Research Report

Therapeutic window of hyperbaric oxygen therapy for hypoxic–ischemic brain damage in newborn rats

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ABSTRACT

Previous studies showed that hyperbaric oxygen (HBO) promoted cell proliferation in hypoxic–ischemic (HI) neonate rats. Neural stem cells (NSC) existed in the brain lifelong and can be activated. This study was undertaken to assess whether HBO treatment promoted the proliferation of NSC and repaired the brain damage regardless of when it is started, thus to explore the therapeutic window of HBO treatment. Seven-day-old Sprague–Dawley rats underwent left carotid ligation followed by 2 h of hypoxic stress (8% O\textsubscript{2} at 37 °C). Hyperbaric oxygen therapy was administered 3, 6, 12, 24, and 72 h after HI. 5-bromo-2′-deoxyuridine and 5-bromo-2′-deoxyuridine/nestin were detected by immunofluorescence and nestin was examined by western blot analysis 10 days after HI. T-maze forced alternation, the foot-fault test, and the radial arm maze were conducted at P 22 days (14 days after HI), P 30 days, and P 34 days. Thereafter, cerebral morphology was examined by Nissl-staining 28 days after HI. There were remarkable increases in the proliferation of neural stem cells in the HBO-treated group, 3, 6, 12, and 24 h after HI, as compared with the HIBD group. The HBO-treated group, 3, 6, and 12 h after HI, performed better in the behavioral test and had less neural loss in the hippocampal CA1 region as compared with the HIBD group. The therapeutic window for effective HBO treatment could be delayed up to 12 h after HIBD, while the effect decreased 24 h after HI.

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1. Introduction

Hypoxic–ischemic encephalopathy (HIE), a common but severe disease, is still the major perinatal cause of neurologic morbidity in full-term newborns (Triulzi et al., 2006). However, at present there is no effective means of repairing hypoxic–ischemic (HI) brain damage (George et al., 2007; Blackmon and Stark, 2007). Therefore, it is important to find an effective treatment for hypoxic–ischemic brain damage (HIBD). Hyperbaric oxygen (HBO) therapy has been used in neonatal hypoxic–ischemic brain damage (HIBD) for several years (Calvert et al., 2002) and it has been shown that HBO therapy (<3.0 atm absolute) was neuroprotective and was not able to result in toxicity of oxygen, such as retinopathy of prematurity ROP (Calvert et al., 2003; Zhang et al., 2005; Calvert and Zhang, 2007). However, the optimal application and therapeutic effect of HBO therapy in neonatal HIBD remains controversial (Zhang et al., 2005).

Previous studies have shown that the therapeutic window is associated with the therapeutic effects and HBO therapy is highly efficient in reducing infarct volume and promoting the recovery of neurobehavioral function within 6 h after brain damage in an adult model of middle cerebral artery occlusion.

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The neonatal brain, however, is in the developmental stage, so it is quite different from the adult brain in both anatomic structure and metabolism. Thus, it is unlikely that the therapeutic window for HBO therapy in the adult can be reliably applied to neonates. Few reports have discussed the therapeutic window of HBO therapy for neonatal HIBD and the 6-hour therapeutic window of HBO therapy as determined from the adult model of MCAO.

Fig. 1 – Effects of HBO treatment at different therapeutic window on the proliferation of endogenous NSC. a. Proliferation of endogenous NSC in the SVZ at different therapeutic window. The proliferating endogenous NSC were double labeled by BrdU/nestin, red in the nucleus and green in the cytoplasm (arrows). The first two pictures were the color separation of the third picture, which were stained red by BrdU in the nucleus and green by Nestin in the cytoplasm respectively (arrows). The immunofluorescence results of the left SVZ were analyzed by laser scanning confocal microscopy (LSM 510; Zeiss) and demonstrated the distribution of proliferating NSC in the SVZ at different therapeutic window. Scale bar is 100 μm. b. The bar chart of BrdU+ nestin+ cells in the subventricular zone. Error bars show standard deviation; * indicates \( P < 0.05 \) vs. group HIBD; # indicates \( P < 0.01 \) vs. all other group.
dramatically limits the application of HBO therapy in HIE. Therefore, it would be of great value to find the appropriate therapeutic window of HBO therapy for neonatal rats after HI.

