NEURAL REGENERATION RESEARCH
Volume 8, Issue 13, May 2013

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2013.13.007 [http://www.nrronline.org; http://www.sjzsyj.org]
Feng ZC, Liu J, Ju R. Hyperbaric oxygen treatment promotes neural stem cell proliferation in the subventricular zone of neonatal rats with hypoxic-ischemic brain damage. Neural Regen Res. 2013;8(13):1220-1227.

Hyperbaric oxygen treatment promotes neural stem cell proliferation in the subventricular zone of neonatal rats with hypoxic-ischemic brain damage☆

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Abstract

Hyperbaric oxygen therapy for the treatment of neonatal hypoxic-ischemic brain damage has been used clinically for many years, but its effectiveness remains controversial. In addition, the mechanism of this potential neuroprotective effect remains unclear. This study aimed to investigate the influence of hyperbaric oxygen on the proliferation of neural stem cells in the subventricular zone of neonatal Sprague-Dawley rats (7 days old) subjected to hypoxic-ischemic brain damage. Six hours after modeling, rats were treated with hyperbaric oxygen once daily for 7 days. Immunohistochemistry revealed that the number of 5-bromo-2′-deoxyuridine positive and nestin positive cells in the subventricular zone of neonatal rats increased at day 3 after hypoxic-ischemic brain damage and peaked at day 5. After hyperbaric oxygen treatment, the number of 5-bromo-2′-deoxyuridine positive and nestin positive cells began to increase at day 1, and was significantly higher than that in normal rats and model rats until day 21. Hematoxylin-eosin staining showed that hyperbaric oxygen treatment could attenuate pathological changes to brain tissue in neonatal rats, and reduce the number of degenerating and necrotic nerve cells. Our experimental findings indicate that hyperbaric oxygen treatment enhances the proliferation of neural stem cells in the subventricular zone of neonatal rats with hypoxic-ischemic brain damage, and has therapeutic potential for promoting neurological recovery following brain injury.

Key Words

neural regeneration; brain injury; neonatal hypoxic-ischemic encephalopathy; hypoxic-ischemic brain damage; hyperbaric oxygen; neural stem cells; neurons; proliferation; subventricular zone; neonatal rats; nestin; grants-supported paper; neuroregeneration

Research Highlights

(1) Hyperbaric oxygen treatment can improve the pathological change to brain tissue in neonatal rats with hypoxic-ischemic brain damage.
(2) Hyperbaric oxygen treatment may enhance the proliferation of neural stem cells in neonatal rats with hypoxic-ischemic brain damage.
(3) Hyperbaric oxygen has therapeutic potential for promoting neurological recovery following hypoxic-ischemic brain damage.

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Received: 2012-11-15
Accepted: 2013-01-10
(N20120314002)

INTRODUCTION

Hypoxic-ischemic encephalopathy is a common cause of neonatal brain injury due to perinatal hypoxic-ischemia with an incidence of 2 per 1 000 term infants in the developed world[1]. However, the incidence of hypoxic-ischemic brain damage is higher in developing nations and reaches approximately 1.0% in China[2]. Although various strategies have been shown to improve the outcome of hypoxic-ischemic brain damage, such as neuroprotective agents and hypothermia[3-7], there is no effective treatment for alleviating the debilitating sequelae to perinatal asphyxia, especially those infants with moderate-to-severe hypoxic-ischemic brain damage. These infants are at a higher risk of mortality and developing severe, long-term disabilities, such as cerebral palsy, cognitive impairment, learning disabilities, and epilepsy[4-8]. Therefore, treatment strategies are of great importance to perinatologists and neonatologists[9-11].

