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Mild hypothermia combined with neural stem cell transplantation for hypoxic-ischemic encephalopathy: neuroprotective effects of combined therapy

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Abstract

Neural stem cell transplantation is a useful treatment for ischemic stroke, but apoptosis often occurs in the hypoxic-ischemic environment of the brain after cell transplantation. In this study, we determined if mild hypothermia (27–28°C) can increase the survival rate of neural stem cells (1.0×10^5 /μL) transplanted into neonatal mice with hypoxic-ischemic encephalopathy. Long-term effects on neurological functioning of the mice were also examined. After mild hypothermia combined with neural stem cell transplantation, we observed decreased expression levels of inflammatory factor nuclear factor-kappa B and apoptotic factor caspase-3, reduced cerebral infarct volumes, increased survival rate of transplanted cells, and marked improvements in neurological function. Thus, the neuroprotective effects of mild hypothermia combined with neural stem cell transplantation are superior to those of monotherapy. Moreover, our findings suggest that the neuroprotective effects of mild hypothermia combined with neural stem cell transplantation on hypoxic-ischemic encephalopathy are achieved by anti-inflammatory and anti-apoptotic mechanisms.

Key Words: *nerve regeneration; brain injury; hypoxic-ischemic encephalopathy; neural precursor cells; hypothermia; neural stem cells; cell transplantation; hippocampus; neuron; cell apoptosis; astrocytes; oligodendrocytes; neuroprotection; NSFC grants; neural regeneration*

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Introduction

Neonatal cerebral hypoxia-ischemia is a common cause of death and neurological disability in children, causing long-term delayed cognitive and behavioral deficits (Edwards et al., 2010; Liu et al., 2012; Yan et al., 2012). Supportive care is the most effective clinical strategy for recovery, but has not yet been successful. Potential use of stem or progenitor cells to reduce brain damage or promote regeneration is a promising strategy that has been tested in different central nervous system disorders and ischemic diseases (Chen et al., 2006). Neural stem cells (NSCs) have the potential to generate cells of both glial and neuronal lineages following loss of primary cells due to cerebral hypoxia-ischemia (Hu et al., 2013; Tu et al., 2013; Zhao et al., 2013; Zhou et al., 2013). NSCs derived from human embryonic stem cells and grafted into the forebrain of postnatal day 7 C57/BL6 mice at 24 hours after cerebral hypoxia-ischemia were able to disperse, integrate, fully differentiate, and show marked sprouting (Daadi et al., 2010). Moreover, there is evidence that cell replacement may be one of the most important mechanisms for improvements in learning and memory abilities (Ma et al., 2007). Nevertheless, we have found that after cerebral hypoxia-ischemia, grafted cells are often induced to be apoptotic by the microenvironment. Therapeutic mild hypothermia exerts strong neuroprotective effects through many mechanisms *e.g.*, decreasing metabolic rate, reducing glutamate release, reducing reactive oxygen species formation, preventing blood-brain barrier destruction, and regulating inflammatory and apoptotic factor expression (Yenari et al., 2008). In the ischemic neonatal rat brain, hypothermia reduces infarct size and functional deficits for prolonged periods of time (Wagner et al., 2002). Thus, the aim of this study was to investigate the underlying mechanism for effectiveness of mild hypothermia combined with NSC transplantation in hypoxic-ischemic encephalopathy (HIE) treatment.

Materials and Methods

Experimental animals and groups

One-week-old C57/BL6 mice (both genders) weighing 6.3 ± 0.3 g were purchased from the Chinese Academy of Sciences, Shanghai, China, and randomly divided into five groups with 50 mice per group: control, HIE, hypothermia, NSC, and hypothermia + NSC.

HIE model preparation and treatment

After anesthesia, the right common carotid artery was ligated through a ventral midline neck incision using a surgical silk suture (Ma et al., 2007). Mice were then placed in a cage for 1.5 hours at a hypoxic atmosphere of 8% O_2 and 92% N_2. The cage was kept in a temperature-controlled water bath to maintain ambient temperature (37°C) inside the cage (Ma et al., 2007). Mice in the control group were subjected to anesthesia only without hypoxia-ischemia intervention. Mice in the HIE group also underwent right common carotid artery ligation and 1.5 hours of 8% oxygen inhalation. Mice in the NSCs group received NSC transplantation after hypoxia-ischemia intervention. Mice in the hypothermia group were placed in a case that was submerged in a water bath at a stable temperature of 27–28°C for 24 hours after hypoxia-ischemia intervention. Mice in the hypothermia + NSCs group received combined therapy of mild hypothermia and NSC transplantation after hypoxia-ischemia intervention. NSC transplantation was performed 1 day after cerebral hypoxic-ischemic injury. The study protocol received approval from the Animal Care and Use Committees of Xinhua Hospital, China.

