**Original Article**

**Intravenously delivered neural stem cells migrate into ischemic brain, differentiate and improve functional recovery after transient ischemic stroke in adult rats**

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**Abstract:** Stem cell transplantation may provide an alternative therapy to promote functional recovery after various neurological disorders including cerebral infarct. Due to the minimal immunogenicity and neuronal differentiation potential of neural stem cells (NSCs), we tested whether intravenous administration of mice-derived C17.2 NSCs could improve neurological function deficit and cerebral infarction volume after ischemic stroke in rats. Additionally, we evaluated the survival, migration, proliferation, and differentiation capacity of transplanted NSCs in the rat brain. Intravenous infusion of NSCs after middle cerebral artery occlusion (MCAO) showed better performance in neurobiological severity scores after MCAO compared to control. However, the volume of cerebral infarction was not different at 7 days after MCAO compared with control. Transplanted NSCs were detected in the ischemic region but not in the contralateral hemisphere. NSCs differentiated into neurons or astrocytes after MCAO. These data suggest that intravenously transplanted NSCs can migrate, proliferate, and differentiate into neurons and astrocytes in the rat brain with focal ischemia and improve functional recovery.

**Keywords:** Ischemic stroke, cell transplantation, neural stem cells, intravenous administration

**Introduction**

Ischemic stroke resulting from abrupt interruption of cerebral blood flow remains one of the most devastating neurological diseases with high incidence and mortality [1]. Despite considerable progress including thrombolytic cerebrovascular recanalization in the first hours after stroke onset and secondary stroke prevention therapies, there still exist few neuroprotective therapies that effectively reduce brain damage and improve neurological recovery once ischemic stroke has occurred [2].

The results of numerous preclinical and clinical studies suggest that cell transplantation therapy may promote functional recovery after neurological disorders including cerebral infarct. A variety of cell types including embryonic stem cells, bone marrow-derived mesenchymal stem cells, induced pluripotent stem cells, and NSCs have demonstrated efficacy in ischemic stroke animal models [3]. Of these cell types, the use of NSCs is most attractive as they are minimally immunogenic [4]. NSCs can also differentiate into neurons, astrocytes, oligodendrocytes, and even endothelium which constitute most of the cerebral cell types affected by ischemic insult [5-7].

The C17.2 NSC line is derived from the neonatal mouse cerebellum and immortalized with v-myc [8]. Transplantation of C17.2 [9] cells has been widely investigated for its therapeutic benefit in central nervous system disorders including Parkinson’s disease, Huntington’s disease [10],...
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traumatic brain injury [11], hypoxic-ischemic brain injury [12], selectively neuro-degenerating adult mouse neocortex [13], and spinal cord injury [14], among others. However, it is still unknown whether C17.2 NSCs possess therapeutic potential for ischemic stroke. Different administration routes for stem cell therapy for cerebral ischemia such as intrastriatal, intraventricular or intravenous delivery have been extensively evaluated [15]. Of these routes, intravenous grafting of stem cells is most attractive because it avoids surgical trauma to healthy brain tissue and other peri-operative complications.

In this study we tested whether intravenously injected C17.2 NSCs could improve the neurological functional recovery and reduction of cerebral infarction volume caused by focal cerebral ischemia in rats. Furthermore, we determined the migration, proliferation and differentiation capabilites of transplanted C17.2 NSCs in the ischemic brain microenvironment.

Materials and methods

Cell culture and preparation

The NSC line C17.2 was maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 5% horse serum along with 2 mM glutamine and penicillin/streptomycin. The cells were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C and passaged at 90% confluence. To identify grafted cells, NSCs were labeled with Cell Tracker CM-DiI (Invitrogen) prior to transplantation by incubating the cells with the dye for 5 min at 37°C and another 15 minutes at 4°C. The labeling efficiency of the cells was verified to be 99-100% prior to all transplantations under fluorescent microscope. Cell viability was determined by trypan blue staining at the end of the harvest and before infusion and the viability was greater than 95%.