Hyperbaric oxygen has shown promise for the treatment of ischemic brain damage in rat models[12-14], in neonatal hypoxic-ischemic brain damage[15-16] and in adult traumatic brain injury[17] by reducing neuronal apoptosis and promoting the recovery of neurological function[12-18]. However, the efficacy of hyperbaric oxygen for the treatment of neonatal hypoxic-ischemic brain damage remains controversial because of a lack of evidence-based medicine[19]. The mechanisms of hyperbaric oxygen therapy for the treatment of hypoxic-ischemic brain damage remain unknown. Endogenous neural stem cell proliferation can attenuate brain injury, which in turn promotes cell proliferation; however, the cell numbers are not high enough to induce a neuroprotective effect.

In the present study, we aimed to investigate whether hyperbaric oxygen can promote neural stem cell proliferation in the subventricular zone of neonatal rats following hypoxic-ischemic brain damage, thus providing support for the clinical application of hyperbaric oxygen.

RESULTS

Quantitative analysis of experimental animals

A total of 108 rats at 7 days of age were randomly divided into three groups with 36 in each group: control group (normal feeding without anesthesia, carotid ligation, hypoxia, or hyperbaric oxygen exposure); hypoxic-ischemic group (hypoxic-ischemic brain damage model); and hyperbaric oxygen group (hypoxic-ischemic brain damage model + hyperbaric oxygen intervention). Each group was composed of pups from each litter to obtain parity within the groups. The brains of six pups from each group were removed at 1, 3, 5, 7, 14 and 21 days after insult. All 108 rats were involved in the final analysis.

Hyperbaric oxygen improved pathological changes in neonatal rats with hypoxic-ischemic brain damage

Pathologic changes were observed based on microscopic examination of hematoxylin-eosin-stained sections. In the hypoxic-ischemic group, the hippocampus, cortex, and thalamus exhibited large areas of cell necrosis, swelling of the cell soma, and disrupted tissue architecture. Furthermore, neuronal cell loss, glial cell hyperplasia and glial scar formation were also observed. Pathological changes to brain tissue gradually aggravated as the time of brain tissue undergoing hypoxic-ischemia advanced. In striking contrast, the hyperbaric oxygen group showed decreased nerve cell degeneration and necrosis, nerve cells appeared less disorganized and their structure appeared more normal (Figure 1).

Hyperbaric oxygen promoted 5-bromo-2′-deoxyuridine expression in neonatal rats with hypoxic-ischemic brain damage

Immunohistochemical staining showed that 5-bromo-2′-deoxyuridine positive cells were round or oval in shape, with larger cell bodies and obvious claybank nucleoli. In the hypoxic-ischemic and hyperbaric oxygen groups, the 5-bromo-2′-deoxyuridine positive cell counts increased gradually from day 1 and peaked at day 5. Thereafter, cell number began to decline at day 14 in each group and reached the lowest at day 21. However, in the hyperbaric oxygen group, the 5-bromo-2′-deoxyuridine positive cell counts were higher than that in the hypoxic-ischemic and control groups at each time point (Figure 2).

Hyperbaric oxygen increased nestin expression in neonatal rats with hypoxic-ischemic brain damage

Immunohistochemical staining showed that nestin, a marker of neural stem cells, was present within the cytoplasm of cells, which had a round or spindle shape. As shown in Figure 3, the number of neural stem cells was higher in the hypoxic-ischemic group than in the control group ($P < 0.05$), suggesting that hypoxia-ischemia increases neural stem cell proliferation to a certain degree. Compared with the hypoxic-ischemic and control groups, the hyperbaric oxygen group had

significantly higher nestin positive cell counts ($P < 0.05$ or $P < 0.01$; Table 1). The number of nestin positive cells increased dramatically at day 1, continued to increase at a more moderate rate at day 3 and peaked at day 5. The nestin positive cell counts began to decrease after day 7 and reached its lowest level at day 21.