NSC culture, labeling, and transplantation

The hippocampus was dissected from embryonic C57/BL6 mouse brain and dissociated cells cultured *in vitro* (Wang et al., 2007). Embryonic C57/BL6 mice were obtained from pregnant (13.5 days) female mice (Animal Center of Chinese Academy of Sciences). Cells were seeded on 10 cm^2 dishes coated with fibronectin (Sigma, St. Louis, MO, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 containing B27 (Gibco, New York, NY, USA) and 10 μg/mL mouse recombinant basic fibroblast growth factor (Gibco). After three serial passages, green fluorescent protein (GFP; Invitrogen, Grand Island, NY, USA) was transferred for adenoviral labeling of transplanted cells (Muraoka et al., 2008). NSCs were exposed to infectious viral particles in the culture medium for 12 hours at 37°C. Cells were infected with recombinant adenoviral vectors carrying GFP at a multiplicity of infection (MOI) of 10^2 (Takahashi et al., 2008). GFP expression was confirmed 5 days after transfection and before transplantation. Mice in the NSC and hypothermia + NSC groups received 3-μL NSC transplants (1.0×10^5 cells/μL) 1 day post hypoxia-ischemia intervention. Cells were injected into the right lateral ventricle of the brain (anterior-posterior, 0.02 mm; medial-lateral, 0.75 mm; dorsal-ventral, 2 mm) using a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA, USA).

Brain section preparation and TUNEL assay

At 24, 48, and 72 hours (short-term study) and 1, 2, and 4 weeks (long-term study) after transplantation, mouse brains were post-fixed in 4% paraformaldehyde followed by 30% sucrose (w/v) for 3 days, and then cut into 15-μm sections using a cryostat (MICROM International GmbH, Walldorf, Germany). Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was used to identify cells with nuclear DNA fragmentation in the penumbra and ischemic core. TUNEL staining was performed according to the manufacturer's instructions (Calbiochem, San Diego, CA, USA). TUNEL-positive cells were visualized using a 3,3′-diaminobenzidine kit (Abcam, Cambridge, UK). Sections were counterstained with methyl green and examined using a light microscope (PM-20; Olympus, Tokyo, Japan). For each section, cells were counted in 10 fields of view (400 ×) from four regions of interest.

Western blot analysis

Caspase-3 and nuclear factor-kappa B (NF-κB; p65) protein expression were analyzed by western blots to determine involvement of apoptosis and inflammation in neuroprotection. Cleaved caspase-3 is the biologically active form of intact caspase-3 and induces apoptosis. Brain tissue was homogenized in ice-cold tissue extraction buffer (Invitrogen, containing 1% protease inhibitor cocktail. Samples containing 20 μg protein were loaded into each well of NuPAGE precast 8–16% Bis-Tris gels (Invitrogen) (Lin et al., 2011). After electrophoresis, proteins were transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked in NuPAGE blocking buffer (Invitrogen) and then incubated with primary antibodies, mouse anti-monoclonal antibody β-actin (1:10,000), NF-κB (p65) (1:5,000), and caspase-3 active form (1:5,000) (all Invitrogen), at room temperature for 2 hours. After washing, membranes were incubated with horseradish peroxidase-conjugated sheep anti-mouse antibody at room temperature for 1 hour and processed using ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA). Protein bands were then imaged (Kodak Image Station 179 4000R station, NY, USA).

Measurement of cerebral infarct volume

Brains were sliced into six 2-mm coronal sections at 1, 2, and 4 weeks after hypoxia-ischemia intervention (Sakurazawa et al., 2012). Digital images of all sections stained with 2,3,5-triphenyltetrazorium chloride (TTC; Sigma) were obtained to determine cerebral infarct volumes. Infarct volumes were calculated by integrating lesion areas (Swanson et al., 1990).

Immunofluorescent staining and cell counting

At 1, 2, and 4 weeks after hypoxia-ischemia intervention, mice were perfused as described previously (Kelly et al., 2004). Primary antibodies, mouse anti-NeuN (Billerica, Millipore, MA, USA), rabbit anti-GFAP (Chemicon, Tokyo, Japan), and rabbit anti-CNPase (Abcam) were added and incubated at 4°C overnight. Secondary antibodies were incubated for 90 minutes at room temperature and visualized by Avidin-Alexa 594 (Invitrogen) treatment. Sections were examined using a Nikon Eclipse TS100 fluorescent microscope (Nikon, Tokyo, Japan; Zádori et al., 2011). For each section, cells were counted in 10 fields of view (400 ×) from four regions of interest.

