat model of cerebral ischaemia damage induced by temporary middle cerebral artery occlusion (tMCAO). Western blotting and enzyme-linked immunosorbent assay demonstrated up-regulated BDNF protein expression by rat embryonic NSCs transfected with the human BDNF gene (BDNF–NSCs). BDNF–NSCs stimulated neurite outgrowth in cocultured dorsal root ganglion neurons, suggesting that BDNF increased neurogenesis in vitro. In vivo, BDNF promoted recovery of tMCAO. Phosphate-buffered saline, untransformed NSCs or BDNF–NSCs were introduced into the penumbra zone of the right striatum of tMCAO rats and neurological function deficit was assessed for up to 12 weeks using the neurological severity score (NSS). The NSS was significantly lower in the BDNF–NSC transfected transplant group than in all the other groups from week 10. BDNF–NSCs recovered 1 week after transplantation expressed BDNF protein. Transplanted NSCs had differentiated into mature neurons 12 weeks after transplantation. Transgenic NSCs have potential as a therapeutic agent for brain ischaemia.

KEY WORDS: NEURAL STEM CELLS; BRAIN-DERIVED NEUROTROPHIC FACTOR; GENE TRANSFECTION; TRANSPLANTATION; CEREBRAL ISCHAEMIA; FUNCTIONAL RECOVERY; RATS

Introduction

Cerebral ischaemia is the most common cause of human stroke and the number of cases is increasing every year, but clinical treatment is limited. Transplantation of neural stem cells (NSCs) into the brain is a potential treatment for cerebral ischaemia.1 NSCs are ideal donor cells for neural transplantation and cellular vectors for gene therapy because of their ability to regenerate and to undergo multidirectional differentiation, and their low immunogenicity. However, NSC transplantation itself does not readily have positive effects on the local pathological changes that occur during the early stages after ischaemia. These changes often have a decisive effect on the nerve lesion that occurs after ischaemia. Investigators have found that exogenous neurotrophic factors may play an important role in promoting functional recovery.
role in modulating resistance to ischaemic injury, and brain-derived neurotrophic factor (BDNF) has been demonstrated to mediate neuronal development and function. A recent report showed that intravenous application of BDNF improved sensorimotor recovery after stroke, suggesting a critical role for BDNF in regulating neurogenesis and functional neurological recovery.

In the present study, a rat model of cerebral ischaemia damage induced by temporary middle cerebral artery occlusion (tMCAO) was used to evaluate whether NSCs transfected with the human BDNF gene (BDNF-NSCs) and transplanted into the ischaemic penumbra zone produced functional recovery.

**Materials and methods**

**ANIMAL CARE**

Animal care, usage and all procedures were approved by the Animal Care and Use Committee of Zhejiang University of Medicine, Hangzhou, China.

**IN VITRO STUDIES**

**Cell culture and transfection**

Rat embryo NSCs were cultured and purified in vitro according to a method reported previously. The retroviral plasmid vector pLXSN carrying the human BDNF gene (BDNF) and labelled with green fluorescent protein (GFP), was inserted into the packaging cell line PA317 and transfected into NSCs (BDNF-NSCs).

For co-culture experiments, dorsal root ganglia (DRG) from newborn Sprague–Dawley rats were cultured using routine methods for 24 h and a suspension of NSCs from each of the four groups in the tMCAO model (see below) was then added and co-cultured for 48 h.

**Assessment of BDNF protein expression**

Changes in BDNF protein levels in DRG neurons were measured by assaying the amount of BDNF secreted into the perfusion medium. Western blot analysis was carried out 24 h after transfection and enzyme-linked immunosorbent assay (ELISA) was carried out using a BDNF Emax® Immunoassay System (Promega, Madison, WI, USA) at intervals from 12 to 96 h after transfection, according to the manufacturer’s instructions. β-Actin was used as the control.