A previous study has demonstrated that HBO is capable of promoting brain cell proliferation (Günther et al., 2005). Our initial study also showed that HBO therapy stimulated cells to proliferate in hypoxic-ischemic neonate rats (Yu et al., 2006). As NSC reside in specific areas of the brain lifelong (Gage, 2000) and are capable of being activated by trauma or focal surrounding signals, especially in the developing brain, we hypothesized that HBO treatment is a stimulus resulting in the proliferation of NSC, thereby repairing brain damage at any time. Therefore, in this study we examined the NSC proliferating in the subventricular zone (SVZ) during the earliest period, analyzed the resulting animal behaviors including the working memory, spatial learning and sensorimotor function at different development stages, and determined the brain histomorphology over a long interval to explore the therapeutic window of HBO therapy in neonatal rats after HI.

2. Results

2.1. Effects of HBO therapy at different therapeutic window on endogenous NSC

BrdU-labeled newborn nuclei were primarily observed in the SVZ, especially at the angular lateralis of the lateral ventricle, 10 days after HI. As depicted in Fig. 2, only less proliferating cells (BrdU-labeled) and less proliferating NSC (BrdU– nestin+ cells) were noted within the SVZ of the CON group (54±9 cells/mm²) and the HIBD group (66±12 cells/mm²) and there was no significant difference between the CON group and HIBD group (P>0.05). A greater number of BrdU+ nestin+ cells were observed in group HBO-3h, group HBO-6h, group HBO-12h, and group HBO-24h (128±16, 109±15, 92±12, and 82±13 cells/mm², respectively) as compared with the CON group and the HIBD group (P<0.05; Fig. 1).

2.2. Western blot analysis of nestin protein

Western blots were analyzed for the nestin protein in the left cerebral hemisphere from control rats, HIBD rats and HIBD rats treated with HBO at different therapeutic window. In each case, a representative band is shown as well as a corresponding GAPDH band. Lanes 1–7 denote respectively the CON group, the HIBD group, group HI-3h HBO, group HI-6h HBO, group HI-12h HBO, group HI-24h HBO, group HI-72h HBO. b. The bar chart of the relative concentration of nestin protein from each group. The relative concentration is expressed as the ratio of optical density (OD) nestin to OD GAPDH. Error bars show standard deviation, * indicates P<0.05 vs. group HIBD.

CON group, group HBO-3h, group HBO-6h, and group HBO-12h, the percentage of correct responses increased day-by-day, while the HIBD group, the group HBO-24h, and group HBO-72h had got a bad increase. On the fourth day, the percentages of correct in the CON group, group HBO-3h, group HBO-6h, and group HBO-12h were higher as compared with the HIBD group (P<0.05). There was no significant difference in the percentages correct between the HIBD group and group HBO-24h and group HBO-72h. On the radial arm maze, the time taken to enter the three baited arms in group HBO-3h, group HBO-6h, and group HBO-12h (83.21±11.2, 89.3±13.2, and 101.23±11.5 min, respectively) was shorter and memory errors were less, as compared with the HIBD group (128.11±23.10 min, P<0.001). On the foot-fault test, the difference in the number of foot-faults from the right to the left side in group HBO-3h, group HBO-6h, and group HBO-12h was better than that in the HIBD group (3.21±1.25, P<0.0001; Fig. 3).