Behavioral testing

Each mouse was subjected to a series of behavioral tests

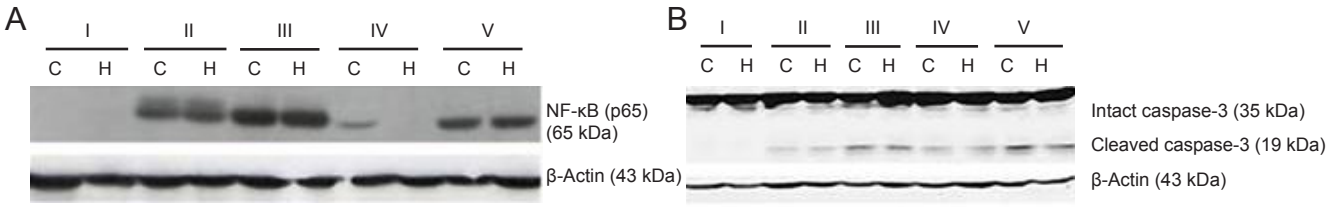

Figure 2 Effect of mild hypothermia combined with NSC transplantation on caspase-3 and NF-κB protein expression in the ischemic cerebral cortex and hippocampus of mice with HIE at 24 hours after cell transplantation (western blot analysis).
Both caspase-3 and NF-κB were virtually absent in the hypothermia and hypothermia + NSCs groups, but expressed in the HIE and NSCs groups. C: Ischemic core of the cortex; H: ischemic core of the hippocampus; HIE: hypoxic-ischemic encephalopathy; NSC: neural stem cell; NF-κB: nuclear factor-κB. I: Control group; II: hypothermia group; III: HIE group; IV: hypothermia + NSCs group; V: NSCs group.

