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Original Article

Neural stem cell transplantation promotes behavioral recovery in a photothrombosis stroke model

Junning Ma[1,2*], Junwei Gao[1,2*], Boru Hou[1,2*], Jixing Liu[1], Sihua Chen[1], Guizhong Yan[1], Haijun Ren[1,2*]

[1]*Department of Neurosurgery, Lanzhou University Second Hospital, Lanzhou 730000, Gansu Province, China;* [2]*Lanzhou University, Lanzhou 730000, Gansu Province, China.* [*]*Equal contributors and co-first authors.*

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Abstract: Stem cell-based therapy provides a promising approach for treat stroke. Neural stem cells isolated from mice hippocampus possessing the capacity of differentiate into neurons and astrocytes both in vitro and vivo. Here, we investigated the capability of neural stem cell transplantation in photothrombosis stroke model. Nissl staining revealed that the cortical infarct significantly decreased by 16.32% (Vehicle: 27.93le: an mm^3, n=6, NSC: 23.37le: ai mm^3, n=6, P<0.05) in the NSC group compared with the vehicle. More over transplantation of neural stem cells significantly (P<0.01) improved neurological performance compared with vehicle. These results indicate that transplantation of neural stem cell is an effective therapy in ischemic stroke.

Keywords: Neural stem cell, transplantation, ischemic stroke, photothrombotic model, functional recovery

Introduction

Stroke, 87% of which is ischemic stroke, is one of the most common cause of death and disability around the globe and 1/6 of all humankind will suffer at least one stroke in their lives [1-3]. Furthermore, death, permanent disability, and declined quality of life are outcomes of the natural process of strokes among patients who suffered from ischemic event [4-6]. These consequences of ischemic stroke causing a huge economic burden for both individual and society. However, developing effective therapies to treat stroke currently constitutes manifold challenge for both basic and clinical researchers. Variability of preclinical evaluation and numerous clinical trial failures culminated in the Stroke Therapy Academic Industrial Roundtable (STAIR) guidelines for the preclinical evaluation of candidate drugs [7-10]. Fortunately, a large body of evidence now suggests that stem cell-based approaches may exert reparative and neuroprotective effects in several experimental stroke models [11-17]. In accordance with previous studies, the neuroprotective efficacy of transplantation of neural stem cell in neurologic performance recovery has been shown in photothrombosis model by our studies. Transplantation of neural stem cells can induce infarct volume and neurological deficits after ischemia stroke by replacing neurons or by trophic actions, including neuroprotection, cell rescue via trophic support, promotion of endogenous neurogenesis, immunomodulation and axonal plasticity [18-20]. In light of the evidence for the efficacy of grafted neural stem cell, stem cell-based therapy may prove to be a novel therapeutic candidate for ischemia stroke.

Materials and methods

Animals

Adult male C57BL/6 mice, weighing 20 to 25 g, were used for the experiments. Animals were housed on a 12: 12-h light/dark cycle and environmental temperatures were maintained at 18-22°C. Food and water were freely available. All animals were handled and cared for in accordance with the Guide of Care and Use of Laboratory Animals approved by the ethic Committee of Experimental Animals of Lanzhou University.

NSC culture

The new born 1-2 day mice were killed by rapid decapitation, followed by immediate removal of the brain and its surrounding membranes.

Primary cultures were established from hippocampus of the brain. Dissociated hippocampus tissue was digested with 0.5% trypsin (Invitrogen, Singapore) for 15 min, dissociated mechanically. After two washing steps with DMEM/F12 (Gibco), cells were exposed to the mitogen EGF in serum-free conditions. Obtained neurospheres of NSCs were grown in DMEM/F12 cultue media with 15 mM HEPES-buffer solution (Hyclone Laboratories, Logan, UT) and antibiotics supplemented with B27-supplement (1:50; Invitrogen) in the presence of EGF (20 ng/ml). Cells were cultured in noncoated 25-cm^2 Nunc (Thermo Fisher Scientific, Roskilde, Denmark), flasks at clonal density (1×10^5 cells/ml), and the media was changed every3 to 4 d.

NSC differentiation

To induce differentiation of progeny derived from NSCs, neuralsphere were plated in dishes coated with a suitable that allowed attachment of cells. For coating the culture dishes we used the following solutions of 5 μg/ml poly-L-lysine (Sigma, St. Louis, MO) (dishes incubated 30 min at 37°C, rinsed twice with distilled water and allowed to air dry). The cells were incubated in DMEM/F12 (devoid of growth factors) supplemented with 10% FBS (Sigma). Under these conditions, neurospheres attached to the substrate and spread in a continuous layer. The media were changed every 3-4 days. Following 8-10 days in vitro, the cells were fixed with 4% paraformaldehyde for 30 min and processed for immuncoytochemistry. For immunocytochemical examination, fixed cells were washed two times in PBS containing 5% Triton X-100 (Sigma). They were the incubated with primary antibody at 4°C overnight. The following antibodies were used to identify cell phenotypes: anti-β-tubulin III antibody (Sigma; 1:200), anti-GFAP (Sigma; 1:200), anti-nestin (Sigma; 1:200), and anti-Brdu (Sigma; 1:500). Following washing with PBS, the cells were incubated with goat anti-mouse or goat anti rabbit secondary antibodies conjugated with FITC (Sigma; 1:200) or TRITC (Sigma). Nuclei were counterstained with Hoechst 33258 (Sigma; 1:200).

