

Changes in Neural Stem Cells in Neonatal Rats with Hypoxic-ischaemic Encephalopathy

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Abstract

Background: Human neural stem cells (hNSC) transplantation has been used for the treatment of neurological injury and neurodegenerative disease and has produced some desired effects, but the beneficial effects are limited and the long-term effects remain unknown because the best time for NSC transplantation after hypoxic-ischaemia (HI) is unclear. **Objectives:** This study was designed to investigate the changes in NSC after cerebral HI-damage, and to provide a theoretical reference for NSC therapeutic benefits to the HI-damaged brain. **Methods:** 210 seven-day-old Sprague-Dawley (SD) rats were randomly assigned into three groups: a HI group, a hypoxic group and a normal control group. They were subdivided into 7 time point groups: 3-hour, 6-hour, 1-day, 3-day, 7-day, 14-day and 21-day. Cells were stained for Nestin, a representative protein of NSC. The counts of immunoreactive cells were made under a light microscope, photographed at 400X magnification and counted in ten random different visual fields per section. **Results:** The results showed that: (1) In normal controls, hypoxic groups and HI groups, there was NSC organisation in SD rat brains at 3-hour, 6-hour or 1-, 3-, 7-, 14- and 21-day time points. (2) NSC were reduced at 2 weeks of life in the normal control group, and reduced to the lowest number at 3 weeks after birth, presenting a trend of NSC gradually reducing with age. (3) Hypoxia can improve NSC proliferation to some degree, but proliferation begins to decrease with development of the HI. **Conclusions:** The results provide reference value for selecting the best treatment time window of NSC transplantation for the treatment of neonatal hypoxic-ischaemic brain damage.

Key words

Hypoxic-ischaemic encephalopathy; Neonatal rats; Neural stem cells

Introduction

Hypoxic-ischaemic encephalopathy (HIE) is a common cause of neonatal brain injury with an incidence rate of 2 in 1000 term infants in the developed world,¹ but it is as high as 1.0% in a developing country such as Mainland China.² There are few effective evidence-based

interventions that can improve the outcomes of perinatal asphyxia with HIE,³⁻⁶ thus it also has a high risk of mortality or severe, long-term neurodevelopmental disabilities, such as cognitive impairment, learning disability, and epilepsy.⁷⁻¹⁰ Therefore, it poses a big challenge to clinicians at present.

Neural stem cell-based therapy may offer the prospect of rescuing damaged tissue, replacing lost nerve cells, and restoring neurologic function after cerebral hypoxic-ischaemic (HI) insult. In recent years, human neural stem cells (hNSC) transplantation has been used for the treatment of neurologic injury and neurodegenerative disease and produced some desired effects.¹¹⁻¹⁴ Jeong et al¹¹ found that intravenously transplanted NSC can enter the rat brain after experimental intracerebral haemorrhage, survive, migrate, and improve functional recovery. Jones et al.¹² demonstrated that NSC transplantation can improve cognition mediated by brain derived neurotrophic factor in a transgenic model

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of Alzheimer disease. Andres et al¹⁵ has demonstrated that human stem cell transplantation can significantly enhance structural plasticity and axonal transport in the ischaemic brain in adult rats. Additionally, a very recent study done by Daadi et al¹⁶ showed that hNSC transplants can enhance endogenous brain repair through multiple modalities in response to HI, and suggested that these hNSC are able to modify the host microenvironment and enhance neuroanatomical plasticity after HI in neonates and improve sensorimotor skills. Luan et al¹⁷ had treated a 75-day-old infant with severe HIE sequelae by transplantation of hNSC and resulting in improved short-term effects, but the beneficial effects on ischaemic brain injury is limited and the long-term effects remain unknown, which may possibly be because the optimal time for NSC transplantation for HI is unclear at this moment. Therefore, in the present study, we sought to investigate the characteristics of changes in NSC after cerebral HI damage, and to provide a theoretical reference for selecting the optimal treatment time window for NSC transplantation for the treatment of neonatal HI-brain damage.