Animal model

All experimental procedures have been approved by the Care of Experimental Animals Committee of Wenzhou Medical University. Adult male Sprague-Dawley rats weighing 270 to 300 grams purchased from Shanghai Laboratory Animal Center were used in all our experiments. Transient focal cerebral ischemia was induced using the intraluminal thread occlusion of middle cerebral artery (MCA) for 120 min in both groups, as previously described [16]. Briefly, the right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were exposed. A monofilament nylon suture with its tip rounded by heating near a flame, which size was determined by the animal weight (18.5 to 19.5 mm in length and 0.26 to 0.28 mm in diameter), was advanced from the ECA into the lumen of the ICA until it blocked the origin of the MCA. Two hours after MCAO, animals were re-anesthetized with 3% sodium pentobarbital, and reperfusion was performed by withdrawal of the suture until the tip cleared the lumen of the common carotid artery. Rectal temperature was maintained at 37 ± 0.5°C using a thermistor-controlled heat blanket. After recovery, rats were assessed for forelimb flexion and contralateral circling to confirm ischemia. Thirty six ischemic stroke rats were randomly divided into two experimental groups: the C17.2 NSCs-treated (n = 18) and the PBS-treated (n = 18). The animals were maintained in each separate cage at room temperature with free access to food and water under a 12 h light-dark cycle.

Transplantation procedure

24 hours after cerebral ischemia, animals received either C17.2 neural stem cells or phosphate buffered saline (PBS). Animals were anesthetized with 3% sodium pentobarbital and then approximately 5×10⁶ NSCs in a volume of 200 µl PBS or equal volume of PBS alone were injected into the rat tail vein at 24 hours after stroke. Immunosuppressants were not used in any animals.

Neurological function test

Neurological/behavioral measurements were performed before MCAO and at 1, 3, 7, 10, 14, 21 and 28 days after intravenous transplantation by an investigator who was blinded to the experimental groups. A set of the Neurobiological Severity Scores (NSS) modified form previous report was used to evaluate sensorimotor behavior including 5 raising the tail tests, 4 placed on the floor tests, 3 sensory tests, and coordination and balance behavior including 3 beam tests [17]. In the severity scores of injury, 1 score point is awarded for the inability to per-
form the test or for the lack of a tested reflex. Thus the higher the score the more severe is the injury. Rats typically had average testing scores between 8-12 prior to cell transplantation in our experiment.

**TTC staining and quantitative analysis**

One week after intravenous transplantation, the rats were deeply anesthetized with 10% chloral hydrate. The brains were removed carefully and series of fresh brain slices were immersed in 0.5% solution of 2, 3, 5-triphenyl-tetrazolium chloride (TTC) in PBS at 37°C for 20 min. The cross-sectional area of infarction in each brain slice was captured and further measured using NIH Image J analysis software. The total infarct volume for each brain was calculated by summation of the infarcted area of all brain slices.

**BrdU labeling**

BrdU (Sigma), the thymidine analog that is incorporated into the DNA of dividing cells during S-phase, was used for mitotic labeling. BrdU

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**Figure 1.** Effect of intravenously transplanted C17.2 neural stem cells on neurological function deficit and cerebral infarction volume in ischemic stroke rats. A. Systemic infusion of $5 \times 10^6$ C17.2 NSCs 24 hours after MCAO significantly improved functional recovery at the indicated days post-MCAO assessed by Neurobiological Severity Scores (NSS) (n = 6 per group). B. Intravenous administration of C17.2 NSCs did not reduce the volume of cerebral infarction at 7 days after MCAO measured by 2, 3, 5-triphenyltetrazolium chloride (TTC) staining. Representative pictures are shown (n = 5 per group). C. The infarction volume was calculated by Image J software and summarized. Values are expressed as means ± SEM. *P < 0.05 and **P < 0.01 between C17.2 NSCs-treated and PBS-treated groups.
(50 mg/kg) was intraperitoneally injected daily for thirteen consecutive days into ischemic rats starting 1 day after MCAO. To quantify BrdU immunoreactive cells, BrdU positive cells co-localizing with or without CM-DiI dye were counted in the infarcted area from each rat. Data was presented as average of BrdU immunoreactive cells in ipsilateral hemisphere from five rats.