Intracerebral transplantation

Hippocampal NSCs were passaged 5-6 times before transplantation. And the NSCs were transplanted 2 day after the onset of stroke, using a 20-ul Hamilton syringe with a 33 G needle attached to a stereotaxic apparatus (David Kopf instruments). Two Days before transplantation, fluorescence immunocytochemistry was carried out to examine the expression of an NSCs marker (anti-nestin antibody; Sigma, 1:200), self-proliferation marker (anti-BrdUa, Ssigma, 1:500) and differentiation markers, including anti-β-tubulin III antibody (Sigma, 1:200) and anti-GFAP antibody (Sigma, 1:200) according to previous studies [21]. On the day of transplantation, neurospheres were centrifuged and resuspended in Hank's Balanced Salt Solution (Gibco). The neurosphere suspension had a concentration of 10x104-10x105 viable cells/μL and was kept on ice throughout the transplantation procedure. The neurosphere suspension (10 μL) was delivered to the right hippocampus of each mouse opposite side to the site of injury (2.0 mm lateral; 1.0 mm posterior of bregma).

Photothrombosis model

Cerebral focal ischemia was induced by photothrombosis [22, 23]. C57BL/6 mice were anesthetized intraperitoneally with ketamine (100 mg/kg body weight) and xylazine (15 mg/kg body weight). Rectal temperature was maintained at 37°C using a heated blanket with feedback control (JR-1/2, Taimeng. China). The scalp was incised from midline and the skull was exposed. To Immobilize the head, the skull glued to a stainless steel plate, which was screwed down to two lateral bars on a metal base. The sensorimototor region of cortex (2.0 mm lateral; 1.0 mm posterior of bregma) was chosen and a~1.0×1.0 mm region of skull was thinned using a high-speed dental drill. Mice were injected with 0.6% rose Bengal (RB) in phosphate-buffered saline (PBS) (24 mg/kg) via the tail vein. The cortical micro vessels of the thinned region were illuminated with a beam of green light for 3 min. The scalp was sutured after stroke induction. Mice were monitored until fully awake and then were returned to their home cages. Sham surgery controls were treated in an identical manner but without green light illumination. The mice ored at 4 h after surgery according to Bederson neurological scores methods (Bederson et al. 1986) by a blinded assessor. Animal reaching score 2-3 were considered to have severe stroke and were chosen for experiments.

Fluoro-Jade C staining

Pretreatment with alcohol-sodium hydroxide mixture, the sections were immersed in a solution containing 1% sodium hydroxide in 80% alcohol for 5 min; pretreatment with potassium permanganate, the sections were then transferred into a solution of 0.06% potassium permanganate for 10 min, and rinsed in distilled water for 2 min; FJC staining, the sections were immersed into 0.0001% solution of FJC dye (FJC, AG325, Lot No. 0602022284, Chemicon, U.S.A) dissolved in 0.1% acetic acid vehicle (PH 3.5) and stained for 10 min; post-treatment with distilled water wash, after incubation in the FJC working solution, the slides were washed three times in distilled water each foe 1 min and left to dry overnight in darkness at room temperature; FJC stain examination under fluorescent microscope, sections were air-dried, dehydrated in ethanol, cleared in xylene and coversliped with D.P.X. Finally, the FJC-stained sections were examined under an epifluorescence microscope or a laser scanning confocal microscope (LSCM, Olympus, FV300). The FJC-positive stain exhibited strong green color by using a filter system the same as that used for activating fluorescein. The images were captured from SNc of individuals for demonstration or semiquantitation analysis.

Rotarod test

The Rotarod test was performed to evaluate motor coordination and balance. Before surgery, all animals were first pre-trained on the Rotarod was accelerated from 10 rpm to 14 rpm within 390 s. Each mouse that fell was returned on the rod until the training was over. Training consisted of three sessions, Each session included three separate trials, and there was a 5 min interval. Mice were evaluated in order to select those able to walk on the rotating rod under the same conditions used in the test. After 3-day training, most animals in our studies surpassed 390 s of walking on the Rotarod. Only those able to walk on the Rotarod for at least 390 s were used in the experiment. The length of time walking on the Rotarod was recorded in the test. Two consecutive passive rotations, without walking, but accompanying the rod were considered as a fall. The mean was used for statistical analysis.