Materials and Methods

Hypoxic-ischaemic rat model: This study was approved by the Animal Care and Use Committee of the General Hospital of Beijing Military Command and Capital Medical University. Timed pregnant Sprague-Dawley (SD) rats were obtained from and reared in the Center of Laboratory Animal Science, Capital Medical University (Beijing, China). 210 seven-day-old rats were randomly assigned into three groups, a HI group, a hypoxic group (sham-operated group) and a normal control group, with 70 rats in each group. After that, they were subdivided into 7 groups: 3-hour, 6-hour, 1-day, 3-day, 7-day, 14-day and 21-day, with 10 pups assigned to each time point. The HI-brain damage model was made according to Vannucci et al¹⁸ and Felling et al¹⁹ and modified properly. Cerebral hypoxic-ischaemic was produced by a permanent unilateral common carotid ligation, followed by systemic hypoxia. Briefly, pups were anaesthetised by inhalation of diethyl ether. Once fully anaesthetised, a midline neck incision was made and the right common carotid artery (CCA) was identified. The CCA was separated from the vagus nerve and ligated using 5-0 silk. The incision was then sutured, and animals were returned to the dam for 3 hours. The pups were prewarmed for 20 minutes in jars submerged in a 37°C water bath. They were then exposed to 2.5 hours of 8% O₂ and 92% N₂

at 37°C. Sham-operated animals were anaesthetised, and the carotid was isolated from the vagus but not ligated. They were not subjected to a hypoxic interval. Control animals were separated from the dam for the same amount of time as experimental animals but were otherwise not manipulated. In all cases, the contralateral and ipsilateral hemispheres from experimental animals were examined separately.

Tissue fixation and frozen section production: Animals were anaesthetised by inhalation of diethyl ether before intracardiac perfusion with 3% paraformaldehyde in PBS. The brain was then extracted and blocked, serial, frozen coronal sections (8 µm) were cut on a cryostat from the middle of optic chiasm, placed in 3% glutaraldehyde phosphate buffer (pH=7.4) and fixed for 2-4 hours, followed by 1% osmium tetroxide fixation, ethanol dehydration, and kept at -20°C for later study.

Nestin immunohistochemistry staining: Frozen sections were brought to room temperature over 20 minutes, then deparaffinised in xylene. This was followed by gradient alcohol dehydration, inactivation in 0.6% methanol-H₂O₂ over 20 minutes, 3 washes in PBS of 5 minutes each, then sheep serum for 20 minutes at room temperature. 1:200 mouse anti-rat Nestin monoclonal antibodies were added and incubated for 90 minutes at 37°C. This was followed by 3 PBS washes of 10 minutes each, a 30 minute incubation at 37°C with biotinylated goat anti-mice IgG, 4 x 5 minute PBS washes and a 5 minute DAB stain. Then the samples were given a final wash, dehydration and fixing. In the negative controls the primary antibody was replaced with 0.01 mol/L PBS and the secondary antibody was replaced with normal sheep serum.

Qualitative and quantitative judgement for NSC: Nestin positive cells were counted as NSC. The counts of immunoreactive cells were made under a light microscope and photographed at 400X magnification and counted in ten random different visual fields of per section.

Statistical methods: All data were presented as mean±standard deviation ($\bar{x}\pm s$) and analysed by one-way ANOVA using SPSS 15.0 statistical software (SPSS Inc, Chicago, IL). $P<0.05$ was considered to be statistically significant.

Results

Brain histopathological changes in different groups:

(1) Normal control groups: The rat brain structure of the control neonates is clear with normal cell contour and pink cytoplasm, nuclei are located in the centres of cells and

nucleoli are distinct (Figure 1A). (2) HI groups: (a) 3-hour after HI: brain structure disorder and focal pyknotic nuclei appear in the cerebral cortex and striatum. (b) 6-hour after HI: the damage increases in the cerebral cortex and striatum, and pyknotic nuclei lesions increase in number. (c) 24-hour after HI: histopathological changes continue to worsen with massive necrosis in the cerebral cortex, striatum, hippocampus, and thalamus, cell dissolution or even disappearance. (d) 3-day after HI: a number of nerve cells dissolve and disappear, glial cell proliferation around lesions, but in the centre of lesions a number of pyknotic nuclei and nucleus pieces still exist. (e) 7-day after HI: only a very few-pyknotic nuclei exist in the centre of lesions, more gliocyte hyperplasia than before. (f) 14- and 21-day after HI: a large degree of neuron loss, glial scar formation in the cortex, striatum, hippocampus and thalamus (Figure 1C-1F). (3) Hypoxic groups: pathological changes are similar to HI groups, but significantly less severe (Figure 1B).