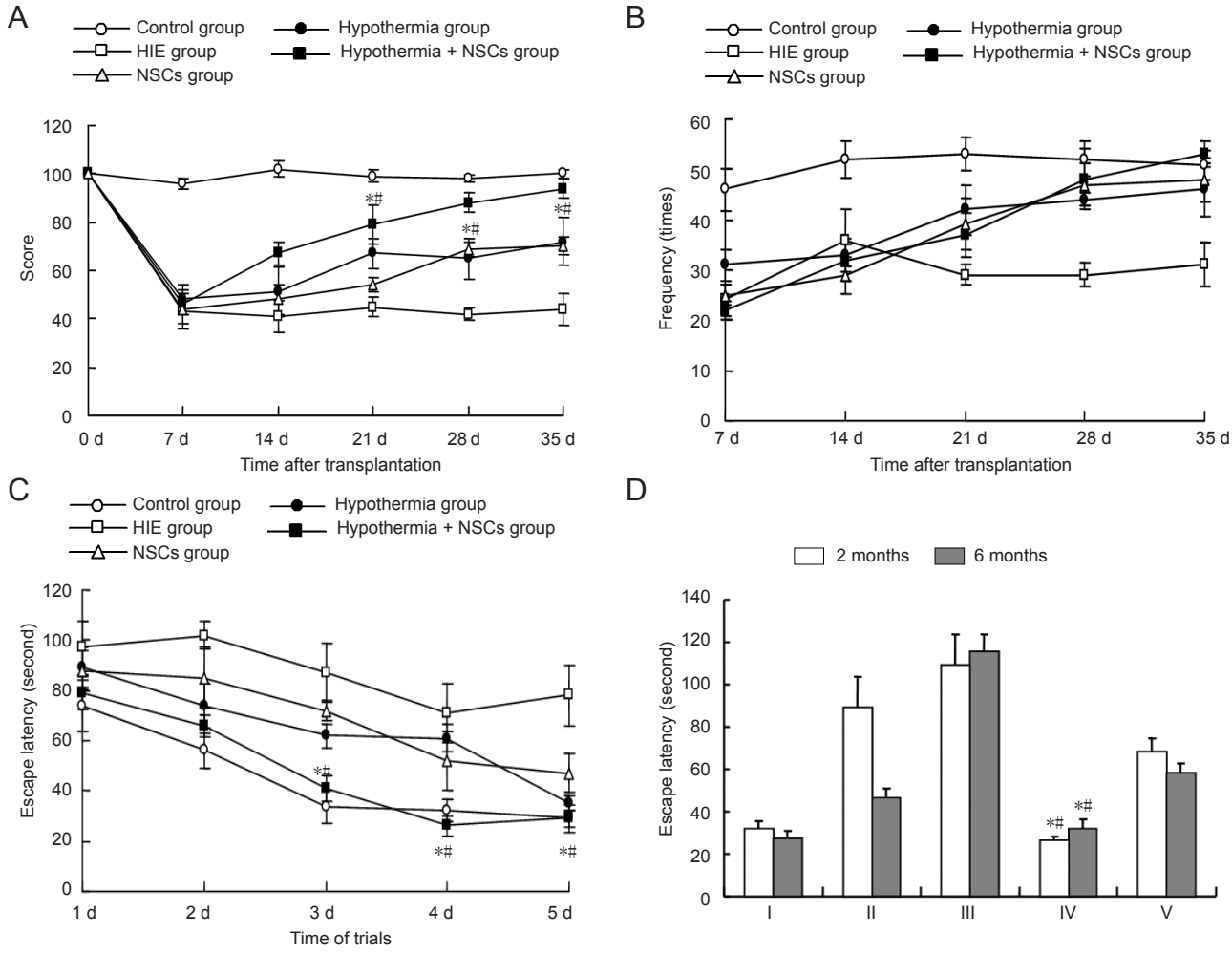

Figure 4 Ameliorated behavior in mice with HIE after treatment with mild hypothermia combined with NSC transplantation.
(A) Rotarod test. Mice in the hypothermia + NSCs group exhibited significantly improved behavior at 7, 14, 21, 28, and 35 days after cell transplantation. (B) Cylinder test. There were no significant behavioral improvements between groups. (C) Morris water maze test. Training experiments were performed at 2 and 6 months after cell transplantation. Escape latencies were significantly shortened by repeated training in the hypothermia + NSCs group. (D) Morris water maze test. The last trial was performed on day 6. $*P < 0.05$, *vs.* HIE group; $\#P < 0.05$, *vs.* NSCs group. Data are expressed as the mean ± SD ($n = 50$ mice per group). Two sample *t*-tests were performed. HIE: Hypoxic-ischemic encephalopathy; NSC: neural stem cell. I: Control group; II: Hypothermia group; III: HIE group; IV: hypothermia + NSCs group; V: NSCs group; d: day(s).

during the 5-week period after cell transplantation. The rotarod test (Shinano-Seisakusyo, Tokyo, Japan) determined hemiparesis and coordinated movements. The rotarod was rotated at a speed that slowly increased from 4 to 40 r/min within 5 minutes. The time the mice remained on the rotarod was measured. Data after hypoxia-ischemia intervention are presented as percentage compared with baseline control before hypoxia-ischemia intervention (Takahashi et al., 2008). The cylinder test examined spontaneous movements (Schallert et al., 2000). Mice were placed in a transparent acrylic cylinder (diameter 6 cm; height 10 cm), and the number of wall contacts made by the forelimbs was counted within 5 minutes. Wall contacts were classified as contralateral forelimb (CF), ipsilateral forelimb (IF), or