2.4. Effect of HBO therapy at therapeutic window on Nissl-stained cells in the CA1 sector

Nissl sections were used for determination of ischemic cell damage by cell counting in the CA1 sector. In the HIBD group, a loss of cytoplasmic Nissl-staining occurred in the CA1 sector; impaired neurons were characterized by cellular shrinkage, cytoplasmic eosinophilia, disappearance of Nissl bodies, and nuclear pyknosis/hyperchromasia. Group HBO-3h, group HBO-
6h, and group HBO-12h lost fewer neurons (245±33 cells/mm², 235±42 cells/mm², and 212±35 cells/mm², respectively) compared with the HIBD group (102±36 cells/mm², P<0.01); in group HBO-24h and group HBO-72h, staining of the cells was lighter and more cells were lost compared with the CON group (261±28 cells/mm², P<0.05; Fig. 4).

3. Discussion

Neural stem cells are immature cells with the capability of self-renewal and differentiation into functional astrocytes and neurons. Neural stem cells reside mainly in the SVZ, hippocampus, and cortex (Gage, 2000), among which the SVZ is identified as an endogenous resource of neuronal precursors that can be recruited to adjacent areas with lesions (Leker and McKay, 2004). 5-bromo-2′-deoxyuridine, a thymidine analog, is a sensitive and specific indicator of DNA synthesis and cell proliferation. In the current study, BrdU was used to evaluate cellular DNA synthesis and label proliferating cells, thus evaluating the proliferation of cells in the SVZ of the lateral ventricle. Nestin, an intermediate filament protein, has also been used as a marker of neural progenitor cells, especially neural stem cells. Thus, BrdU/nestin were used to double-label the proliferating NSC in the SVZ, which were then examined by laser scanning confocal microscopy 10 days after HI. It was found that a greater number of BrdU+ nestin+ cells were in the HBO-treated group within 24 h following HI as compared with the HIBD group. A greater number of BrdU+ nestin+ cells were observed in group HBO-72h, but there was no significant difference. In the Western blot analysis of nestin, the same results were obtained, indicating that HBO therapy stimulates the proliferation of NSC if the treatment is administered within 24 h after HI. As the NSC persist in the brain lifelong and HBO can stimulate the NSC into proliferation and differentiation, why didn’t the NSC proliferate when the HBO therapy was administered 72 h after HI? We have concluded that this occurrence may reflect changes in the niches of the brain. It has been reported that microglia proliferate and release inflammatory factors after HI (Oechmichen and Meissner, 2006), preventing the recovery of brain tissue and inducing apoptosis and death of newborn NSC. In addition, 48 – 72 h after HI is the peak time of apoptosis and death of NSC (Yu et al., 2004; Oechmichen and Meissner, 2006). If HBO therapy is administered at this time, less NSC remain, thus less neurons would be seen compared with the HBO-treated group at an earlier time period. Researchers have found that multiple applications of HBO treatment may salvage the injured brain and promote recovery of neurobehavioral function if the therapeutic window was delayed to

Fig. 3 – Effects of HBO treatment at different treatment window on behavior test. a: Effect of HBO treatment at different therapeutic window on a T-maze forced alteration task at 22 days of age. Results represent the mean percent of correct responses of each group. The bar chart showed the percent of correctness in different groups on the fourth day. Error bars show standard deviation. * indicates P<0.05 vs. the HIBD group. b: Effect of HBO treatment at different therapeutic window on radial arm maze performances. Animals were tested at 30 days of age. This bar chart shows the time taken to visit the 3 baited arms. Error bars show standard deviation. * indicates P<0.01 vs. the HIBD group. c: Effect of HBO treatment at different therapeutic window on radial arm maze performances. The bar chart showed the working memory errors in each group. Error bars show standard deviation. * indicates P<0.05 vs. group HIBD. d: Effect of HBO treatment at different therapeutic window on the foot-fault test at 35 days of age. The bar chart showed the number of foot-faults right-left side in each group. * indicates P<0.0001 vs. the HIBD group.
24 h after acute brain damage in the adult (Yin and Zhang, 2005). Therefore, if the therapeutic window is delayed and repeated treatments are given after neonatal HI, whether HBO treatment can drive NSC to proliferation needs further study.