Changes in Nestin positive NSC: There were Nestin positive NSC in the three different groups, which were distributed mainly in the hippocampus, the subependymal germinal matrix area, the outside wall of the lateral ventricle, the subventricular zone and cortex and corpus striatum.

It can be seen from Figures 2-8 and Table 1 that there was no difference in the number of NSC between HI and hypoxic groups at 1, 3 and 7 days after HI or hypoxia, but there was significant difference in the number of NSC between control and hypoxic groups. There was a greater significant difference in the number of NSC between control and HI groups at 3 days. There was the most significant difference in the number of NSC between control and HI groups at 7-, 14- and 21-day. It can be seen that hypoxia and HI will help the proliferation of NSC, but proliferation begins to decrease with the development of the HI.

Discussion

Neural stem cells are the precursors that generate the astonishing cell diversity found in the central nervous system, they are multipotent and can exhibit a high mitotic index.²⁰ However the optimal treatment time-window is very different in different diseases.^{15,21-23} Johann et al²¹ demonstrated that the optimal treatment window for Huntington's disease was less than 2 days after lesion. Li et al²² found that the optimal treatment time period for intracerebral haemorrhage was 7-14 days after injury. Darsalia et al²³ showed that NSC transplanted shortly

after stroke (within 48 hours) resulted in better cell survival than did transplantation 6 weeks after stroke, while Andres et al¹⁵ demonstrated that human NSC transplantation at 1 week after stroke can significantly improve functional recovery in several behavior tests through enhancing the changes in dendritic and axonal structural plasticity and can enhance recovery of stroke-impaired axonal transport. Luan et al¹⁷ had treated a severe HIE patient with adverse neurologic sequelae by transplantation of hNSC and observed improved short-term effects. However, the beneficial effects of NSC transplantation on HI brain injury are limited and the long-term effects are not well documented because the best treatment time window for NSC transplantation after HI is unclear.

The results of the present study show the dynamic characteristics of changes in neural stem cells in neonatal rats with HIE, which are the following: (1) No matter whether in normal controls, hypoxic groups or HI groups, there was NSC organisation in SD rat brain at 3-hour, 6-hour or 1-, 3-, 7-, 14- and 21-day time points. (2) NSC were reduced at 2 weeks of life in normal control groups, and reduced to the lowest point at 3 weeks after birth, presenting a trend of gradual reduction with age. This is similar to the case in adult animals reported by Maslov et al²⁴ in which a comparison of the NSC populations in the subventricular zones of young and old mice demonstrates a significant reduction in the older mice. (3) The number of NSC was higher in hypoxic groups than that in normal control groups and HI groups, which hints that hypoxia can improve NSC proliferation to some degree. (4) With the extension of time and the development of HI, NSC will become fewer and fewer with increasing numbers of gliocytes and then formation of a glial scar in the brain tissues. This kind of change gradually appears within 3 days of life and becomes increasing severe afterwards.

According to our present results, in order to: (1) improve native NSC proliferation and differentiation; (2) replace the lost and necrotic native NSC; (3) exert delayed post-ischaemic neuroprotection,²⁵ we believe that the syngenic NSC transplantation should be performed as soon as possible after the HI insult, and a period of 24-48 hours may be the optimal time-window for NSC transplantation. Thus, in the following study, investigators should be to test this hypothesis and to find the optimal therapeutic dosage of injected NSC.