Grip strength test

The forelimb grip strength test was performed with a homemade grip device attached to a highly sensitive force transducer (Xinhang, China, JZ300) that measures peak force of the forelimbs. The mouse tail was dragged backwards at constant speed when both forelimbs gripped the grasping-bar of a metal triangle stand. When mouse forelimbs loosened, the maximal force was recorded by the Biological Data Acquisition & Analysis System (Taimeng BL-420F in China). Grip strength was measured five times for each animal and the mean was recorded and used for statistical analysis. Before surgery, the mice with the forelimb grip strength surpassing 108 g were chosen to use for experiments.

Nissl staining

Animals were transcardially perfused and decapitated at day 7 post-surgery. The brains were dipped in 4% paraformaldehyde for 3 days and sectioned

Into 30 um coronal slices in a vibrating microtome (LEICA TV 1000S). Brain sections were collected serially at 120 um intervals and stained with cresyl violet. Infarct volume was measured using Image J software. To eliminate the contribution of post-ischemic edema to the volume of infarct, infarct volumes were corrected for swelling according to the method of Loihl et al [24]. The resulting Nissl staining displayed dark blue color in a normal region, but light bluecolor in the infarct area of the brain.

Statistical analysis

All results were expressed as the mean ± the standard error of the mean (SEM). All analysis of variance for repeated measures were performed with SPSS 1.70 statistical software. The behavioral tests were subjected to two-way ANOVA. Mean comparisons were used for Post Hoc Tests analyses of repeated ANOVAs. Cerebral ischemic volumes were analyzed by One-way ANOVA. Mean comparisons were used for Post Hoc Tests analyses of One-way ANOVA. Value of $P<0.05$ was considered statistically significant and $P<0.001$ was considered statistically highly significant.

Results

Hippocampal neurosphere formation and differentiation in vitro

The dissociation of single cells (**Figure 1Aa**) isolated from hippocampus proliferated in res-