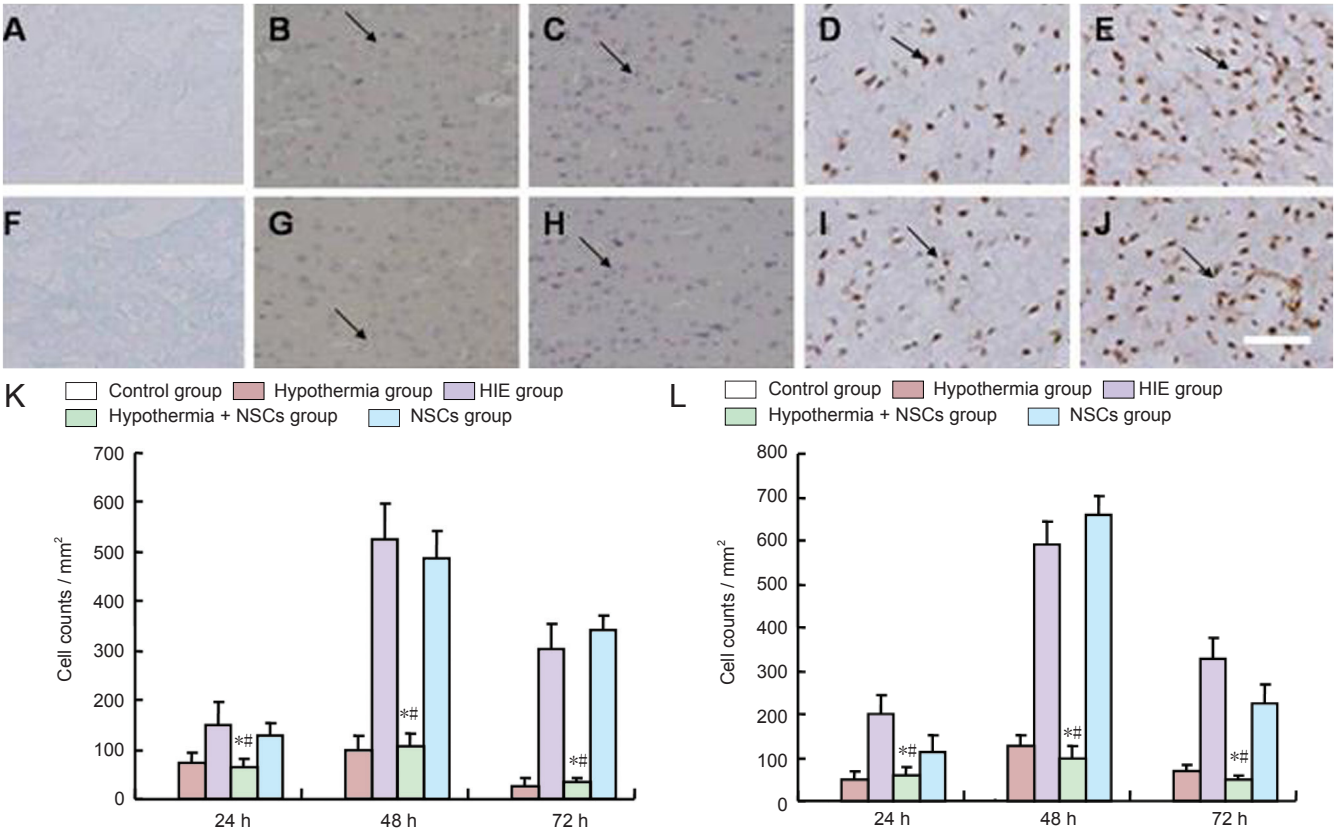

Figure 1 Effect of mild hypothermia combined with NSC transplantation on TUNEL-positive cells in the penumbra and ischemic core of mice with HIE at 24 hours after cell transplantation (TUNEL staining).
Ischemic core of (A–E, K) cerebral cortex and (F–J, L) hippocampus; (A, F) control group; (B, G) hypothermia group; (C, H) HIE group; (D, I) hypothermia + NSCs group; (E, J) NSCs group. Arrows indicate TUNEL-positive cells. Scale bar: 100 μm. (K, L) TUNEL-positive cell number in each group. ∗$P < 0.05$, *vs.* HIE group; ♯$P < 0.05$, *vs.* NSCs group. Data are expressed as the mean ± SD ($n = 50$ mice per group). Two sample *t*-tests were performed. NSC: Neural stem cell; HIE: hypoxic-ischemic encephalopathy; h: hours.

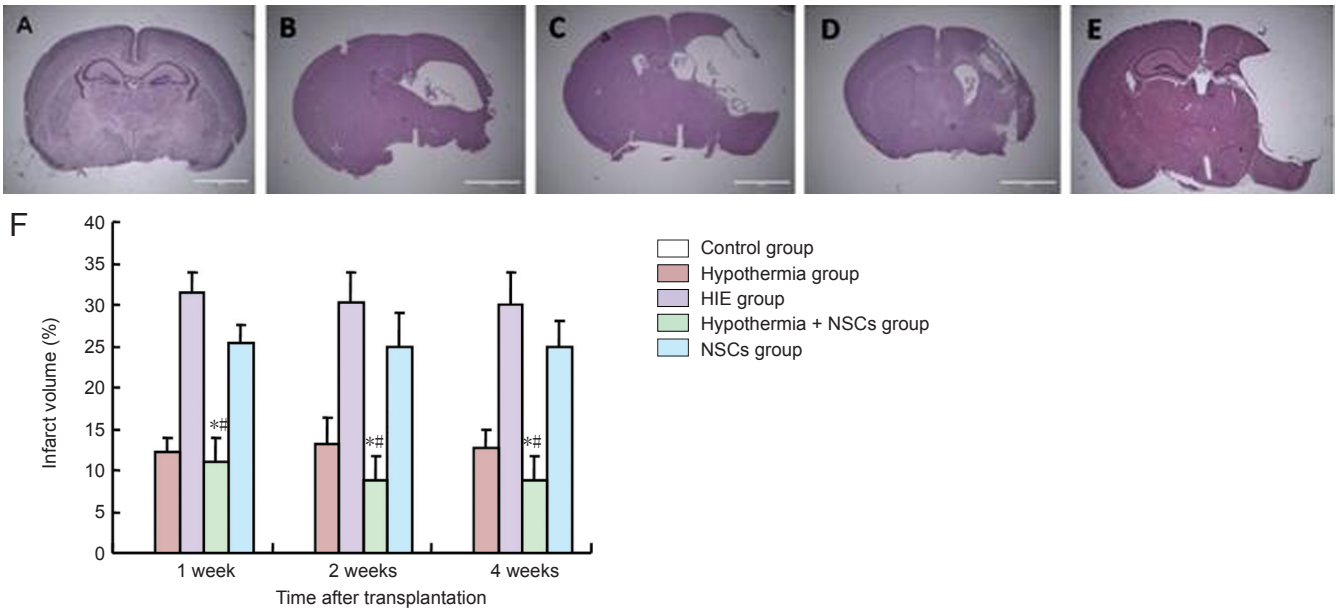

Figure 3 Effect of mild hypothermia combined with NSC transplantation on infarct volume in mice with HIE.
Hematoxylin-eosin staining at 4 weeks: (A) control group; (B) hypothermia group; (C) HIE group; (D) hypothermia + NSCs group; (E) NSCs group. Scale bars: 2 mm. (F) Significantly smaller infarct volumes in the hypothermia + NSCs group compared with the NSCs group. Data are expressed as the mean ± SD ($n = 50$ mice per group). Two sample *t*-tests were performed. ∗$P < 0.05$, *vs.* HIE group; ♯$P < 0.05$, *vs.* NSCs group. HIE: Hypoxic-ischemic encephalopathy; NSC: neural stem cell.

