Proliferation of neural stem cells correlates with Wnt-3 protein in hypoxic-ischemic neonate rats after hyperbaric oxygen therapy

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Hyperbaric oxygen therapy promoted brain cell proliferation. Wnt-3 is closely associated with the proliferation of neural stem cells. We examined whether hyperbaric oxygen promoted neural stem cells to proliferate and its correlation with Wnt-3 protein in hypoxic-ischemic neonate rats. Hyperbaric oxygen therapy was administered 3 h after hypoxia ischemia daily for 7 days. The proliferating stem cells and Wnt-3 protein were examined dynamically in the subventricular zone. Results showed that stem cells proliferated and peaked 7 days after hyperbaric oxygen therapy. Wnt-3 protein increased to the higher levels 3 days after therapy. Linear regression analysis showed that nestin protein correlated with Wnt-3 protein. We propose that hyperbaric oxygen treatment promote stem cells to proliferate, which is correlated with Wnt-3 protein. *NeuroReport* 18:1753–1756 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: hyperbaric oxygen, hypoxic-ischemic brain damage, neural stem cells, Wnt-3

Introduction

Hypoxic-ischemic encephalopathy is the major recognized perinatal cause of neurological morbidity in full-term newborns [1] and can result in mental impairment, seizures and permanent motor deficits, such as cerebral palsy [2]. At present, however, there are no effective means of repairing hypoxic-ischemic brain damage (HIBD). Therefore, it is necessary to develop a suitable therapy for brain damage.

Increasing knowledge of the renewal of cells within the central nervous system has shed new light on HIBD. Previous studies showed that endogenous neural stem cells might be activated [3] and hypoxia ischemia itself could stimulate the stem cells to proliferation. Its capacity for recruiting endogenous stem cells is, however, limited [4,5]. Thus, other strategies have been described to modify neurogenesis after brain damage [6].

Several studies have indicated that hyperbaric oxygen therapy may alleviate neuronal injury and promote the recovery of HIBD in rats [7–10]. Recent studies have shown that hyperbaric oxygen is capable of promoting brain cell proliferation [11]. We also have found that hyperbaric oxygen therapy can protect the neural stem cells and stimulate cell proliferation in the hippocampal dentate gyrus and subventricular zone initially [12]. All these observations prompted us to ask whether hyperbaric oxygen therapy can stimulate neural stem cells to proliferation. If this is the case, what is the mechanism for the proliferation of stem cells after hyperbaric oxygen therapy?

Wnt signaling is implicated in the control of cell growth and differentiation during central nervous system development [13], which regulates the proliferation and differentiation of neural stem cells. Wnt-3, as the starting protein of Wnt signaling pathway, is closely associated with the proliferation of neural stem cells [14,15]. Therefore, in this study we dynamically examine the proliferation of neural stem cells at different times after hypoxia ischemia and explore the correlation between the proliferation of stem cells and Wnt-3 protein.

Materials and methods

Animals

One hundred and fifty 7-day-old Sprague–Dawley rats of both sexes (purchased from the Animal Department, Xiang Ya School of Medicine, Central South University, China), weighing 12.6–15.8 g, were randomly divided into three groups: (i) a normal control group, (ii) HIBD group, and (iii) a HBO group in which hypoxic-ischemic rats were treated with hyperbaric oxygen (n=50 rats in each group). Each group was divided into five subgroups according to the time after hypoxia ischemia as follows: 6 h, 24 h, 3 days, 7 days, and 14 days (n=10 rats per subgroup).

Animal model of hypoxic-ischemic brain damage

The protocol was evaluated and approved by the Animal Department of XiangYa School of Medicine, Central South University. Seven-day-old rats were subjected to the Rice procedure [16]: rats were anesthetized with ether, and the left carotid artery was sectioned permanently between double ligatures. After recovering for 1–2 h, the rats were

exposed to 2 h of hypoxia in a plastic container that was perfused with a mixture of 8% oxygen balanced with nitrogen. The temperature inside the container was maintained at 36±2°C. The pups were then returned to their mothers.

Hyperbaric oxygen therapy
The HBO groups were administered hyperbaric oxygen therapy within 3 h after hypoxia ischemia. Hyperbaric oxygen was administered for 60 min in a baby hyperbaric oxygen chamber [YLC0.5/1A (a symbol for a baby hyperbaric oxygen chamber with the outer diameter of 0.5 meter and the length of 1 meter), Wuhan, China] pressured with 100% oxygen to 2.0 atm absolute. A constant oxygen flow was given to maintain the oxygen concentration in the chamber at 85% or greater.