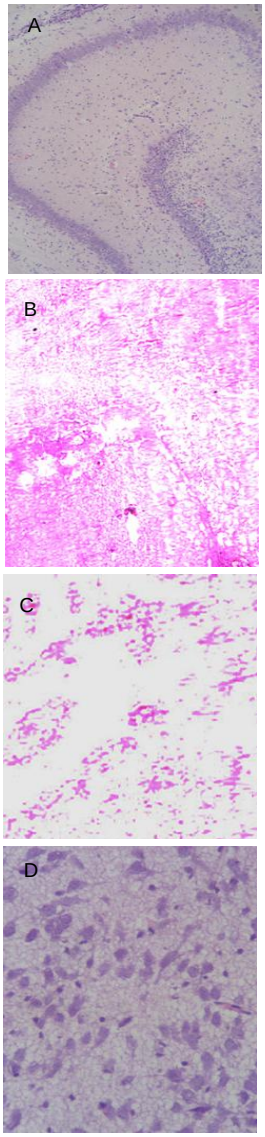

Figure 1 Effect of hyperbaric oxygen (HBO) on pathological changes of brain tissue in neonatal rats with hypoxic-ischemic (HI) brain damage (hematoxylin-eosin staining, light microscope).

(A) Control group showed normal brain structures and regularly arranged brain cells (× 40).

(B) HI group showed disrupted tissue architecture at day 1 after modeling (× 40).

(C) Large areas of cell necrosis and neuronal cell loss could be seen in the HI group at day 3 after modeling (× 400).

(D) These pathological changes were improved in the HBO group at day 3 after modeling (× 400).

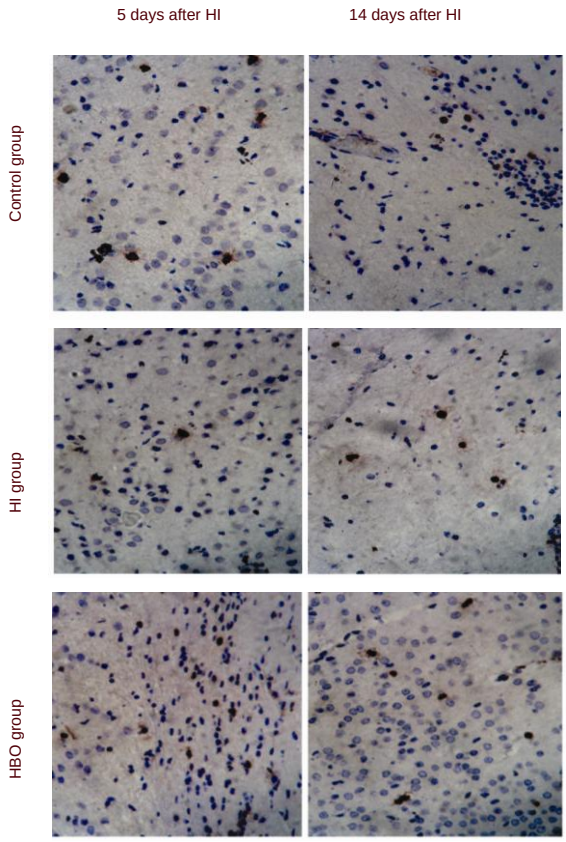

Figure 2 Effect of hyperbaric oxygen (HBO) on 5-bromo-2′-deoxyuridine expression in the subventricular zone of neonatal rats with hypoxic-ischemic (HI) brain damage (SABC immunohistochemistry staining, optical microscope, × 400).

5-Bromo-2′-deoxyuridine positive cell counts were higher in the HBO group than in the HI and control groups at each time point.

Hyperbaric oxygen promoted the proliferation of neural stem cells in neonatal rats with hypoxic-ischemic brain damage

The 5-bromo-2′-deoxyuridine and nestin dual positive cells were round or irregular in shape with claybank endochylema and blue nucleoli. The 5-bromo-2′-deoxyuridine and nestin dual positive cells were considered to be proliferating neural stem cells. As shown in Figure 4, hyperbaric oxygen treatment significantly increased the number of 5-bromo-2′-deoxyuridine and nestin dual positive cells in brain tissue at day 1 after injury ($P < 0.05$). Thereafter, the number of 5-bromo-2′-deoxyuridine and nestin dual positive cells increased gradually and peaked at day 5 ($P < 0.05$). The number of 5-bromo-2′-deoxyuridine and nestin dual positive cells started decreasing at day 14 overall, but more dual positive cells were seen in the hyperbaric oxygen group than in the hypoxic-ischemic group and control group.