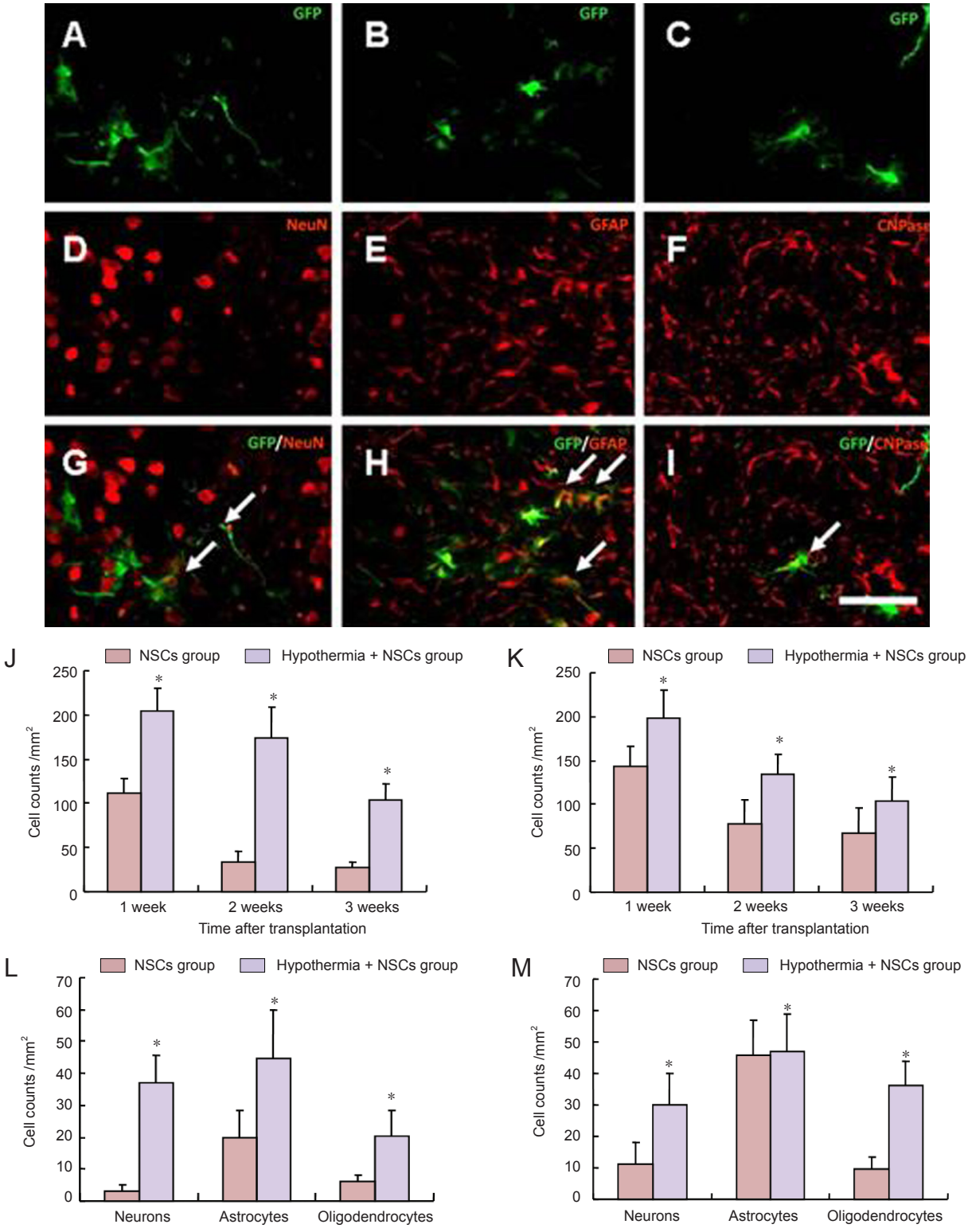

Figure 5 Effect of mild hypothermia combined with NSC transplantation on survival and differentiation of transplanted cells (GFP$^+$) in the brain of mice with HIE.

NeuN (A, D, G), GFAP (B, E, H), and CNPase (C, F, I) immunostaining demonstrates that transplanted cells can differentiate into neurons, astrocytes, and oligodendrocytes, respectively. (A–C) GFP (green); (D–F) NeuN, GFAP, and CNPase staining (red); (G–I) merged images. Arrows indicate GFP$^+$ cells that have differentiated into neurons (NeuN$^+$), astrocytes (GFAP$^+$), and oligodendrocytes (CNPase$^+$). Scale bar: 100 μm. (J, K) Effect of hypothermia combined with NSC transplantation on survival of transplanted cells in the cortex and hippocampus, respectively. (L, M) Effect of hypothermia combined with NSC transplantation on cell differentiation in the cortex and hippocampus, respectively. $*P < 0.01$, *vs.* NSCs group. Data are expressed as the mean ± SD ($n = 50$ mice per group). Two sample *t*-tests were performed. GFP: Green fluorescent protein; NeuN: neuronal nuclear antigen; GFAP: glial fibrillary acidic protein; CNPase: 2′,3′-cyclic nucleotide 3′-phosphodiesterase; HIE: hypoxic-ischemic encephalopathy; NSC: neural stem cell.