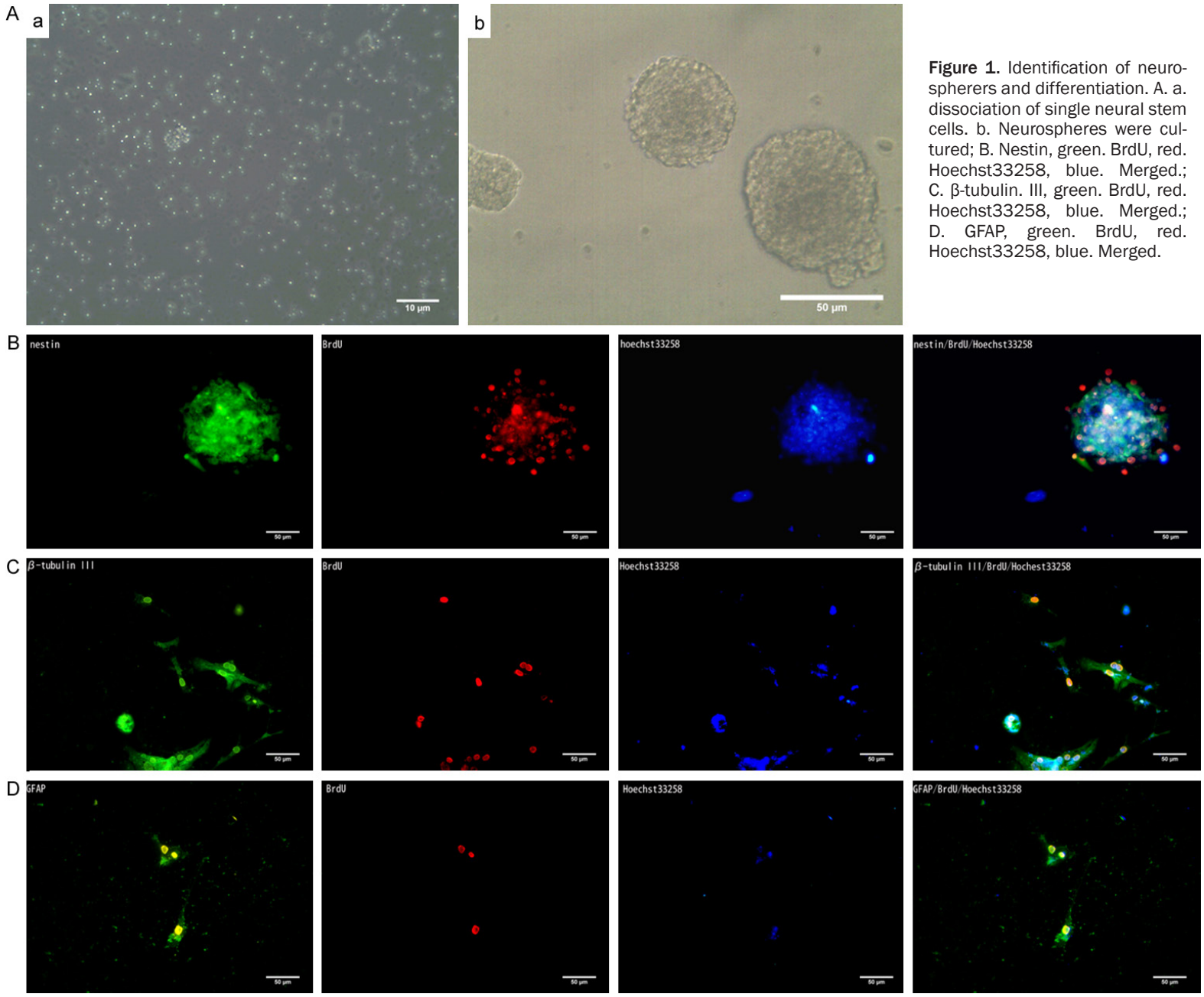

Figure 1. Identification of neurospherers and differentiation. A. a. dissociation of single neural stem cells. b. Neurospheres were cultured; B. Nestin, green. BrdU, red. Hoechst33258, blue. Merged.; C. β-tubulin. III, green. BrdU, red. Hoechst33258, blue. Merged.; D. GFAP, green. BrdU, red. Hoechst33258, blue. Merged.