In brief, the results showed that hypoxia can improve the proliferation of NSC, that is, NSC organisation changes as a response to hypoxic-ischaemic insult. But, such ability is limited. As the illness worsens, this kind of proliferation

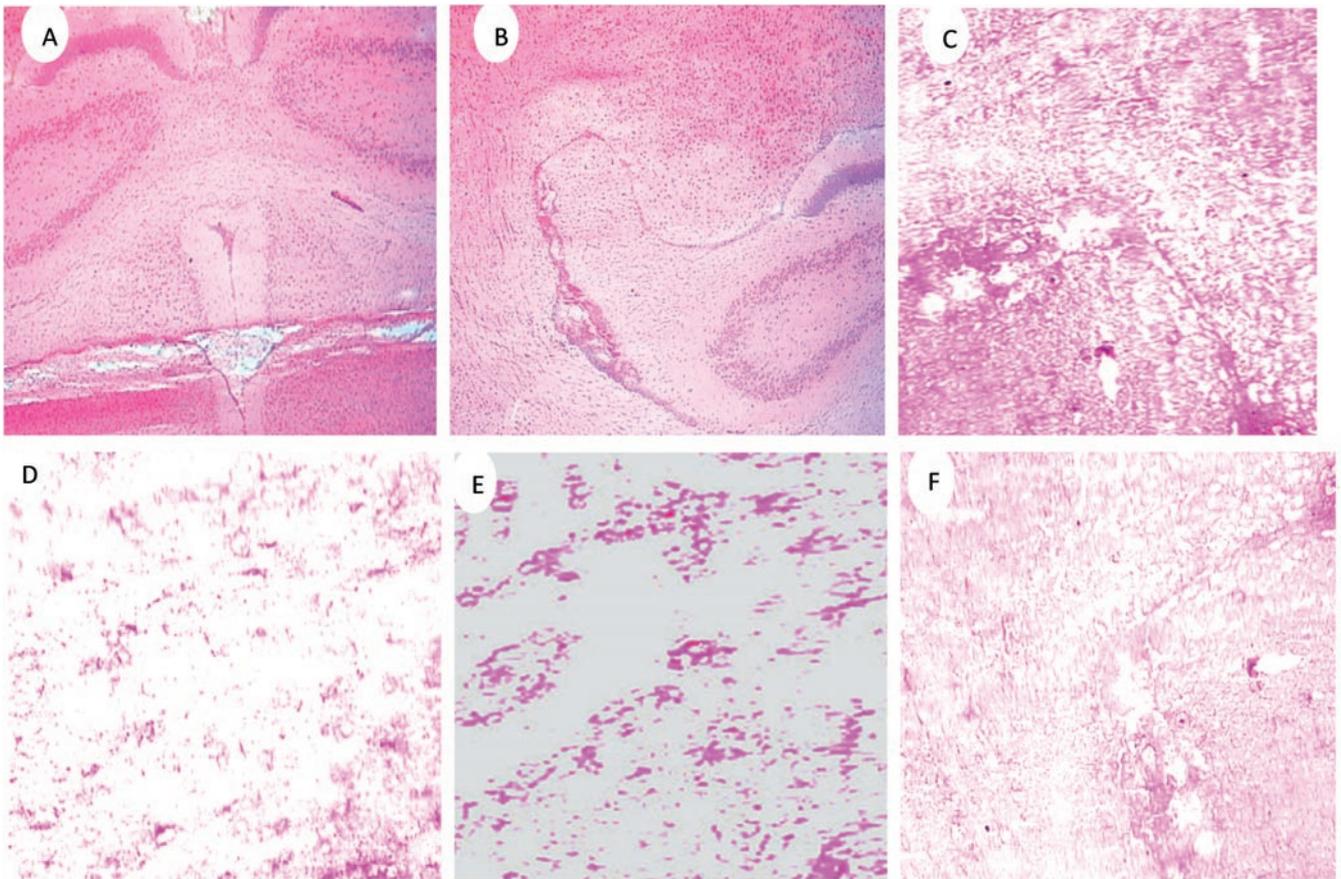


Figure 1 Brain histopathological changes in different groups (HE). (A) Control group: show the rat brain structure is clear (x25); (B) hypoxic group at 24 hours (x25); (C) 3-hour after HI: show brain structure disorder (x400); (D) 24-hour after HI: show massive necrosis (x200); (E) 3-day after HI: show a number of nerve cells dissolve and disappear (x200); (F) 14-day after HI: show more gliocyte hyperplasia and glial scar formation (x100).