both forelimbs (BF). Percentage of CF and BF use relative to the total number of contacts was calculated as follows: total percentage (CF + BF/2) / (CF + BF + IF) × 100 % (Lee et al., 2010). The Morris water maze test examined cognitive function. Morris water maze training experiments were performed at 2 and 6 months after cell transplantation. Mice were trained and tested in a 1-m diameter and 50-cm-high circular pool on 4 consecutive days. On the fifth day, mice were released into the water under the same conditions and the ability to find the platform evaluated as a test of memory recall. Time to find the platform is described as the escape latency. Latency was recorded for each trial (Johnston et al., 2001). Swimming trajectories were recorded using a computerized video system (San Diego Instruments, San Diego, CA, USA) that enabled the time spent in various zones of a standard, computerized grid design to be calculated and used to score animal performance in recalling platform location.

Statistical analysis

Data were statistically analyzed using SPSS 11.0 statistical software (SPSS, Chicago, IL, USA) and expressed as the mean ± SD. Differences between groups were compared using two sample *t*-tests. Statistical significance was set at $P < 0.05$.

Results

Effect of mild hypothermia combined with NSC transplantation on TUNEL-positive cells in the cerebral cortex and hippocampus of mice with HIE

TUNEL staining was performed 24 hours after cell transplantation. TUNEL-positive cell number significantly increased in the cerebral cortex and hippocampus in HIE, hypothermia, NSCs, and hypothermia + NSCs groups compared with controls. However, TUNEL-positive cell number significantly decreased in hypothermia and hypothermia + NSCs groups compared with NSCs and HIE groups ($P < 0.05$; **Figure 1**). In the ischemic core of the cortex and hippocampus, TUNEL-positive cell number decreased in the hypothermia + NSCs group compared with HIE and NSCs groups ($P < 0.05$).

Effect of mild hypothermia combined with NSC transplantation on caspase-3/NF-κB expression in the cerebral cortex and hippocampus of mice with HIE

At 24 hours after cell transplantation, there were striking differences between the five groups in NF-κB protein expression in the cerebral cortex and hippocampus. Moreover, caspase-3 (which mediates the apoptotic mechanism) was cleaved and activated, as shown by western blot analysis of cell lysates. In ischemic cerebral cortex and hippocampus of HIE mice at 24 hours after cell transplantation, cleaved caspase-3/NF-κB were virtually absent in the hypothermia and hypothermia + NSCs groups, but more strongly expressed in the HIE and NSCs groups (**Figure 2**).

Effect of mild hypothermia combined with NSC transplantation on infarct volume in mice with HIE

Significantly smaller infarct volumes were observed in the hypothermia + NSCs group than the hypothermia, HIE, and NSCs groups at 1, 2 and 4 weeks after cell transplantation (**Figure 3**).

Effect of mild hypothermia combined with NSC transplantation on functional recovery in mice with HIE

In the rotarod test, mice in the hypothermia + NSCs group showed marked behavioral improvements compared with the hypothermia, HIE, and NSCs groups (**Figure 4A**). In the cylinder test, some functional recovery was observed in the early stage after cell transplantation in the hypothermia + NSCs, hypothermia, and HIE groups, but there were no significant differences between these groups at 4 weeks after cell transplantation (**Figure 4B**). Morris water maze training experiments were performed at 2 and 6 months after cell transplantation. Training experiments included 5 days of training with a final test on day 6. Escape latencies of mice in the hypothermia + NSCs group were markedly shorter than in the hypothermia, HIE, and NSCs groups at 4 and 5 days after cell transplantation (**Figure 4C**). At 6 months after cell transplantation, mice in the hypothermia + NSCs group showed marked behavioral improvement compared with the hypothermia, HIE, and NSCs groups. There was no obvious difference in escape latency between hypothermia + NSCs and control groups (**Figure 4D**).

Effect of mild hypothermia combined with NSC transplantation on survival and differentiation of transplanted cells

NSCs and NSC-derived cells were visible after GFP labeling, appearing green when observed with a fluorescence microscope. Transplanted cells were reduced in the right cerebral cortex and hippocampus. To verify this reduction, cells were counted (at a magnification of 400 ×) in the cerebral cortex and hippocampus using a double-blinded method. The mean cell number in 10 sections was used as the cell number for each mouse. Transplanted cells in the NSCs group were significantly reduced compared with the hypothermia + NSCs group at 1, 2, and 4 weeks after cell transplantation (**Figure 5**). In all groups at 4 weeks after cell transplantation, GFP-positive cells in the mouse brain (*i.e.*, NSC-derived cells) were mostly found in the hippocampus and cerebral cortex. NSCs differentiated into mature neurons, astrocytes, and oligodendrocytes as shown by expression of appropriate phenotypic markers, specifically, neuronal nuclear antigen (NeuN: mature neuronal marker), glial fibrillary acidic protein (GFAP: astrocyte marker), and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase: early oligodendrocyte marker) (**Figure 5**). There was an obvious difference in neuronal differentiation between NSC and hypothermia + NSCs groups at 4 weeks after transplantation (**Figure 5**), with NSCs in the hypothermia + NSCs group tending to differentiate into NeuN-positive mature neurons compared with the NSCs group.