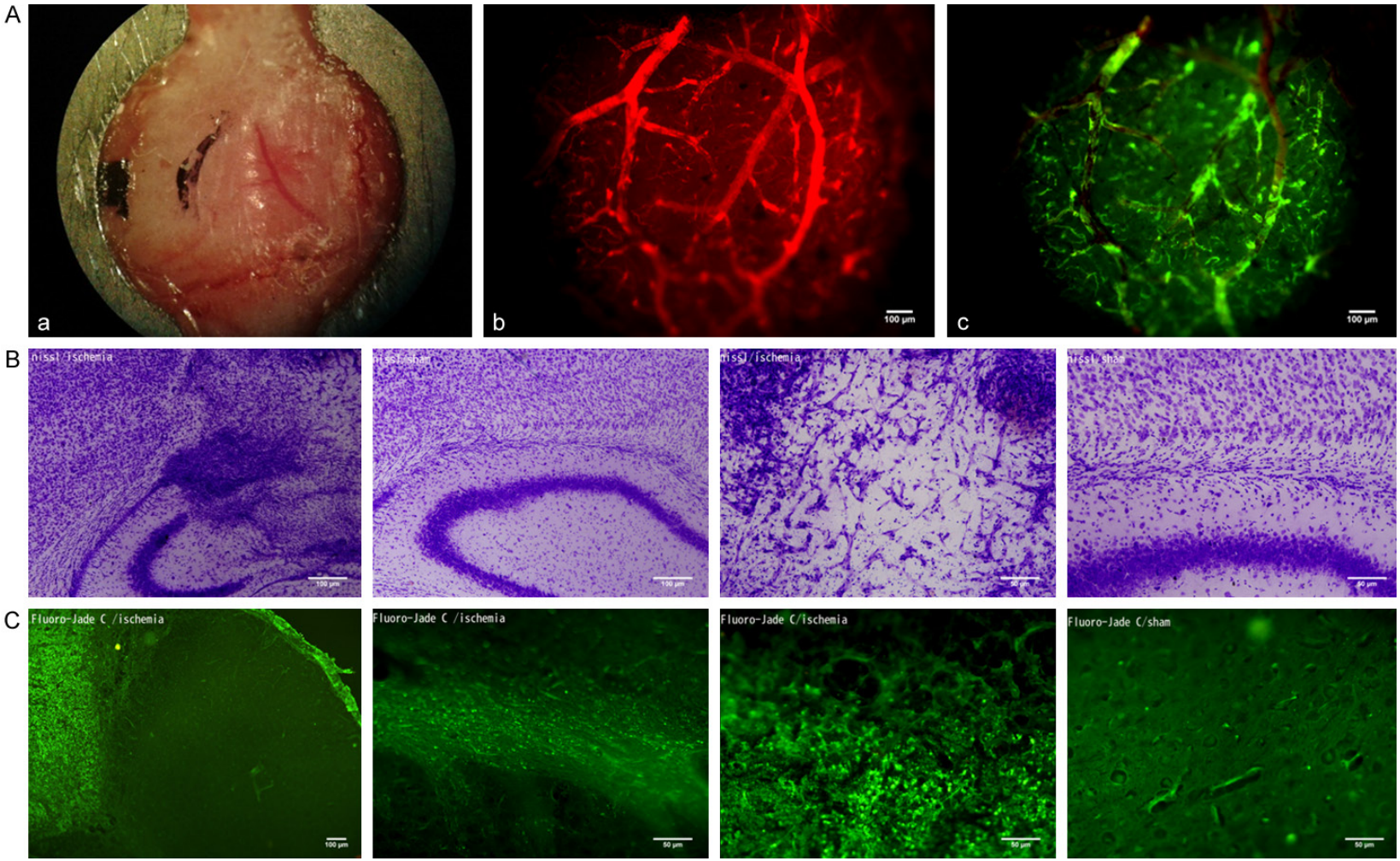

Figure 2. Pathology of photothrombosis model. A. a. The sensorimotor region of cortex. b. Sensorimotor cortex was illuminated by green light. c. Thrombosis obstructed blood vessels; B. Morphology of ischemia area by Nissl staining; C. Neuronal degeneration in ischemia region after the insult.

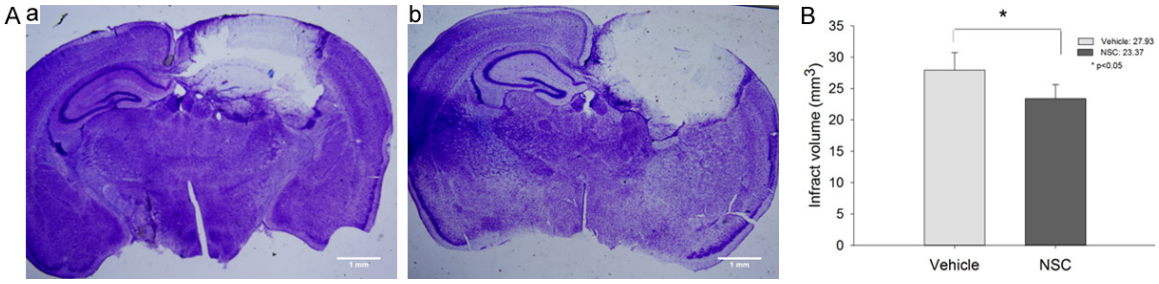

Figure 3. Infarct volume shown by Nissl staining at 7 days after transplantation of neural stem cell. A. a. Representative Nissl stained coronal section at 7 days after transplantation of NSC. b. The coronal section of Vehicle group by Nissl staining; B. Calculated infarct volumes at 7 days after transplantation. Transplantation of neural stem cells significantly reduced infarct size compared to Vehicle group (n=6, P<0.05).

ponse to growth factors in culture medium and formed neurospheres (**Figure 1Ab**). After seven days in vitro, this cell clusters measuring about 50-80 um in diameter consisting of several hundred cells were positive for nestin and BrdU by immunohistochemical stain (**Figure 1B**), showing the potential of neural stem cell for proliferation. Neurosphere can also be differentiated into β-tubulin III-positive neurons (**Figure 1C**) and GFAP-positive astrocytes (**Figure 1D**), which demonstrates the multipotent properties of differentiation of neural stem cells.

Pathology of photothrombosis model

Photothrombotic lesion model was used to induce cerebral ischemia. The right sensorimotor cortex was illuminated by green light for 3 min. Then, we detected glowing thrombosis blocked blood vessels by used red light to illuminate the cortex (**Figure 2A**). To observe histopathological outcome after ischemia, we used Nissl and Fluoro-Jade C staining. The result of Nissl indicative of neuronal loss in ischemia area, particularly in the infarct core at 7 day after the insult. In the sham group, most of the pyramidal neurons had a round or oval nucleus, located in the center of perikarion that are surrounded by pale cytoplasm. In comparison with the control animals, the ischemic group showed the neuronal changes were triangular in shape mostly exhibiting a dark staining due to condensation of cytoplasm and karyoplasms (**Figure 2B**). Fluoro-Jade C-positive staining was not detected in brain sections of sham group. In lesioned animals, Fluoro-Jade C staining showed neuronal degeneration in ischemia region at 7day after stroke. Some degenerating cells were also observed in hippocampus. In the peri-infarct area, there were sporadically distributed degenerating neurons after ischemia (**Figure 2C**).

Transplantation of neural stem cells reduced infarct size after ischemic stroke

To investigate whether transplantation of neural stem cells could facilitate amelioration of ischemic stroke, we measured infarct size at day 7 after NSCs transplantation. We used Nissl staining to measure infarct volume at day 7 after transplantation. Measurements from Nissl staining sections shown that infarct volume in ischemia plus vehicle group is 27.93-num mm^3 (**Figure 3B**). Seven days after transplantation, the cortical infarct significantly decreased by 16.32% (Vehicle: 27.93cle: mm^3, n=6, NSC: 23.37cle: c mm^3, n=6, P<0.05) in the NSC group compared with the vehicle group (**Figure 3B**). These data indicated that transplantation of neural stem cells reduced infarct size after ischemic stroke.