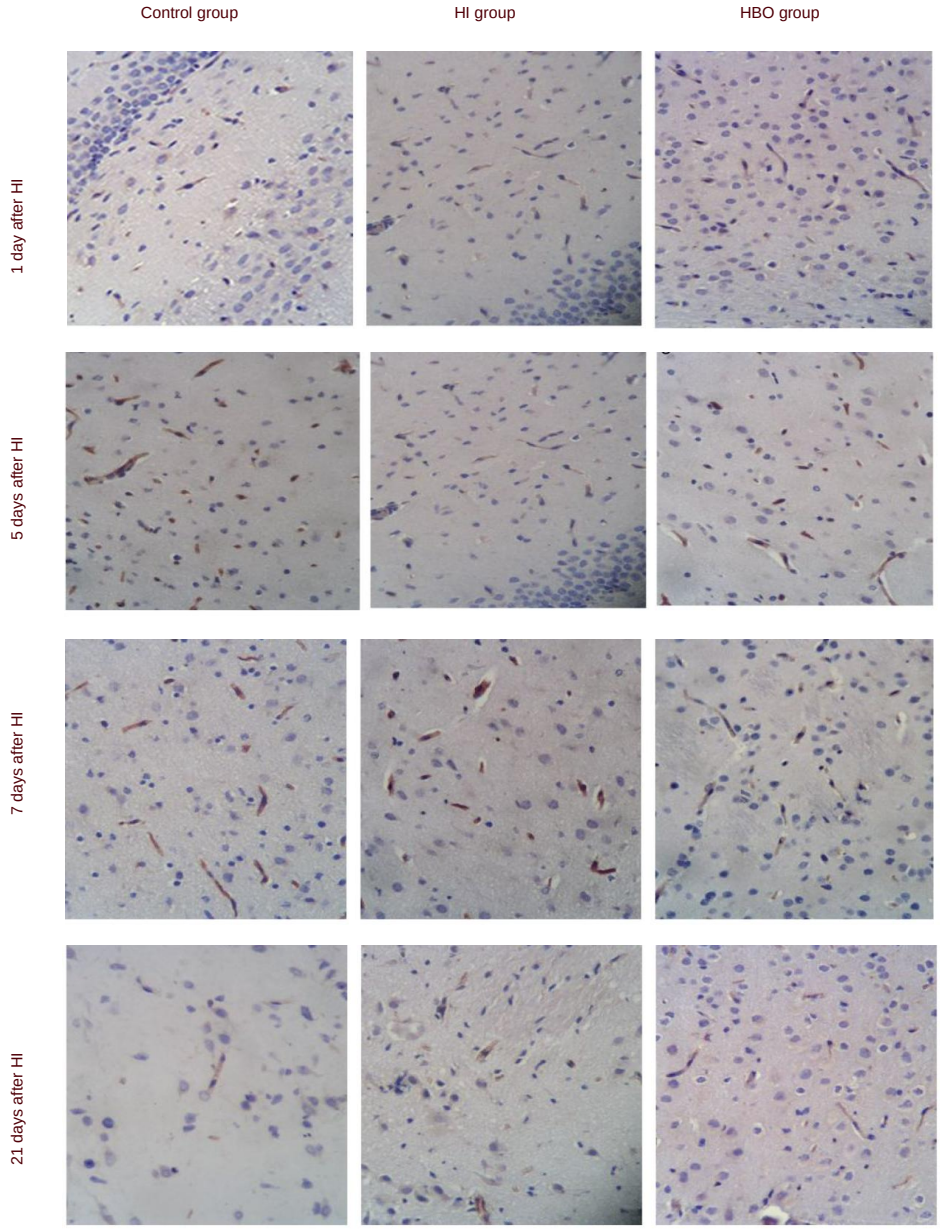

Figure 3 Effect of hyperbaric oxygen (HBO) on nestin expression in the subventricular zone of neonatal rats with hypoxic-ischemic (HI) brain damage (SABC immunohistochemistry staining, optical microscope, × 400).