**Histological and immunofluorescent assessment**

Animals were anesthetized with 10% chloral hydrate 14 or 28 days after MCAO. Rat brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, and embedded in optimal cutting temperature compound. A series of adjacent 5 µm-thick cryosections were cut from each block in the coronal plane. Immunofluorescent staining was used to identify cells derived from C17.2 NSCs. To visualize the cellular colocalization of Cell Tracker CM-DiI and cell type specific markers in the same cells, CY5 and FITC were used for double-label immunoreactivity. Each coronal section was treated with cell type-specific antibodies: a neuronal nuclear antigen (NeuN for neurons; dilution in 1:200; Abcam); an astrocytic marker, glial fibrillary acidic protein (GFAP; dilution in 1:100; Santa Cruz); a neural stem cell marker nestin (dilution in 1:100; Abcam); a thymidine analogue BrdU (dilution in 1:1000; Abcam). Negative control sections from each animal received identical preparations for immunofluo-
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rescent staining, except that primary antibodies were omitted.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was determined with Student’s t-test when there were two experimental groups. For all tests, a value of $P < 0.05$ was taken as significant.

Results

Administration of NSCs improves functional recovery after MCAO

Functional analysis of ischemic rats was performed at different time points after intravenous NSCs transplantation. Neurological Severity Scores (NSS) were calculated based on a series of motor sensory, reflex, and balance tests. Treatment with intravenously injected C17.2 NSCs 24 hours after MCAO significantly improved functional recovery, as evidenced by improved NSS scores (Figure 1A). There were no significant differences among the groups in NSS scores before MCAO or 1, 3 and 7 days after MCAO. At 10, 14 and 21 days post-MCAO, rats in C17.2 NSCs-treated group showed greater sensorimotor deficit improvement as compared to PBS-treated control ($P < 0.05$). The greatest improvements were observed in C17.2 NSCs-treated rats at 28 days after MCAO ($P < 0.01$). These results demonstrated that functional deficits following ischemia produced by transient MCAO in rats were improved by intravenous transplantation of C17.2 NSCs.

NSCs treatment does not reduce cerebral infarction volume

To clarify whether improvement of neurological function was correlated with reduction of cerebral infarction volume, the animals at 7 days after MCAO were perfused and stained with 2, 3, 5-triphenyltetrazolium chloride (TTC) to measure the infarction volume. Normal brain tissue typically stains with TTC, but infarcted lesions show limited or no staining. Lesion volume was calculated by measuring the area of reduced TTC-staining in the fresh brain coronal sections. TTC-staining obtained one week after MCAO with or without intravenous C17.2 NSCs transplantation is shown in Figure 1B. The infarction volume was calculated by Image J software and is summarized in Figure 1C. There was no significant reduction in infarct size between C17.2 NSCs-treated group and PBS-treated control.

NSCs migrates into ischemic brain and maintains proliferative capability

To investigate whether intravenously transplanted NSCs could enter into the brain of rats with MCAO injury, NSCs pre-labeled with red fluorescent dye CM-DiI were identified in brain sections at 28 days after MCAO. As shown in Figure 2A, transplanted NSCs were found in the ischemic region but were not detectable in the contralateral hemisphere. The cells were mainly limited to the infarcted area, although some were sparsely observed throughout the affected hemisphere. These results suggested that intravenously injected NSCs transversed the blood-brain barrier and migrated into the brain of ischemic stroke rats.

The proliferative capability of transplanted NSCs in ischemic region was evaluated by the S-phase marker BrdU. As shown in Figure 2B, more BrdU-positive cells were observed in the ischemic hemisphere of C17.2 NSCs-treated rats compared with PBS-treated control. BrdU immunoreactive cells were further co-localized with CM-DiI. Approximately 86% of transplanted NSCs (red fluorescent dye by CM-DiI) were overlapped with BrdU suggesting that most of them still maintained strong proliferative capability in the ischemic environment for at least 14 days after MCAO.

However, most of the BrdU-positive cells were not co-localized with CM-DiI in NSCs-treated rats, indicating that they were proliferating endogenous cells. As shown in Figure 2C, there was a significant increase of proliferating endogenous cells in NSCs-treated ischemic regions compared to PBS-treated control. These results demonstrated that transplanted NSCs could not only maintain their proliferative capacity in vivo, but also enhanced the proliferation of endogenous cells. However, identification of the specific cell types that were proliferating requires further investigation.

Transplanted NSCs differentiate into neurons and astrocytes in vivo

Immunofluorescent studies were carried out to identify neural stem cells (Nestin), neurons (NeuN) and astrocytes (GFAP) in the lesion zone...
of rats transplanted with C17.2 NSCs at 28 days after MCAO. As shown in Figure 3, few NeuN (1.93 ± 2.38%) and GFAP positive cells (34.36 ± 7.00%) were co-stained with CM-Dil, and most transplanted C17.2 NSCs (55.00 ± 12.29%) appeared undifferentiated as shown by double-labeling with nestin, a specific marker for neural progenitor cells. These data suggested the C17.2 NSCs may differentiate into neurons and astrocytes in vivo.