Transplantation of neural stem cells improved behavioural performance after ischemic stroke

We monitored neurologic performance using the Rotarod test and grip strength test. Rotarod test was used to evaluate brain functional recovery and neurological deficits after brain injury. Measures of latency to remain on the Rotarod showed significant group and time effects. There were significant (P<0.01) deficits (**Figure 4A**) in rotator performance (time of walking on the Rotarod) in vehicle group (192.7±17.82 s, 211.67±19.58 s, 219.33±16.66 s, 239.56±19.26 s, 250.44±22.99 s, 247.9±17.27 s, 255.2±17.29 s, 251.8±15.6 s, n=9) and NSC group (189.1±18.14 s, 201.5±14.18 s, 225.7±15.34 s, 246.6±12.1 s,

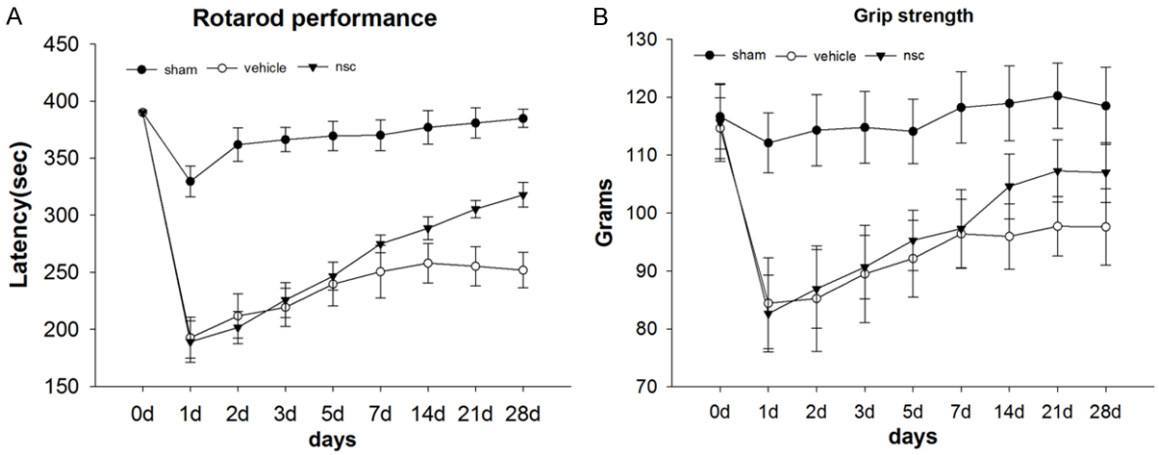

Figure 4. Transplantation of NSCs enhanced functional recovery. A. Motor impairment as assessed using the Rotarod after surgery: transplantation of neural stem cells (n=10, P<0.01) increased the ability of remaining on the Rotarod at all time points after photothrombosis. B. Grip strength: transplantation of NSCs (n=10, P<0.01) has significantly promoted grip strength recovery at day 14, 21, and 28 after ischemia compare with vehicle group (n=9, P<0.01).

274.8±7.57 s, 288.6±10.15 s, 305.3±7.51 s, 317.9±10.75 s, n=10) at all time points after surgery compared with shams (329.6±18.14 s, 361.8±14.18 s, 366.2±15.34 s, 369.4±12.1 s, 370.1±7.57 s, 377±10.15 s, 380.8±7.51 s, 384.8±10.75 s, n=10). Compared with vehicle group performance, transplantation of neural stem cells significantly (*P*<0.01) improved the ability of remaining on the Rotarod at all time points within 28 days after ischemia. We next evaluated the effect of transplantation of neural stem cell on forelimb grip strength. Transplantation of neural stem cells improved grip strength after ischemic stroke (**Figure 4B**). There were significant group effects. The grip strength in sham group had a slight decrease at the first day after surgery due to surgery effect, and then returned to pre-surgery level on the second day after surgery. The forelimb grips strength decreased significantly (P<0.01) in vehicle group (84.42±7.87 g, 85.24±9.11 g, 89.48±8.42 g, 92.16±6.63 g, 95.96±5.65 g, 97.91±5.15 g, 97.61±6.59 g, n=9) and NSC group (82.65±6.65 g, 86.90±6.8 g, 90.65±5.46 g, 95.27±5.18 g, 97.30±6.73 g, 104.60±5.6 g, 107.29±5.38 g, 106.99±5.15 g, n=10) at all the time points after surgery compared with sham group (112.99±5.18 g, 114.30±6.15 g, 114.79±6.19 g, 116.99±5.56 g, 118.23±6.13 g, 118.94±6.46 g, 120.24±6.46 g, 118.50±6.67 g, n=10). Transplantation of neural stem cells significantly improved forelimb grip strength at day 14, 21, and 28 after ischemia compared with vehicle group. These dada suggested that transplantation of neural stem cells improved behavioural performance after ischemic stroke.