5-Bromo-2′-deoxyuridine labeling
Two days before killing, animals were administered with 5-bromo-2′-deoxyuridine (BrdU; Sigma, St Louis, Missouri, USA) intraperitoneally every 8 h for five times (50 mg/kg, dissolved in saline).

Tissue preparation for microscopy
Animals were deeply anesthetized with chloral hydrate (450 mg/kg, intraperitoneally) at different times (6 h, 24 h, 3 days, 7 days, and 14 days) after hypoxia ischemia and underwent a transcardiac perfusion with 0.9% saline, followed by cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). The bregma was labeled with methylene blue. The brain was removed and postfixed in paraformaldehyde for 24 h. Tissue taken from 1.0 to −0.8 mm anterior to the bregma was processed, embedded in paraffin wax, and cut into 5-μm sections coronally (SM2000R, Leica, Nussloch, Germany) on polylysine-coated slides.

Immunohistochemistry
Sections were deparaffined and antigen was restored by microwaving. Sections were then blocked in 5% bovine serum albumin (Sigma) for 1 h at 37°C. Sections were subsequently incubated with the primary antibodies at 4°C overnight. The specific primary antibodies used to identify the proliferating neural stem cells were mouse anti-nestin (1:200, Chemicon, Temecula, California, USA) and rat anti-BrdU (1:200, Accurate Chemicals, Westbury, New York, USA). Mouse anti-nestin (1:200, Chemicon) and rabbit anti-Wnt-3 (1:100, Santa Cruz, California, USA) primary bodies were used to examine the expression of Wnt-3 protein in the stem cells. For BrdU processing, sections were incubated in 2 M HCl for 30 min at 37°C followed by 0.04% pepsin for 6 min at room temperature. Sections were incubated subsequently for 1 h at 37°C in the dark with the following secondary antibodies: fluorescein isothiocyanate (FITC)-conjugated or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (1:100, Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, China). Sections were rinsed and coverslips were placed.

Western blot analysis of Wnt-3 and nestin protein
Animals were deeply anesthetized and fresh tissue samples (approximately 100 mg) were obtained from the lesioned brain at five time points (6 h, 24 h, 3 days, 7 days, 14 days) after hypoxia ischemia. The whole protein was extracted and sodium dodecyl sulfate–polyacrylamide gel (10%) was performed. The protein on the gel was subsequently transferred to the polyvinylidine diflouride transfer membrane (Bio Lab, Hercules, California, USA). The membrane was blocked for 1 h. The membrane was then incubated with a mouse monoclonal antibody to nestin (1:1000; Chemicon) or a rabbit multiclonal antibody to Wnt-3 (1:2000; Santa Cruz) at 4°C overnight. The membrane was then washed and incubated with horseradish peroxide-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1:10 000, BioRad, Hercules, California, USA) for 1 h. After thorough washing, the positive band was revealed by Western blotting detection reagents (KPL, Gaithersburg, Maryland, USA) and autoradiography film. Glyceraldehyde-3-phosphate dehydrogenase (Kangchen Biology, Shanghai, China) was used as a reference protein for the densitometric analyses.

Cell counting and semiquantitative protein estimation on blots
The $BrdU^+nestin^+$ cells in the subventricular zone were counted using laser scanning microscope (LSM 510; Zeiss, Jena, Germany). The fluorescence intensity of Wnt-3 and nestin protein was quantified by using laser scanning confocal microscope software (LSM 510, Zeiss); The Wnt-3, nestin and glyceraldehyde-3-phosphate dehydrogenase proteins were estimated directly from radiograph film by imaging densitometry (TANON 2020, Shanghai, China).

Statistical analysis
Data were expressed as mean±standard deviation. The differences between groups were examined for statistical significance using one-way analysis of variance and the difference between two groups was examined for statistical significance by Newman–Keuls method. The correlation between nestin protein and Wnt-3 protein was analyzed by linear regression.

Results

Proliferation of neural stem cells at different times after hypoxia ischemia
The $BrdU^+nestin^+$ cells were considered to be proliferating stem cells. $BrdU^+nestin^+$ cells were observed in the dorsal angulus lateralis of the subventricular zone in each group with proliferating cells migrating outwards. Hyperbaric oxygen therapy significantly increased the number of $BrdU^+nestin^+$ cells 24 h after hypoxia ischemia (Q=4.9252, $P<0.05$). Thereafter, the $BrdU^+nestin^+$ cells increased gradually 3 days after hypoxia ischemia and reached their highest levels 7 days after hypoxia ischemia. The $BrdU^+nestin^+$ cells decreased gradually 14 days after hypoxia ischemia. Even so, more $BrdU^+nestin^+$ cells were observed in the HBO group than controls (Q=12.2474, $P<0.01$) and HIBD group (Q=8.4619, $P<0.01$; Fig. 1).