We obtained different results from the neurobehavioral tests and Nissl-staining during the later time period, which is of great significance in evaluating the therapeutic effect of HBO. Based on the neurologic scores, we found that HBO therapy administered within 12 h after HI improved neurologic performance as compared with the HIBD group. On the contrary, HBO therapy administered 24 h after HIBD resulted in poor neurologic performance and could not alleviate the brain damage compared with the HIBD group. The Nissl-staining gave rise to the same results. In the HBO-treated group 12 h after HIBD, more neurons were lost and the morphology was abnormal as compared with the CON group. We thus conclude that the HBO therapeutic window can be expanded to 12 h after HIBD, which is somewhat different from the results we obtained with the proliferating NSC.

Hyperbaric oxygen therapy administered within 24 h after HIBD may promote the proliferation of NSC in the SVZ, while HBO therapy within 12 h after HI may improve the neurologic performance and reduce brain damage, indicating that the treatment window could be delayed for 12 h after HI. Hyperbaric oxygen therapy administered 24 h after HI may significantly promote the proliferation of NSC, but it has a poor prognosis, indicating that the newly proliferating NSC did not differentiate into neurons or migrate to the impaired areas. Proliferation and differentiation are regulated by external factors, such as cytokines, neurotransmitters, hormones, and local niches, and internal factors, such as autogenes (Park et al., 2006; Aguirre et al., 2005; Günther et al., 2005). After HI, great changes have occurred in the local niches, and more inflammatory transmitters might be produced, which hamper the survival of NSC. Moreover, external signals target the internal genes by signal conduction pathways (Bouhon et al., 2005; Cai et al., 2006), interfere with migration and differentiation and result in the dysmaturity of NSC (Felling et al., 2006), which might account for
the poor prognosis when HBO was delayed to 24 h after HI. Hyperbaric oxygen treatment can promote endogenous NSC to proliferate, whether repeated HBO treatment can improve the brain niches, promote the endogenous NSC to proliferate and differentiate, thus reverse brain damage and extended the therapeutic window needs further study.

In conclusion, HBO therapy administered within 12 h after HI is capable of stimulating NSC to proliferate, improving some of the neurologic performances, and alleviating the brain damage.

4. Experimental procedures

4.1. Study designs

Seven-day-old Sprague–Dawley rats (purchased from the Animal Department of Xiang Ya School of Medicine of Central South University), weighing 12.8–15.6 g, were randomly divided into 7 groups: 1) a normal control group (CON), 2) a HBBD group, 3) a HBO-3h group (HBO treatment was administered 3 h post HI), 4) a HBO-6h group (HBO treatment was administered 3 h post HI), 5) a HBO-12h group (HBO treatment was administered 12 h post HI), 6) a HBO-24h group (HBO treatment was administered 24 h post HI), and 7) a HBO-72h group (HBO treatment was administered 72 h post HI; n=30 for each group; Fig. 1).

4.2. Hypoxia–ischemia model and HBO treatment

The protocol was evaluated and approved by the Animal Department of Xiang Ya School of Medicine, Central South University. Unsexed 7-day-old Sprague–Dawley rats were subjected to the Rice procedure (Rice et al., 1981), as described previously. Briefly, rats were anesthetized with ether, and the left carotid artery was sectioned permanently between double ligatures. After recovery for 1–2 h, the rats were exposed to 2 h of hypoxia in a plastic container that was perfused with a mixture of humidified 8% oxygen balanced with nitrogen. The temperature inside the container was maintained at 37 °C. The pups were then returned to their dams after the hypoxic exposure. The HBO groups were administered HBO treatment 3, 6, 12, 24, and 72 h after HI. The HBO treatment was administered for 60 min in a baby HBO chamber (YLCD-5/1A, WuHan, China) with a concentration in the chamber at 85% or greater. HBO treatment was administered for 60 min in a baby HBO chamber (YLCD-5/1A, WuHan, China) with a concentration in the chamber at 85% or greater.