**IN VIVO STUDIES**

**Establishment of the tMCAO model**

Adult male Sprague–Dawley rats (250 – 300 g) were given food and water under a 12 h light – 12 h dark cycle and maintained at room temperature (20 – 25 °C). Surgery to establish the temporary middle cerebral artery occlusion (tMCAO) model was then performed between 08.00 and 12.00 h, using a method described previously. Under anaesthesia using 10% chloral hydrate administered intraperitoneally, the right common carotid artery was exposed and the branches of the external carotid artery, including the occipital terminal lingual and maxillary arteries, were isolated and coagulated. The internal carotid artery was then isolated, and its extracranial
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branch and the pterygopalatine artery were ligated close to the origin. The internal carotid artery, which is the only extracranial branch of the common carotid artery, remained patent. A 3 cm length of 4-0 nylon suture with a slightly enlarged and rounded tip was introduced into the transected lumen of the external carotid artery. The suture was gently advanced from the internal carotid artery to the opening of the middle cerebral artery. The distance from the tip of the suture to the bifurcation of the common carotid artery was 19 – 20 mm. Reperfusion was performed by withdrawing the surgical suture from the external carotid artery 120 min after the establishment of tMCAO. Blood gases, blood glucose and arterial blood pressure were monitored during the surgical procedure. Gentamicin (20 000 U/day) was administered intraperitoneally for 3 days following resuscitation.

Preparation of NSC suspension
The NSCs were labelled with 10 µmol/l of 5-bromodeoxyuridine (BrdU) for 3 days and were then suspended in 0.01 M phosphate-buffered saline (PBS), pH 7.2, to a density of 5 × 10⁷ cells/ml before transplantation.

Transplantation technique
The method used for transplantation into the brain was that described by Fukunaga et al.⁷ All rats were anaesthetized using 10% chloral hydrate administered intraperitoneally and the head was fixed in a Kopf stereotaxic frame. A burr hole (1 mm) was made on the right side of the cranium and injection was carried out into the ischaemic penumbra zone (including the ischaemic perifocal territories) of the right striatum using a 20 µl Hamilton microinjection needle at a rate of 5 µl/min (0.5 mm posterior and 3.5 mm to the right of the bregma; 4.5 mm below the dura). The needle was retained in the brain for an additional 5 min after injection to prevent donor reflux.

Randomization and treatment groups
Rats that survived at least 3 days after surgery were used to establish the tMCAO model. They were randomly divided into four groups on day 3 for treatment as follows: group 1, sham transplantation; group 2, injected with 20 µl of 0.01 M PBS pH 7.2; group 3, the untransformed NSC transplanted group in which 20 µl of NSC suspension was injected; and group 4, the BDNF–NSC transplantation group in which 20 µl of transfected BrdU-labelled BDNF–NSC suspension was injected. Half the rats in the BDNF–NSC transplantation group (group 4) were used to assess BDNF protein expression 1 week after transplantation (as described below). The remaining rats in group 4 and all rats in groups 1 – 3 were used to monitor neurological function deficit (see below) from weeks 2 to 12 after transplantation; rats in group 4 were also used to evaluate the survival, migration and differentiation of the transplanted NSCs at week 12.

Evaluation of neurological function deficit
Neurological function deficit was evaluated in all groups immediately before transplantation (3 days after the establishment of tMCAO [0 weeks after transplantation]) and at weeks 2, 4, 6, 8, 10 and 12 after transplantation by means of the neurological severity score (NSS), as described previously.⁸ The NSS grades the results from a series of motor, sensory, balance and reflex tests on a scale of 0 – 18 (0, no neurological function deficit; 18, maximum neurological function deficit).

Assessment of BDNF protein expression
Those rats in the BDNF–NSC transplantation group (group 4) to be used to assess BDNF
protein expression were selected randomly 1 week after transplantation and were killed under anaesthesia. The brains were fixed by transcardial perfusion with saline and they were then perfused again using 4% paraformaldehyde. Brain sections 10 µm thick were cut near the transplantation site and double-stained for BrdU and BDNF using an immunofluorescence method with specific antibodies. For BrdU the primary antibody was antirat BrdU (Abcam [Hong Kong] Ltd, Hong Kong, China) and the secondary antibody was goat antirat IgG–cyanine 3 (CY3) (GeneTex Inc., Irvine, CA, USA). For BDNF the primary antibody was antirabbit BDNF (Chemicon) and the secondary antibody was goat antirabbit IgM–fluorescein isothiocyanate (FITC). Sections were evaluated according to: (i) the distribution of BrdU-positive cells; and (ii) BDNF protein expression in BrdU-positive cells; and (iii) BrdU–neurofilament double-positive cells as a percentage of BrdU-positive cells.