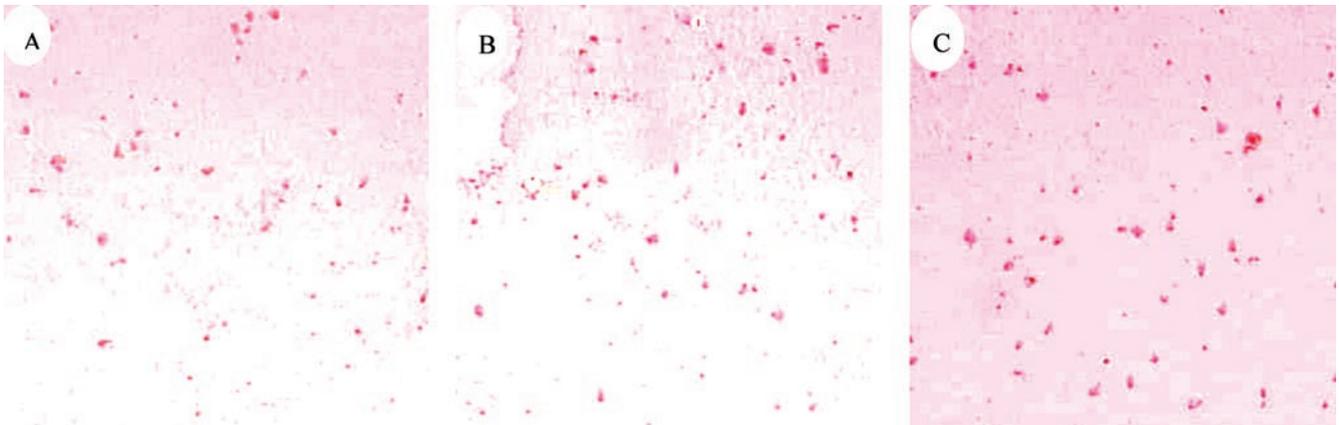


Figure 2 3-hour after animal model was made, Nestin positive NSCs showed claybank with DAB colouration (x100). (A) HI-group; (B) Hypoxic group; (C) Normal control group.

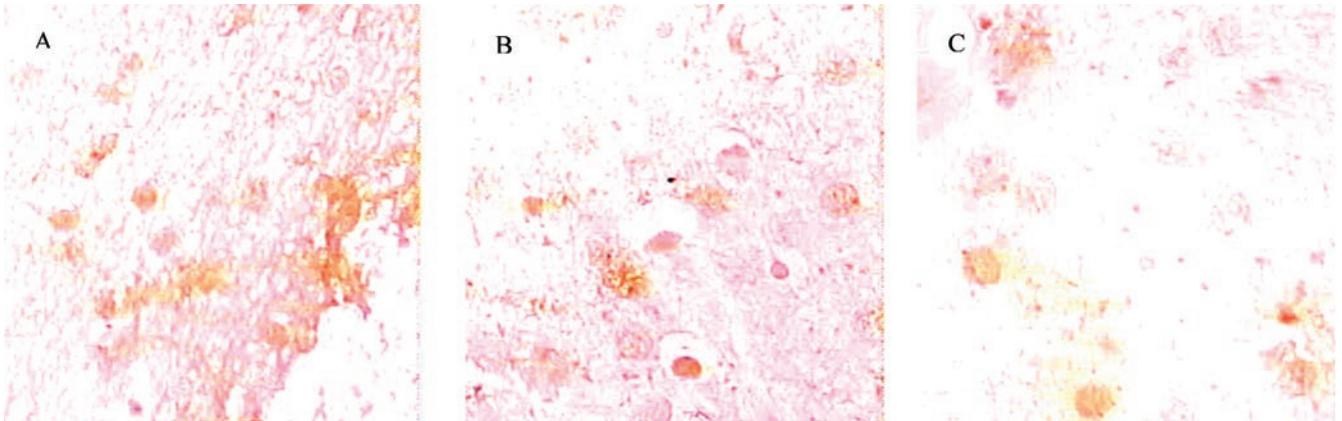


Figure 3 6-hour after animal model was made, Nestin positive NSCs showed claybank with DAB colouration (x100). (A) HI-group, (B) Hypoxic group; (C) Normal control group.