Survival and differentiation of grafted neural stem cells in vivo

Twenty-eight days after transplantation, fluorescent staining of BrdU-labeled grafts results demonstrated the survival of the grafts (**Figure 5A**). Grafted NSCs appeared around the site of the needle track and migrated to the ischemia region. No signs of tumor formation caused by the grafted NSCs were detected in any of the mice. To investigate the proliferation capacity and differentiation profiles of the grafted NSCs, we used double immunofluorescence labeling with an anti-BrdU antibody specific grafted NSCs and various neural specific markers. The grafted NSCs elaborated neuronal lineages as demonstrated by the coexpression of BrdU and β-tubulin III (**Figure 5B**). And the immunocytochemistry for BrdU and the astrocytic marker GFAP confirmed the differentiation into astrocytes (**Figure 5C**).

Discussion

Stem cell therapies are a promising approach to treat stroke and other neurodegenerative disorders because of the potential to replace lost brain cells [12, 25-29]. In the present study, we evaluated the effects of transplantation of

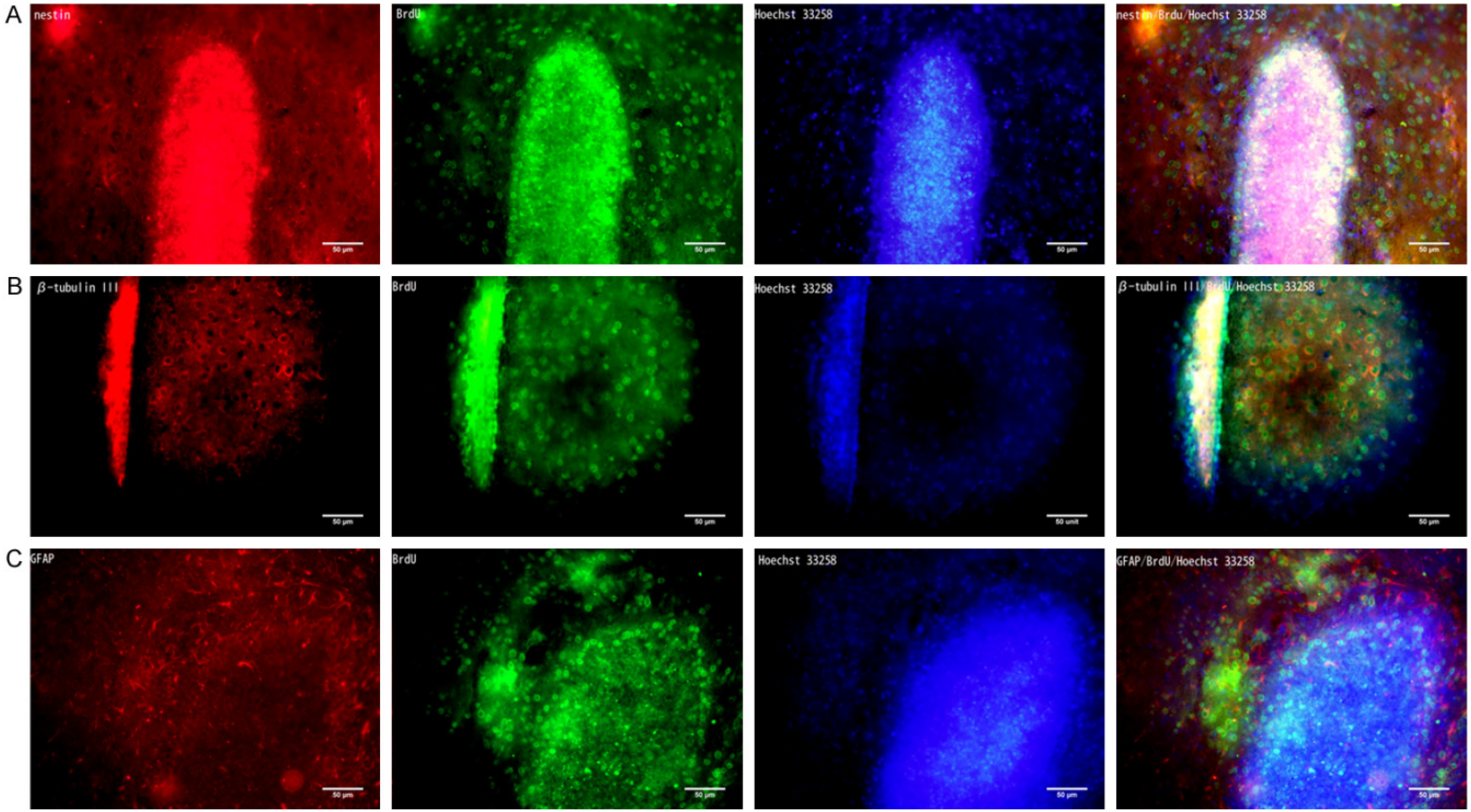

Figure 5. Survival and differentiation of grafted neural stem cells in vivo. A. Nestin (red) and Brdu (green) positivity was detected in around the site of the needle track demonstrated the survival of the grafts. B. Fluorescent staining with β-tubulin III (red) and BrdU (green) revealed that the grafted NSCs differentiated into neurons 28 days after transplantation. C. Fluorescent staining with GFAP (red) and BrdU (green) indicated that grafted NSCs differentiated into astrocytes 28 days after transplantation. Nuclei were counterstained with Hoechst 33258 (blue).