**STATISTICAL ANALYSIS**

Statistical analysis was carried out on the NSS data which were expressed as mean ± SD and compared by analysis of variance using SPSS® software, version 11.5 (SPSS Inc., Chicago, IL, USA). A *P*-value < 0.05 was considered statistically significant.

**Results**

**IN VITRO STUDIES**

**BDNF protein expression**

Western blotting showed greater BDNF protein expression in BDNF–NSCs compared with untransformed NSCs and NSCs transfected with empty vector plasmids (Fig. 1A). After transfection, the level of BDNF protein secreted into the perfusion medium was measured by ELISA and was found gradually to increase between 12 h and its peak at 72 h (Fig. 1B). These results showed that BDNF–NSCs express high levels of BDNF protein and secrete BDNF as early as 12 h after transfection.

**Promotion of neurite outgrowth in co-cultured DRG neurons**

No neurite extension was detected when NSCs from the sham transplantation group (group 1) were co-cultured with DRG, whereas transfected BDNF–NSCs (group 4) greatly promoted dendrite and axon outgrowth of DRG neurons during 2 days of co-culture (Fig. 2), indicating that BDNF–NSCs may stimulate neurite outgrowth of DRG neurons by secreting BDNF protein.

**IN VIVO STUDIES**

**Establishment of the tMCAO model**

Of the 61 rat models of tMCAO established, 50 animals survived beyond 3 days after the
Five rats died from subarachnoid haemorrhage 1–2 days after the operation, two rats died 3 days after the operation because of infection and four rats died 2–3 days after the operation because of severe cerebral oedema. Of the 50 surviving rats, 14 showed hemiparesis, 24 exhibited paralysis of the forelimbs, 11 had paralysis of the hind limbs and one did not display any obvious motor dysfunction.

Numbers in each of the four groups were: group 1, sham transplantation, \( n = 10 \); group 2, PBS injected, \( n = 10 \); group 3, untransformed NSC transplanted group, \( n = 10 \); and group 4, BDNF–NSC transplantation group, \( n = 20 \). Ten rats in the BDNF–NSC transplantation group (group 4) were used to assess BDNF protein expression 1 week after transplantation.

**Neurological function deficit**

Neurological function deficit, evaluated using the NSS, was compared between groups by analysis of variance and is shown in Table 1. At 0 weeks after transplantation (baseline), the NSS did not differ significantly between
the four groups (mean ± SD ranging from 6.6 ± 1.8 to 6.8 ± 1.6). The scores demonstrated progressive recovery during the period from week 2 to week 12 after transplantation in all groups. There were no statistically significant differences in NSS between the four groups during weeks 2 – 6. From 8 to 12 weeks after transplantation, however, NSS in the BDNF–NSC transfected group (group 4) was lower than in the sham transplantation group (group 1), the PBS injected group (group 2) and the untransformed NSC transplantation group (group 3); the differences were statistically significant for group 4 compared with groups 1 and 2 at week 8 – 12 (P < 0.05) and for group 4 compared with group 3 at weeks 10 and 12 (P < 0.05) after transplantation (Table 1).

![Control vs BDNF co-culture](image.png)

**FIGURE 2:** Co-culture of brain-derived neurotrophic factor (BDNF)-modified neural stem cells (NSCs) with dorsal root ganglion (DRG) neurons for 48 h promoted neurite outgrowth: control, co-culture of DRGs with NSCs from the sham-transplanted group (group 1); BDNF, co-culture of DRGs with NSCs transfected with the human BDNF gene (group 4) (scale bars 100 µm)
**BDNF protein expression**

At 1 week after transplantation, immunofluorescence staining showed that the BDNF–NSCs had migrated from the transplantation site to the ischaemic region of the brain and that some of the surviving cells were expressing BDNF protein (Fig. 3A). The number of BrdU–BDNF double-positive cells was about 25% of the number of BrdU-positive cells. This suggests that the transplanted transgenic NSCs survived in the ischaemic areas and functionally expressed BDNF protein in vivo.

**Differentiation of BDNF–NSCs**

At 12 weeks after transplantation, BDNF–NSCs had migrated from the NSC transplantation site to the area around the lesion site. Some of the cells were positive for both BrdU and neurofilament (Fig. 3B). The absolute number of BrdU-positive cells varied between different rats and the number of BrdU/neurofilament double-positive cells as a percentage of BrdU-positive cells ranged from 20 to 38%. This finding suggests that the transplanted BDNF–NSCs were able to survive in the ischaemic areas and were capable of migration and differentiation into neurons.