Hyperbaric oxygen treatment significantly increased the number of nestin positive cells. The number of nestin positive cells increased gradually at day 3 and peaked at day 5, and than began to decrease at day 14 and reached the lowest level at day 21.

Table 1 Effect of hyperbaric oxygen (HBO) on nestin positive cell counts (/400-fold visual field) in the subventricular zone of neonatal rats with hypoxic-ischemic (HI) brain damage

Group	Time after HI (day)					
	1	3	5	7	14	21
Control	91.5±7.3	99.7±8.1	83.3±5.3	72.8±7.3	67.6±7.1	55.1±7.2
HI	99.9±7.5	107.3±9.3[a]	119.5±9.3[a]	89.3±8.3[a]	71.8±9.3[a]	61.6±7.1[a]
HBO	113.7±9.3[bc]	135.5±10.2[bc]	153.9±9.2[bc]	112.9±9.2[bc]	97.9±9.2[bc]	87.7±9.1[bc]

Data are expressed as mean ± SD; n = 6 in each group at each time point. [a]$P < 0.05$, [b]$P < 0.01$, *vs*. control group; [c]$P < 0.05$, *vs*. HI group using Student's *t*-test.

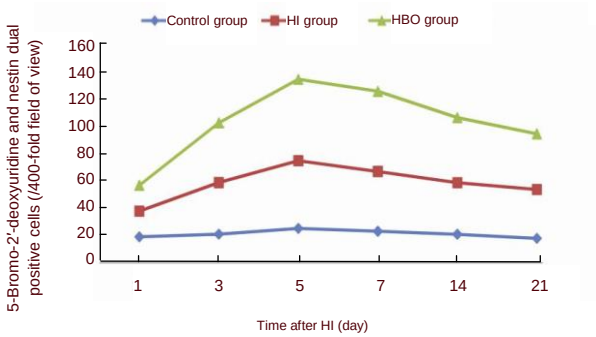

Figure 4 Effect of hyperbaric oxygen (HBO) on the proliferation of neural stem cells in brain tissues of neonatal rats with hypoxic-ischemic (HI) brain damage.

Data are expressed as the mean. HBO treatment significantly increased the number of 5-bromo-2′-deoxyuridine and nestin dual positive cells in brain tissue at day 1 after injury ($P < 0.05$) and peaked at day 5 ($P < 0.05$). The number of 5-bromo-2′-deoxyuridine and nestin dual positive cells began to increase from day 14, but more dual positive cells were seen in the HBO group than the HI and control groups.

DISCUSSION

The results of this study showed that hypoxia-ischemia can result in disrupted tissue architecture, large areas of cell necrosis and neuronal cell loss in rat brain tissues, while hyperbaric oxygen can improve pathological changes to rat brain tissues after hypoxia-ischemia. Thus, we believe that hyperbaric oxygen can be used for the treatment of neonatal hypoxic-ischemic brain damage, however, the mechanism of this neuroprotective effect remains unknown. Therefore, we investigated whether hyperbaric oxygen can promote neural stem cell proliferation in neonatal rats following hypoxic-ischemic brain damage.

It was believed that 5-bromo-2′-deoxyuridine positive cells represented proliferating cells. In the developing brain, however, the proliferating cells included neural stem cells and astrocytes, thus an increase in 5-bromo-2′-deoxyuridine positive cells cannot reflect the proliferation of neural stem cells alone. Nestin, an intermediate filament protein expressed in neural stem cells, lineage-constricted progenitors, and immature neurons is widely used to identify neural stem cells[20-21]. Thus, we used 5-bromo-2′-deoxyuridine and nestin double staining to distinguish between proliferating neural stem cells. In this study, we found that hyperbaric oxygen treatment considerably increased the number of neural stem cells in rats with hypoxic-ischemic brain damage, especially during the first 5 days of treatment. Even with the gradual decrease in proliferating neural stem cells after day 14, the number of these cells in the hyperbaric oxygen group was significantly higher than in the control or hypoxic-ischemic group for up to 21 days following brain injury. This information in conjunction with evidence reported by Yang *et al* [22] that hyperbaric oxygen significantly improves endogenous neural stem cell proliferation in neonatal rats following hypoxic-ischemic brain damage, provides additional support for the clinical application of hyperbaric oxygen for the treatment of neonatal hypoxic-ischemic brain damage.