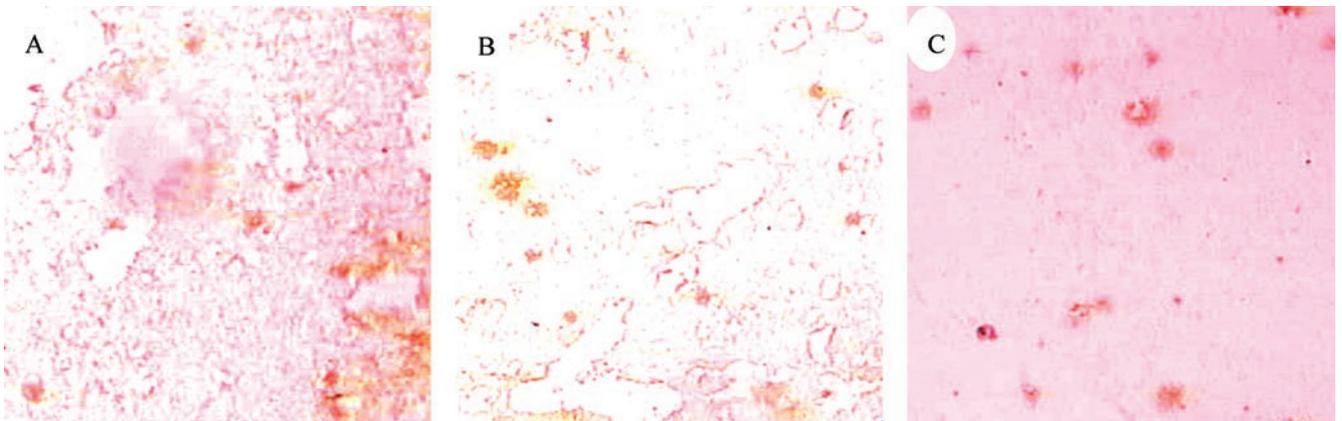


Figure 4 1-day after animal model was made, Nestin positive NSCs showed claybank with DAB colouration (x200). (A) HI-group; (B) Hypoxic group; (C) Normal control group.

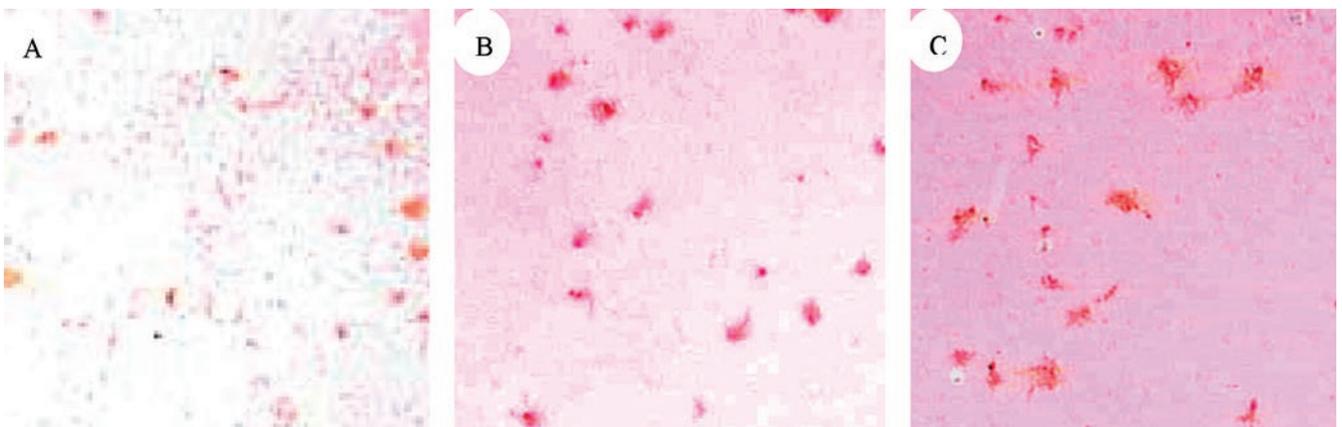


Figure 5 3-day after animal model was made, Nestin positive NSCs showed claybank with DAB colouration (x200). (A) HI-group, (B) Hypoxic group, C-Normal control group.