**Discussion**

The apoptosis and necrosis of localized areas of nerve tissue in the early stage of cerebral ischaemia and the lack of regeneration capability of the adult brain in the later stage play essential roles in the progression of this disease. Existing clinical therapies, including thrombolysis, nutritional support and convalescent care, do not provide a fundamental solution to these two problems, and their curative effectiveness is therefore far from satisfactory. In the present study,

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**FIGURE 3:** In vivo protein expression of bromodeoxyuridine (BrdU)-labelled brain-derived neurotrophic factor (BDNF) and differentiation of BDNF-modified neural stem cells (NSCs) recovered after NSC transplantation in a rat model of cerebral ischaemia induced by temporary middle cerebral artery occlusion. (A) Double staining for BrdU and BDNF in NSCs recovered 1 week after transplantation, showing that BDNF-modified NSCs had survived and expressed BDNF protein at this time-point. (B) Double staining for BrdU and neurofilament in NSCs recovered 12 weeks after transplantation, showing that BDNF-modified NSCs had differentiated into neurons in the ischaemic region (scale bar 100 µm applies to all images).
recovery from cerebral ischaemia damage was investigated following the transplantation of BDNF-modified NSCs into rats in order to try and reveal the function of BDNF in promoting neurogenesis.

The discovery of NSCs has challenged the conventional idea that adult neural cells cannot renew themselves. NSCs offer an alternative way to treat cerebral ischaemia because of a number of distinct biological features. First, NSCs have very low immunogenicity and do not express mature cellular antigens, such as major histocompatibility complex (MHC) class 1 and 2 molecules. Thus, immune rejection is very rare after transplantation of these cells, providing the possibility of long-term survival and restoration of pertinent neurological functions. Secondly, NSCs have a self-renewal function. The cloning of NSCs has been found to result in regeneration persisting for >3 years in vitro, the final stem cells having the same biological properties as the original stem cells. Thirdly, the multipotency of NSCs is critical during the process of recovery from cerebral ischaemia. Under regulation by local cytokines in vivo, NSCs can differentiate into the new neurons that are needed in the disease-affected domain, thereby participating in the repair of nerve tissue, and they can also differentiate into non-nerve cells through straddled germinal layer differentiation. Fourthly, studies have demonstrated that NSCs can pass through the blood–brain barrier, migrate into brain tissue and integrate both structurally and functionally with host cells. Fifthly, migration potency could play a role in recovery from cerebral ischaemia; under the influence of signals released from the damaged neurological region, NSCs migrate towards the target region after transplantation.

These features make NSCs ideal as donor cells for neural transplantation and as cellular vectors for gene therapy. Gray et al. transplanted immortalized NSCs (MHP36 cells) into the ischaemic domain of rats and found that these cells had migrated from the transplantation site to surrounding areas 1 week after transplantation. Surprisingly, the NSCs had formed an array along the impaired CA1 layer and had reconstructed the tissue 3 months after transplantation. In the present study, an untransformed NSC transplantation group, a sham transplantation group and a PBS-injected group showed no significant differences in NSS up to week 6 after transplantation compared with the transfected BDNF–NSC transplantation group. From weeks 8 to 12 after transplantation, which corresponds to the migratory period of NSCs, marked decreases in the NSS in the transfected BDNF–NSC transplantation group were seen, whereas there was no difference in NSS between the sham transplantation group and the PBS-injected group during the whole 12 weeks following transplantation, thereby excluding any influence by the grafting procedure.