Several mechanisms appear to contribute to the efficacy of hyperbaric oxygen for the treatment of hypoxic-ischemic brain damage: (1) Hyperbaric oxygen can lead to a remarkable increase in the amount of O_2 delivered to brain tissue even in injury patients. An increase in blood oxygenation occurs not only during hyperbaric oxygen treatment, but also persists after treatment for at least 6 hours[17]. (2) Hyperbaric oxygen increases the oxygen dispersion distance in edematous brain tissue. (3) Hyperbaric oxygen significantly reduces cerebrospinal fluid levels of lactate, thereby improving cerebral aerobic metabolism and the cerebral metabolic rate of oxygen (an index reflecting mitochondrial function)[17]. (4) Hyperbaric oxygen promotes blood-brain barrier integrity and reduces cerebral edema and hyperemia, thereby decreasing intracranial hypertension. (5) Hyperbaric oxygen reduces neuronal apoptosis, promotes cell survival, reduces cerebral infarct volume, and promotes the recovery of neurological function[12-14, 18]. (6) Increased oxygenation suppresses the expression of cyclooxygenase-2 and its downstream targets after a global ischemic insult[23]. (7) Increased oxygenation increases the activation of peroxisome proliferator-activated receptor-gamma, and subsequent increased production of 15-Deoxy-Delta(12,14)-prostaglandin J(2), which in turn increases downstream antioxidant enzymatic activities[24]. We also noted that the increase in the number of neural stem cells is most robust during the first 5 days of treatment. It appears that immediate or early hyperbaric oxygen administration improves prognosis for patients with hypoxic-ischemic brain damage. A question that can be investigated in the future is if longer treatment causes further improvement in prognosis for patients who suffer from hypoxic-ischemic brain damage.

In conclusion, our experimental findings indicate that hyperbaric oxygen treatment promotes the proliferation of neural stem cells and is likely to improve recovery following brain injury. The evidence supports the clinical

application of hyperbaric oxygen for treatment of neonatal hypoxic-ischemic brain damage.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

This experiment was performed from June 2010 to May 2011 in the Central Laboratory, Bayi Children's Hospital Affiliated to General Hospital of Beijing Military Command, China.

Materials

A total of 108 Sprague-Dawley rats at 7 days of age were obtained and reared in the Center of Laboratory Animal Science, Bayi Children's Hospital Affiliated to General Hospital of Beijing Military Command, China (license No. 200A036). All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[25].

Methods

Establishment of hypoxic-ischemic brain damage model and hyperbaric oxygen intervention

Hypoxic-ischemic brain damage was induced by the method of Vannucci *et al* [26], which involves permanent, unilateral ligation of the common carotid artery, followed by systemic hypoxia[27]. Rats were anesthetized with diethyl ether inhalation. Once fully anesthetized, a lateral neck incision was made, the right common carotid artery was separated from the vagus nerve, and the artery was ligated using 5-0 silk. The incision was then sutured, and the animals were returned to the dam for 3 hours. Pups were warmed for 20 minutes in jars submerged in a 37°C water bath. They were then exposed for 2.5 hours to 8% O_2 and 92% N_2 at 37°C. In the hyperbaric oxygen group, pups received hyperbaric oxygen treatment 6 hours after the hypoxia-ischemia insult for 1 hour in a baby hyperbaric oxygen chamber (YLC 0.5/1A, Wuhan Shipbuilding Design and Development Institute, China) with 100% O_2 at 3 atmospheres of pressure. Pups were returned to their cages following treatment. Hyperbaric oxygen treatment was given once daily for 1 hour for 7 consecutive days. Control animals were separated from the dam for the same amount of time as experimental animals but were otherwise not manipulated. The contralateral and ipsilateral hemispheres from experimental animals were examined separately.