Activation of Wnt-3 in stem cells after hyperbaric oxygen therapy
The Wnt-3 protein, stained green on the membrane, was observed in the neural stem cells (labeled by nestin protein, red). An initial increase of Wnt-3 protein was observed at 6 h after hypoxia ischemia (Q=4.2098, $P<0.05$) in the HBO

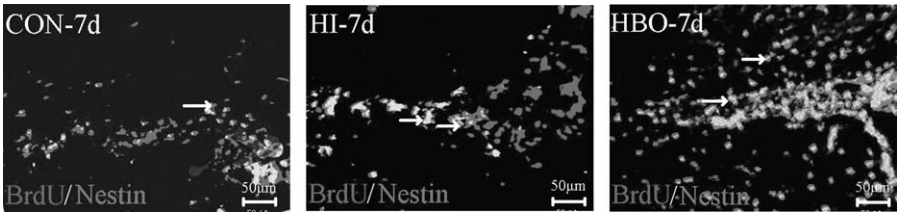

Fig. 1 Effects of hyperbaric oxygen therapy on neural stem cells. The proliferating neural stem cells in the subventricular zone were double labeled by BrdU/nestin immunofluorescence. More $BrdU^+nestin^+$ cells were seen in the HBO group as compared with controls and the HIBD group 7 days after hypoxia ischemia. The scale bar was labeled in each figure. BrdU, 5-bromo-2′-deoxyuridine; HIBD, hypoxic-ischemic brain damage; HBO, hypoxic-ischemic rats were treated with hyperbaric oxygen.

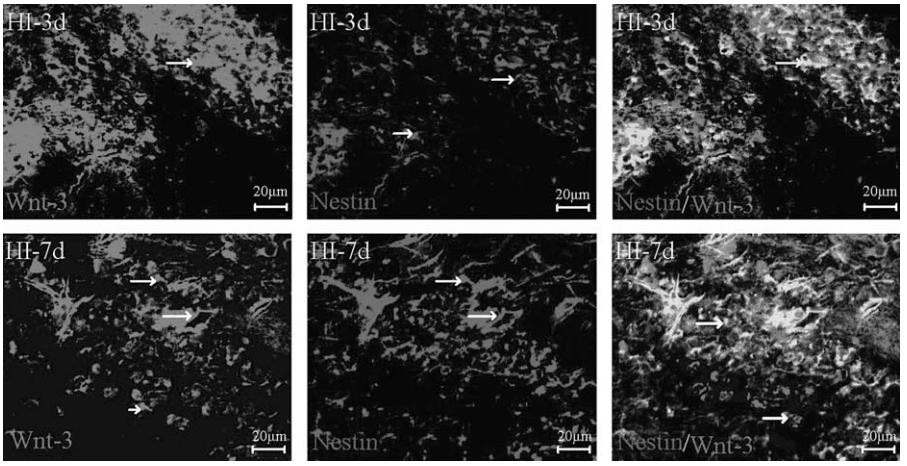

Fig. 2 Effects of hyperbaric oxygen therapy on Wnt-3 and nestin protein. Wnt-3 protein and nestin protein increased to the higher level at 3 days and 7 days after hypoxia ischemia, respectively. The scale bar was labeled in each figure.

group, as compared with controls; then Wnt-3 protein increased to a higher level 3 days after hypoxia ischemia (Q=3.7900, $P<0.01$) and maintained at the higher level 7 days after hypoxia ischemia. Wnt-3 protein then declined at 14 days after hypoxia ischemia (Q=6.7186, $P<0.01$, Fig. 2).

Expression of nestin protein after HBO therapy

The nestin protein, stained red in the cytoplasm, increased 24 h after hypoxia ischemia initially (Q=3.1200, $P<0.05$), then increased gradually 3 days after hypoxia ischemia (Q=8.0229, $P<0.01$) and peaked 7 days after hypoxia ischemia (Q=6.2401, $P<0.01$, as compared with nestin protein 3 days after hypoxia ischemia). It declined 14 days after hypoxia ischemia (Fig. 2).

Correlation between Wnt-3 protein and nestin protein

The scatterplot graph was made according to the fluorescence intensity of Wnt-3 and nestin protein at all time points in the animals of HBO group. Linear regression analysis showed that Wnt-3 protein and nestin protein were linear correlated (r^2=0.64, $P<0.05$).