Discussion

Neonatal cerebral hypoxia-ischemia can cause death or neurological deficits (including seizures, altered reflexes, distorted levels of consciousness, developmental delay, epilepsy, mental

retardation, and cerebral palsy) within 12–24 hours (Du Plessis et al., 1997). Although the incidence of HIE is 2.5/1,000 live births (Graham et al., 2008), present management of HIE is at a standstill and bound to supportive measures.

Currently there is increasing interest in NSC transplantation as a restorative approach in neurodegenerative disorders characterized by neural cell death. NSCs have the capacity to divide and differentiate into mature neurons and glia cells (Gage et al., 1995), but are absent in the developing and mature central nervous system (Johansson et al., 1999). NSCs migrate preferentially to the area of injury and can express neuronal markers 14–21 days after transplantation in hypoxic-ischemic brain, demonstrating that the brain influences these cells to induce neuronal differentiation (Zheng et al., 2006). However, at later stages, the number of neurons formed is less and possibly only 5% of grafted cells differentiate into neurons. Moreover, poor cell capabilities post-transplantation are a serious problem for clinical application. Even though > 90% of cells are viable at the time of transplantation, approximately only 5–10% of cells survive after the procedure (Zawada et al., 1998). Similarly, another study found that < 50% of transplanted NSCs continue to survive in mouse brain at 2 weeks post-transplantation (Lee et al., 2007).

Enhancement of graft survival is a fundamental strategy for advanced clinical value of cell therapies. During implantation, cells are subjected to hypoxic-ischemic injury. One beneficial way to promote differentiation and increase survival of transplanted NSCs (thereby improving neurological function in hypoxic-ischemic injury mice) is to adjust the microenvironment of the hypoxic-ischemic brain. The effect of mild hypothermia on diminishing damage after hypoxia-ischemia, and infarct volume in transient focal ischemia, is well established in the laboratory (Colbourne et al., 2000). Mild hypothermia is known to exert physically powerful neuroprotective effects through several mechanisms, including reducing metabolic rate, diminishing glutamate release, reducing formation of reactive oxygen species, preventing blood-brain barrier breakdown, and regulating expression of inflammatory and apoptotic factors (Yenari et al., 2008). Indeed, one of these mechanisms is reduction of ischemia-induced death of apoptotic cells by suppressing caspase-3 activation (Ohmura et al., 2005). In this study, we hypothesized that combined therapy of mild hypothermia and NSC transplantation would be more effective than monotherapy, and additionally investigated the mechanism underlying the neuroprotective effect. First, we used TUNEL staining to detect apoptotic cells. TUNEL-positive cells were markedly reduced in the hypothermia + NSCs group compared with the NSCs group. In addition, NF-κB and caspase-3 protein levels were significantly lower in the hypothermia + NSCs group than in the NSCs group.

Therefore, mild hypothermia decreases apoptosis and NF-κB activation in the ipsilateral hemisphere following hypoxia-ischemia intervention. We also found significantly smaller cerebral infarct volumes in mice receiving mild hypothermia and NSC transplantation than those receiving only NSC transplantation at 1, 2, and 4 weeks after cell transplantation. In behavioral tests (rotarod test and Morris water maze), mice subjected to mild hypothermia and NSC transplantation showed behavioral amelioration over time compared with NSC transplantation only. We also investigated the effect of mild hypothermia combined with NSC transplantation on cell survival and differentiation to determine if transplanted cells exert effects through neural differentiation to replace lost neurons. Our results suggest significant differences in reduction of grafted cell number between NSC transplantation and mild hypothermia + NSC transplantation groups at 1, 2, and 4 weeks after cell transplantation. Recently, it has been found in postnatal rats that caspase-3 inhibition influences the direction of NSC differentiation, and increases neuronal production (Levison et al., 2000, Ceccatelli et al., 2004). Our results are consistent with these results.

In conclusion, our results suggest that mild hypothermia has neuroprotective effects on grafted cells by decreasing cell apoptosis and attenuating NF-κB activation in the ipsilateral hemisphere following hypoxia-ischemia intervention. Moreover, mice subjected to mild hypothermia performed better in long-term behavioral tests.

Our present findings provide support for using mild hypothermia combined with NSC transplantation for the treatment of cerebral hemorrhage and ischemia lesions over prolonged periods of time.

Author contributions: *Ma J designed the study, Jiang F and Li QF performed the research, He XG analyzed the data, and Wang L drafted the manuscript. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*

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