5-Bromo-2′-deoxyuridine labeling

Two rats in each group, at each interval, were randomly selected for intraperitoneal administration of 5-bromo-2′-deoxyuridine (200 mg/kg; Sigma, St. Louis. MO, USA) for 8 hours before euthanasia and fixation of the brain.

Brain tissue preparation

Animals were anesthetized with chloral hydrate and underwent transcardial perfusion with 50–100 mL 0.9% (w/v) saline followed by 50–100 mL cold 4% (w/v) paraformaldehyde in 0.1 mol/L PBS (pH 7.4). The bregma was labeled with methylene blue, and the brain was removed and post-fixed in paraformaldehyde for 24 hours. Coronal sections were taken from 1.0 mm to –0.8 mm and –3.0 mm to –4.5 mm from the midline to the anterior fontanelle, processed, embedded in paraffin wax and cut coronally into 5-μm sections, and transferred onto polylysine-coated slides.

Hematoxylin-eosin staining

After dewaxing, slides were stained with hematoxylin-eosin to examine pathological changes to brain tissue under the optical microscope (Nikon Corporation, Tokyo, Japan).

Immunohistochemistry for nestin and 5-bromo-2′-deoxyuridine expression

Frozen sections from the subventricular zone were placed at room temperature for 20 minutes, treated with xylene to remove lipids, and subjected to gradual alcohol dehydration. Sections then underwent 0.6% (v/v) methanol-H_2O_2 treatment for 20 minutes, three PBS washes at 5 minutes intervals, and incubation in sheep serum for 20 minutes at room temperature. Subsequently, sections were incubated with mouse anti-rat nestin (1:200; Chemicon, Temecula, CA, USA) and mouse anti-rat 5-bromo-2′-deoxyuridine (Accurate Chemicals, Westbury, NY, USA) monoclonal antibodies for 90 minutes at 37°C, and washed three times with PBS at 10 minutes intervals. They were then incubated with biotinylated goat anti-mouse IgG (1:100; Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, China) for 30 minutes at 37°C, washed four times with PBS at 5 minutes intervals, stained with 3-diaminobenzidine tetrahydrochloride (Beijing Unique Biotechnology Co, Beijing, China) for 5 minutes, washed, dehydrated, and cleared for observation. For negative controls, primary antibody was replaced with 0.01 mol/L PBS, and secondary antibody was replaced with normal sheep serum.

Counting neural stem cells

The dual positive 5-bromo-2′-deoxyuridine and nestin

cells were considered to be proliferating neural stem cells. Neural stem cell counts were performed at a magnification of 400 × under the optical microscope (Nikon). Five random sections of each sample were examined at 10 different visual fields to obtain cell counts for each group.

Statistical analysis

Data are presented as mean ± SD and the difference between two groups was analyzed by the Student's *t*-test using SPSS 15.0 statistical software (SPSS, Chicago, IL, USA). A $P < 0.05$ value was considered to be statistically significant.

Acknowledgments: We would like to thank Dr. Xiaoying Zhang and Dr. Shen Zang from Bayi Children's Hospital Affiliated to General Hospital of Beijing Military Command for their excellent technical assistance.

Funding: This work was supported by Guangdong Province Science Research Project, No. B30502.

Author contributions: Zhichun Feng was responsible for the study design and funds. Jing Liu analyzed the data and wrote the paper. Rong Ju finished the experiments. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Care and Use Committee of the General Hospital of Beijing Military Command in China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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(Reviewed by Diwakarla S, Norman C, Chen ZL, Chen X)
(Edited by Wang LM, Yang Y, Li CH, Song LP)