Discussion

In present study, we demonstrated that intravenous administration of C17.2 NSCs 24 hours after cerebral ischemia could significantly promote the recovery of neurological deficits in MCAO rats compared to PBS-treated control. However, the improvement of neurological function was not accompanied by a reduction in cerebral infarction volume detected by TTC staining. Intravenous infusion of stem cells has been shown to reduce infarct volume post-MCAO in rats using human umbilical cord blood cells [18], adipose tissue-derived mesenchymal stem cells [19], and rodent fetus forebrain-derived neural stem cells [20]. Nevertheless, these findings are consistent with our results in this study and others’ reports using placenta-derived mesenchymal stromal cells [21]. These differences may be attributed to the stem cells source and their distinct neurotrophic factor production, the administration timeline, number of cells administered, or differences in the ischemic stroke models with either transient or permanent MCAO.

Intravenously injected C17.2 NSCs pre-labeled with red fluorescent dye CM-Dil were found in the ischemic border and infarct region of the ischemic hemisphere. These results suggest that infused C17.2 NSCs can transverse the blood-brain barrier without the use of a permeabilizer such as mannitol. Ischemic stress likely resulted in a partial disruption of the blood-brain barrier which may have allowed the transplanted C17.2 NSCs access to the parenchyma. However, there was no fluorescent signal detected on the contralateral side. It was reported that stroma derived factor 1 (SDF-1) and C-X-C chemokine receptor 4 (CXCR4) mediated the migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesions in rats [22]. Intracerebrally transplanted human NSCs into the contralateral hemisphere also migrated to the injured cortex with increased SDF-1 expression in a hypoxic-ischemic cerebral injury model [23]. Since approximately 55% of C17.2 NSCs are undifferentiated (Figure 3), we speculate that the homing of intravenously injected C17.2 NSCs into infarcted brain may also involve the interaction of SDF-1 and CXCR4 [24]. However, this requires further investigation in the future.
Considering that the improvement in neurological function was not correlated with a reduction of cerebral infarction volume, we next investigated the proliferative capacity of infiltrated C17.2 NSCs in the ischemic region 2 weeks after transplantation. Approximately 86% of C17.2 NSCs in the ischemic hemisphere were identified as BrdU-positive. Thus, most intravenously engrafted C17.2 NSCs maintained their aggressive proliferation for at least 14 days after MCAO in the ischemic microenvironment. However, most BrdU-positive cells were not colocalized with CM-DiI, indicating that they were proliferating endogenous cells. We observed a significant increase of proliferating endogenous cells in C17.2 NSCs-treated rats compared to PBS-treated controls (Figure 2). These results demonstrate that transplanted C17.2 NSCs can promote the proliferation of endogenous cells. It has been previously reported that intravenously transplanted human bone marrow mesenchymal stem cells can promote angiogenesis and intrinsic neurogenesis in MCAO rats [25, 26]. The endogenous proliferating cells in the infarct area in our study may therefore be endothelial cells and intrinsic NSCs. However, these identifications require additional experiments in the future.

Injected human NSCs have been shown to differentiate into astrocytes (GFAP) and neurons (NeuN) in the adult rat brain with experimental focal ischemia [27, 28]. We have now identified the differentiation potential of transplanted C17.2 NSCs in rat brains injured with MCAO. Although some grafted C17.2 NSCs expressed proteins specific for neurons and astrocytes, most transplanted C17.2 NSCs still maintained NSC properties at 28 days after MCAO.

Taken together, our results show that intravenously transplanted mouse-derived C17.2 NSCs 24 hours after induction of stroke can improve neurological function recovery although lesion sizes did not differ significantly from PBS-treated rats. The C17.2 NSCs in the ischemic brain were partly differentiated into neurons and astrocytes, which may contribute to the improvement in neurological function of ischemic stroke rats. In a conclusion, the NSCs could migrate into the damaged areas, maintain their proliferative capacity and also enhance the proliferation of endogenous cells in the ischemic rat brain.

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Disclosure of conflict of interest

None.

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