The straight transplantation of unmodified NSCs has limited effect on the functional recovery of neurons after ischaemic cerebral injury, which is why efforts were channelled into studying BDNF-modified NSCs in the present study. Many investigators have found up-regulation of expression of endogenous BDNF and its receptor, TrkB, in various pathological conditions involving cerebral ischaemia. For example, after the establishment of a tMCAO model, Yang et al. found 4.07- and 2.84-fold increases at 6 and 48 h, respectively, in the BDNF mRNA level in the hippocampus compared with a sham transplantation group. Miyake et al. also detected an elevation of BDNF mRNA expression in the ischaemic penumbra of the
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hippocampus. Larsson et al.\textsuperscript{16} reported that neuron survival decreased significantly when the activity of BDNF was blocked by Trk–Fc fusion protein. These findings suggest that endogenous BDNF may protect local ischaemic neurons. Several mechanisms have been proposed to be involved in the neuroprotective effect of endogenous BDNF. The first of these is the stabilization of Ca\textsuperscript{2+} concentration by up-regulating the expression of calcium binding protein\textsuperscript{17} and, furthermore, BDNF may down-regulate the function of \textit{N}-methyl-\textit{d}-aspartic acid receptors in a manner that resembles the toxicity of excitatory amino acids.\textsuperscript{18} Secondly, is an effect on the metabolism of free radicals. It has been shown that BDNF can inhibit not only the expression of induced nitric oxide synthase but also the activity of glial cells and infiltration of macrophages in the hippocampal region after cerebral ischaemia, resulting in a decrease in free radicals and the protection of neurons.\textsuperscript{19} Thirdly, is the protection of neurons against apoptosis. Han et al.\textsuperscript{20} found that BDNF was able to inhibit cell apoptosis through a specific death signal transduction molecule, caspase-3. Fourthly, is an increase in the activity of protein kinase C. The rapid inactivation of protein kinase C is a typical manifestation of cerebral ischaemia. When pre-treated with protein kinase C inhibitor, BDNF does not protect neurons,\textsuperscript{21} which indicates that BDNF may provide a better protective effect when protein kinase C activity is increased. Fifthly, is the restoration of damaged neurons by changing their plasticity. When neurons and axis cylinders are damaged, local protein expression of BDNF and TrkB increases to stimulate the budding of the axis cylinders and the formation of synapses. BDNF, basic fibroblast growth factor and nerve growth factor can promote the dissociation and differentiation of endothelial cells and stimulate angiogenesis, leading to restoration of nerve function.\textsuperscript{22} Lastly, is promotion of the proliferation and differentiation of NSCs. Some investigators have reported that around 34% of NSCs can differentiate into neurons with the help of BDNF and that neuronal precursor cells can acquire the characteristics of mature neurons in the presence of BDNF.\textsuperscript{23} These neuroprotective characteristics of endogenous BDNF may play an important role in nerve regeneration and repair following cerebral ischaemia.

Study of the function of exogenous BDNF has also made great progress. Zhang and Pardridge\textsuperscript{24} reported that administration of BDNF decreased the area of cerebral infarction by 68% and 70% after 24 h and 7 days of cerebral ischaemia, respectively. Kiprianova et al.\textsuperscript{25} found that continuous intraventricular administration of BDNF prevented the death of CA1 neurons in the hippocampus. In these studies, however, the amount of drug given and the time interval between individual doses were limited. It is possible that frequent administration may cause complications such as infection.

Since NSCs are well suited for use as vectors in gene therapy, transplantation of BDNF-modified NSCs into the brain may provide stable and long-lasting expression of BDNF. The present study demonstrated that transplantation of \textit{BDNF}–NSCs plays a dual role in the regeneration of nerve system function after cerebral ischaemia. Transplanted NSCs took part in the reconstruction of nerve tissue through migration, proliferation and differentiation, leading to replacement of lost neurons. Secreted BDNF derived from NSCs was able to promote the repair of damaged neurons in the early stages after ischaemic damage. In addition, secreted BDNF stimulated not only
endogenous NSCs but also transplanted NSCs to differentiate into specific types of neuron and the present study showed that the curative effect in the BDNF–NSC transplantation group was better than that in the untransformed NSC transplanted group in the later stages after tMCAO.

The present study demonstrated that transplantation of BDNF-modified NSCs has a better curative effect than the transplantation of untransformed NSCs in the treatment of a rat model of cerebral ischaemia. The transplanted BDNF–NSCs differentiated and replaced the lost neurons induced by ischaemic damage in vivo. The BDNF produced by the transplanted cells also improved the local tissue environment and stimulated neurogenesis. Thus, the transplantation of transgenic NSCs and, particularly, BDNF modification of NSCs may provide a valuable means of improving the effectiveness of treatment of cerebral ischaemia.

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Conflicts of interest
The authors had no conflicts of interest to declare in relation to this article.

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