neural stem cells on lesion volume and behavioral outcomes after ischemic stroke. Consistent with previous studies [16, 20, 30, 31], we found that transplantation of neural stem cells degraded infarct volume and neurological deficits. In addition, our data demonstrate that neural stem cells derived from hippocampus possessing the capacity of generating both neural and glial cell lineages in vitro and maintained these characteristics upon transplantation in vivo. Moreover, 4 weeks after transplantation, fluorescent staining showed migration of the grafted NSCs from injection sites to the ischemic lesion. Thus, we believe that grafted neural stem cell is an effective and novel approach for the treatment of stroke.

Previous findings showed that time window and appropriate dosage of transplanted cells are critical for the cell survival and phenotypic fate [15, 28, 32-34]. And three studies confirmed that compared with a later time point (6 weeks), better cell survival is achieved via transplantation of the stem cells shortly after stroke (24, 48, 72 hours), and differentiated in a more even distribution between neuronal and astroglial [15, 28, 34]. In consideration of therapeutic efficacy, we implanted NSCs at 48 hours after insult with a dosage of $1x10^6$ cell number. In line with previous studies, our results showed that neural stem cells grafted by this time point and concentration was effective in reducing neurological deficit and infarct volume [28, 34].

Different with previous studies, we have previously used photothrombotic model to evaluate the effect of NSCs transplantation on stroke. This model induces a cortical infarct by the systemic injection of Rose Bengal (a photoactive dye) in combination with irradiation by a light beam at a specific wavelength [22]. By contrast with other stroke models, photothrombotic model has a lot of advantages; it is simpler and much less invasive than surgical model; it is highly reproducible and the size and location of the infarct can be controlled by researchers, provided surface brain regions are targeted [35]. Altogether, photothrombotic model is a valuable tool in the stem cell based therapy for stroke research field. We performed Nissl and FJC staining to observe the pathological outcome of photothrombotic model after stroke. In the present data, neuronal damage and loss in the infarct core has showed by Nissl result, and Fluoro-Jade C stain has successfully identified the neuronal degeneration in the ischemia region at 7 days after stroke. Additionally, we monitored behavioural performance after ischemic stroke by using Rotarod test and grip strength test. The time walking on the Rotarod and the forelimb grip strength are sharply declined by sensorimotor cortex damage following photothrombosis stroke. However, our data of neurological deficit in vehicle group showed a process of limitedly spontaneous recovery. This phenomenon might be explained by endogenous neurogenesis. In the adult mammalian brain, neural stem cells in the subventricular zone and dentate gyrus continue to generate neuronal precursors [36]. In pathological conditions, these neuronal precursors can migrate to the infarct area and differentiate into functional mature neurons that could be integrated into the neuronal circuitry [37, 38]. Nevertheless, only about 0.2% of dead neurons are reportedly replaced by newly generated neurons [39]. Therefore, this spontaneous regeneration is insufficient to induce neurological improvement.

In the present study, we observed that neural stem cell therapy enhanced functional outcome after ischemic stroke, and reduced infarct volume. Moreover, 4 weeks following transplantation, grafted neural stem cells differentiate into neurons and astrocytes. This finding provides evidence that the application of cell therapy is an effective approach to treat ischemia stroke. However, the detailed cellular reparative regeneration mechanisms of transplantation of neural stem cell in ischemic stroke remain largely unclear. Previous studies suggest that neural stem cell transplantation may exert reparative and neuroprotective effects by acting on multiple ways. Aside from its characteristics of multipotency and self-renewal, grafted NSCs has been reported to increasing axonal sprouting and dendritic plasticity following stroke [40], modulating inflammation to altered the environment encountered of the new neurons [20], and promoting endogenous neurogenesis by the stimulation of endogenous neural stem cell [20, 41]. Interestingly, a series of studies showed NSC transplantation may protect the infarct tissue from inflammatory damage via a bystander mechanism rather than direct cell replacement [12, 42, 43].

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Haijun Ren and Junning Ma, Department of Neurosurgery, Lanzhou University Second Hospital, Lanzhou 730000, Gansu Province, China. Tel: +86 13893668727; E-mail: baiyunguan@hotmail.com (HJR); Tel: +86 13679407700; E-mail: 929963181@qq.com (JNM)

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