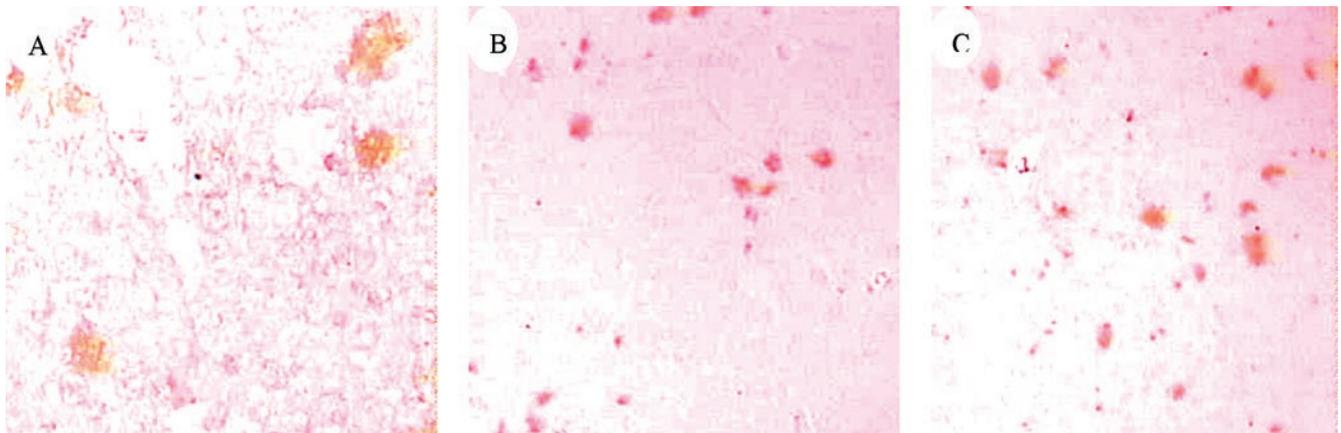


Figure 6 7-day after animal model was made, Nestin positive NSCs showed claybank with DAB colouration (x400). (A) HI-group, (B) Hypoxic group, (C) Normal control group.