4.3. 5-Bromo-2′-deoxyuridine labeling

Eight days after HI, animals were administered 5-bromo-2′-deoxyuridine (BrdU; Sigma, St. Louis, MO, USA) intraperitoneally once every 8 h for 5 doses (50 mg/kg, dissolved in saline). Two hours after the last injection, the animals were sacrificed (Hirabayshi and Gotoh, 2005; Iwai et al., 2002).

4.4. Tissue preparation for microscopy

Animals were deeply anesthetized with chloral hydrate (450 mg/ kg, intraperitoneally) at 2 sequential time intervals after HI (10 days or 4 weeks; n=10/sub-group) and underwent a transcardiac perfusion with 50 ml 0.9% saline, followed by 50 ml 4% paraformaldehyde (PFA) in 0.1 M PBS (pH=7.4). The bregma and the left hemisphere were labeled with methylene blue. Then the brain was removed and post-fixed in paraformaldehyde for 24 h. Tissue, 1.0 to −0.8 mm anterior to the bregma, was processed, embedded in paraffin max, and cut into 5 μm sections coronally (SM2000R, Leica, Nussloch, Germany) on polylysine-coated slides.

4.5. Immunohistochemistry

Sections were deparaffinized and antigen was restored by microwaving, then sections were blocked in 5% bovine serum albumin (BSA, 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 NSC were mouse anti-nestin (1:200, Chemicon, Temecula, CA) and rat anti-BrdU (1:200, Accurate Chemicals, Westbury, NY, USA). 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: FITC-conjugated goat anti-mouse (1:100, Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, China) for nestin, and TRITC-conjugated goat anti-rat (1:100, Beijing Zhongshan Golden Bridge Biotechnology Co.) for BrdU. Sections were rinsed and coverslips were placed. Immunoreactivity was demonstrated by using laser scanning confocal microscopy (LSM Invert 510; Zeiss, Jena, Germany).

4.6. Western blot analysis

Ten days after HI, animals were deeply anesthetized and fresh tissue samples (approximately 100 mg) from the lesioned brain (bregma between 1.0 and −4.5 mm) were obtained and placed on ice. Samples were then ground into fine powder and homogenized in a tissue-lysis buffer (0.1 mol/l NaCl, 0.01 mol/l Tris–HCl, 0.001 mol/l EDTA, and 1 μg/ml aprotinin). SDS-gel electrophoresis was performed in 5% polyacrylamide gels under non-reducing conditions. Lysate equivalent to 60 μg of protein from brain tissue samples of every group were run on each gel, together with pre-stained low-molecular-weight markers (BioRad, Richmond, CA, USA). The proteins on the gel were subsequently transferred to the Immobillon PVDF transfer membrane in buffer containing 20% methanol, 39 mmol/l glycine, 48 mmol/l Tris base, and 0.4% SDS (pH=8.3). The membrane was blocked in 5% powdered milk in 0.01 mmol/l PB for 1 h. The membrane was then incubated for 2 h with a mouse monoclonal antibody to nestin (1:1000; Chemicon) at 4 °C overnight. The membrane was washed with 0.1% TBS-Tween and then incubated with horseradish peroxide-conjugated anti-mouse antibody (1:2000) and GAPDH (1:10,000) for 1 h. After thorough washing, the positive band was revealed by using ECL Western blotting detection reagents (KPL, Maryland, USA) and autoradiography film.