Western blot analysis of total cell nestin and Wnt-3 protein

Wnt-3 protein exists in the normal neonatal rats and decreases with age [17]. In the HBO group, Wnt-3 protein increased 6 h after hypoxia ischemia and rose to the higher levels significantly 3 days after hypoxia ischemia (Q=4.3658, $P<0.01$), till 14 days after hypoxia ischemia it decreased significantly (Q=7.7241, $P<0.01$).

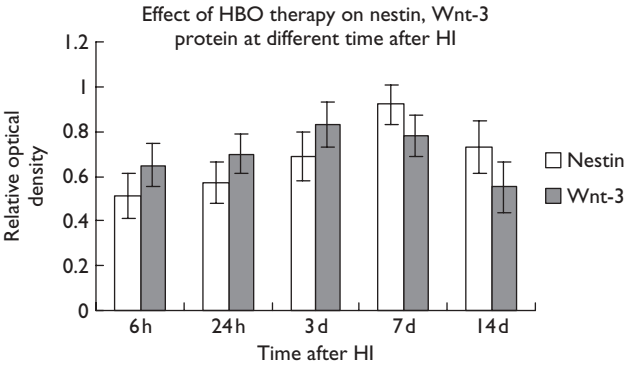

Fig. 3 Western blot analysis of nestin, Wnt-3 protein. The bar graph of Western blots shows the relative optical density of nestin and Wnt-3 protein at different times after hypoxia ischemia, error bars show standard deviation.

An initial increase of nestin protein was observed 24 h after hypoxia ischemia (Q=3.1200, $P<0.05$), the nestin protein increased to the higher levels 7 days after hypoxia ischemia (Q=6.2401, $P<0.01$), then it declined 14 days after hypoxia ischemia (Fig. 3).

Discussion

Neural stem cells are immature cells with the capability of self-renewal and differentiation into functional astrocytes and neurons [3]. Neural stem cells reside mainly in the subventricular zone, hippocampus and cortex, among which

the subventricular zone is identified as an endogenous resource of neuronal precursors [18]. BrdU, a thymidine analog, is a sensitive and specific indicator of DNA synthesis and cell proliferation. In this study, BrdU was used to evaluate cellular DNA synthesis and label proliferating cells [19]. In the developing brain, proliferating cells include neural stem cells and astrocytes, so an increase of $BrdU^+$ cells alone does not indicate the proliferation of stem cells. Nestin, an intermediate filament protein, is widely used to identify, isolate or purify neural stem cells [20]. Thus, in this study, BrdU/nestin immunofluorescence was used to double label the proliferating stem cells.

In the mammalian brain, neurogenesis persists in the subventricular zone of the lateral ventricles [21] and newly generated neural precursors are recruited from the subventricular zone to nearby lesioned areas [22]. This study showed that hyperbaric oxygen therapy increased the number of $BrdU^+nestin^+$ cells in the subventricular zone, significantly higher than that in the controls, indicating that hyperbaric oxygen can promote the proliferation of neural stem cells. Our study also showed that the number of $BrdU^+nestin^+$ cells peaked 7 days after hyperbaric oxygen therapy. Western blot analysis revealed the similar results: in the HBO group, nestin protein began to increase 24 h and peaked 7 days after hypoxia ischemia. These observations indicated that hyperbaric oxygen treatment was neuroprotective to neural stem cells.

The mechanism for the proliferation of stem cells after hyperbaric oxygen therapy is still unknown. Wnt signaling pathway was closely associated with the proliferation of stem cells and neurogenesis [23,24]. Once Wnt proteins are secreted, they are bound tightly to the cell surface, activating the intracellular signal pathway and regulating the target genes, thus promoting cell proliferation [25]. Wnt-3 protein reaches the highest level at embryonic week 12, declines after birth and drops to the minimal level in the adult rat brain [13,14,17]. In this study, Wnt-3 protein was detected in the proliferating neural stem cells and changed dynamically. It reached the higher levels 3 days after hypoxia ischemia, earlier than the time that endogenous NSC increased to the higher levels, and declined 14 days after hypoxia ischemia. The Western blot analysis of Wnt-3 protein got the same results. All these indicated the Wnt signaling pathway was activated before the proliferation of stem cells and the proliferation of stem cells was associated with Wnt-3 protein in hypoxic-ischemic neonate rats after hyperbaric oxygen therapy. To confirm our hypothesis, Wnt-3 and nestin protein at different times were analyzed by linear regression. Results showed that nestin protein was correlated with Wnt-3 protein, indicating the proliferation of stem cells after hyperbaric oxygen therapy was correlated with the Wnt signaling.

Conclusion

Hyperbaric oxygen treatment promotes the proliferation of endogenous neural stem cells in hypoxic-ischemic neonate rats, an effect that is correlated with Wnt-3 protein, a key protein in Wnt signaling.

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