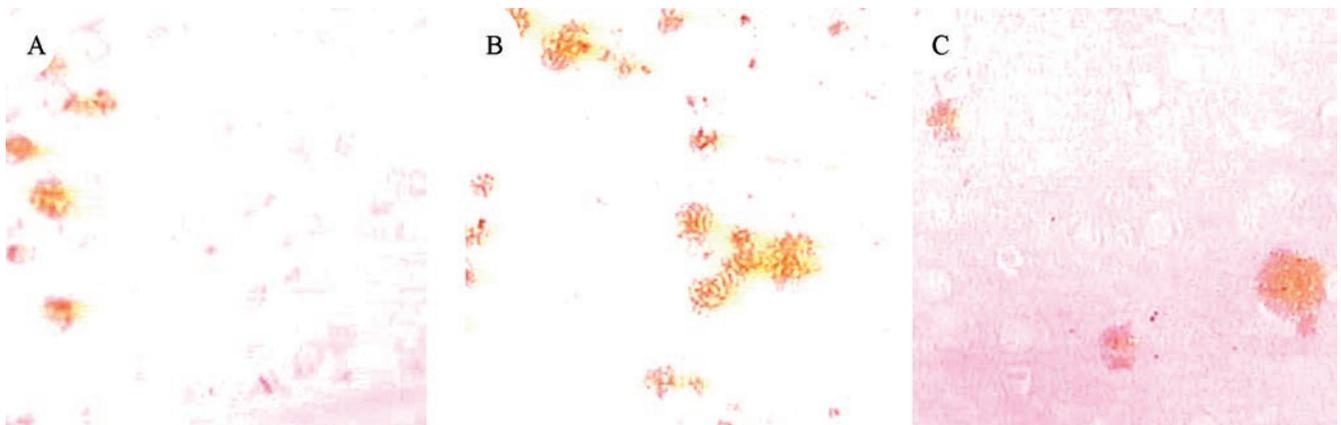


Figure 7 14-day after animal model was made, Nestin positive NSCs showed claybank with DAB colouration. (A) HI-group (x200), (B) Hypoxic group (x400), (C) Normal control group (x400).

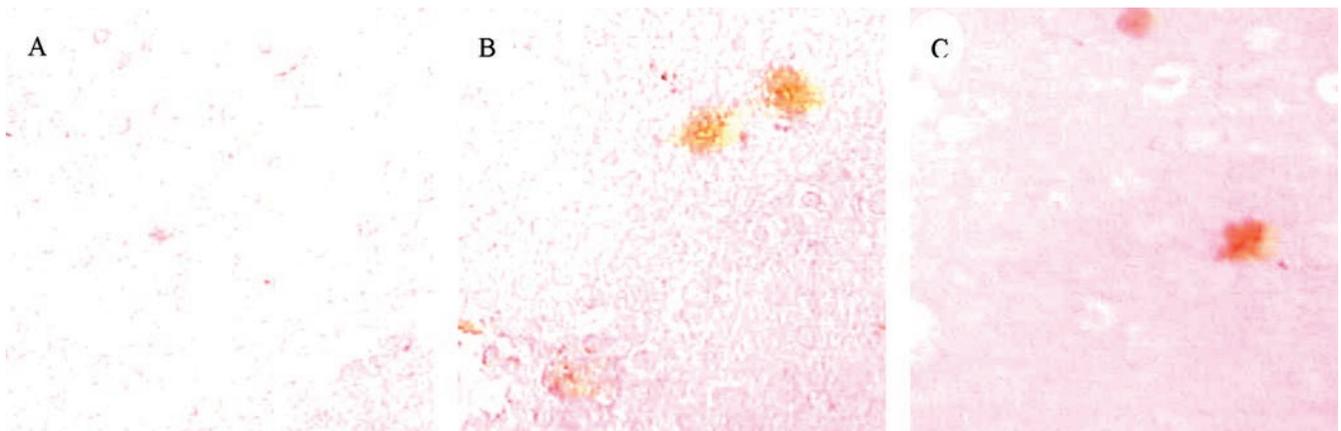


Figure 8 21-day after animal model was made, Nestin positive NSCs showed claybank with DAB colouration. (A) HI-group (x200); (B) Hypoxic group (x400); (C) Normal control group (x400).

Table 1 Changes in the number of nestin positive NSC in the subventricular zone in different groups ($\bar{x}\pm s$, n=10)

Time	Control groups	Hypoxic groups	HI-groups	Compare between different groups (F-value)
3 hours	94.6±12.7	93.7±11.4	92.4±6.8	0.108
6 hours	94.1±6.7	94.0±12.1	93.1±7.2	0.037
1 day	92.6±8.7	99.4±8.1	87.7±9.5	4.55*
3 days	98.6±8.9	105.2±6.0	88.5±6.4	13.54#
7 days	75.5±9.5	92.3±13.1	74.2±8.9	9.02#
14 days	66.2±12.8	76.7±9.9	67.2±7.1	3.21
21 days	54.4±8.1	67.4±7.2	59.6±9.4	6.22#
F-value*	30.02	17.37	27.69	

*Compare between different time after hypoxic or HI; *p<0.05; #p<0.01.

cannot compensate for the nerve cell necrosis and loss, and eventually results in severe brain damage. The results of this study provides a reference for selecting the optimal treatment time-window of NSC transplantation for the treatment of neonatal hypoxic-ischaemic brain damage.

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