4.7. Behavior evaluation

4.7.1. T-maze forced alternation (Balduini et al., 2001)

At 22 days of age (14 d after HI), animals were habituated for 2 consecutive days to the presence of food pellets (40 mg) in a
wooden T-maze, 11 cm in width × 18 cm in height × 40 cm in length. The guillotine doors were placed 15 cm from the end of the start box. Each trial consisted of a sample run and a test run. In the sample run, a wooden block was placed at a selected point in the T-maze to close off one arm. The animal was forced to enter the open arm and allowed to eat the food. The rat was then picked up and confined to the start box for 15 s. The guillotine doors placed at the start box and at the selected point in the T-maze were then removed and the animal started the test run. During this run, the animal was deemed to have made a choice when it had placed a hind foot in one of the two arms; no retraction of the foot was allowed. If the rat alternated, i.e., entered the arm not previously visited on the sample run, the rat was allowed to eat the reward and then returned to its cage. If the other arm was chosen, i.e., the same arm visited on the sample run, the rat was confined to that arm for 10 s and then returned to its cage. Each rat underwent five trials a day separated by an inter-trial time of 15 min for 4 consecutive days and the performance was recorded as a percentage of correct responses.

4.7.2. Radial arm maze
The radial arm maze (Balduini et al., 2000) was performed with 30-day-old animals. The 8-arm radial maze consisted of a central platform (30 cm in diameter) from which 8 arms radiated symmetrically, 50 cm in length and 12 cm in width. A well was present at the outer end of each arm. Animals were deprived of water for 48 h before testing. At the end of each daily session, the animals were individually placed in a cage, allowed to drink ad libitum for 30 min, and then placed back into their home cages. Initially, animals were allowed free exploration sessions for 2 consecutive days, with all arms baited with water (50 ml per well). For spatial discrimination testing, only 3 arms were always baited, and the sequence of angles between the arms was 135°, 90°, and 135°. Rats were tested for acquisition over 3 daily sessions composed of 5 trials, separated by 1-minute intervals. Each trial began with the placement of the animal on the central platform facing the fixed arm and ended when the rat had visited the 3 baited arms. The following data were recorded: (1) the time taken to visit the 3 baited arms and (2) the number of reference memory errors, i.e., each entry into a non-baited arm.

4.7.3. Foot-fault test
Following the radial arm maze, the rat was placed on a horizontal grid floor (50 cm × 40 cm, with a square size of 3 cm × 3 cm, and a wire diameter of 0.4 cm). The foot-fault (Bona et al., 1997) was defined when the animal misplaced a fore- or hind-limb and the paw fell between the grid bars. The number of foot-faults was noted within 2 min. Only the side difference of foot-faults was used for the statistical evaluation to eliminate the influence of the extent of activity on different rats.

4.8. Nissl-staining

The brains from behaviorally-tested animals were used for histology. The tissue preparation was done as described above 4 weeks after HI (P 35 days, that is, 35 days after birth). After deparaffinage, the slides were Nissl-stained with toluidine blue for neuronal cell bodies, the brain sections were then mounted, air-dried, dehydrated, and coverslipped.

4.9. Cell counting and semi-quantitative protein estimation on blots
Quantification of BrdU+ nestin+ cells in the SVZ and the neuronal cell density in the CA1 region in the Nissl-stained slices was done using the confocal scanning microscopy software (LSM 510). A systematic, random counting procedure was used, as described by Williams and Rakic (1988) for quantification. To determine the number of BrdU+ nestin+ cells in the SVZ and neuronal cells in the CA1, separate series of every sixth section of the left hemisphere from each brain were analyzed. The relative amounts of nestin protein were estimated by an imaging densitometer (TANON 2020, Shanghai, China). In every case, results were obtained by calculating a ratio of the nestin protein levels to the GAPDH protein levels and reported as the relative optical density.

4.10. Statistical analysis
Data are presented as the mean±standard deviation (SD). Analysis of variance (ANOVA) was used to compare the mean values among the groups. The T-maze test and radial arm maze results were analyzed by 2-way and 1-way ANOVA, respectively. Mann–Whitney U test was used for the foot-fault test. P value <0.05 was considered significant.

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