Original Article

Hyperbaric oxygenation promotes neural stem cell proliferation and protects the learning and memory ability in neonatal hypoxic-ischemic brain damage

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Abstract: The aim of our study was to evaluate whether hyperbaric oxygenation (HBO) was an effective therapy for neonatal hypoxic ischemic brain damage (HIBD). Seven-day-old rat pups were divided into 3 groups: sham, hypoxia-ischemia (HI) control and HI-HBO group. HBO was administered for HI rats daily. The pathologic changes in brain tissues were observed by hematoxylin-eosin (H-E) staining. The immunohistochemical staining was applied to detect the Nestin and 5-bromo-2-deoxyuridine (BrdU) positive cells in hippocampal dentate gyrus region. The learning and memory function of rats was examined by Morris water maze. The HI rats showed obvious pathologic changes accompanied by levels decreasing and disorder arrangement of pyramidal cells, glial cells proliferation in postopertive, and nerve nuclei broken, while pathologic changes of rats in sham group was approximate to that in the HI + HBO group that was opposite to the HI group. Compared with the sham group, the Nestin and BrdU positive cells in HBO + HI group at different time points increased significantly ($P < 0.01$). Learning and memory function of rats in HI group was poor compared with the sham/HI + HBO group ($P < 0.01$), while that in HI + HBO group was approximate to that in sham group ($P > 0.05$). HBO treatment improved the learning and memory ability of the HI rats. HBO therapy may be effective for neonatal HIBD treatment.

Keywords: Hypoxic ischemic brain damage, hyperbaric oxygenation, hematoxylin-eosin staining, Nestin, 5-bromo-2-deoxyuridine, Morris water maze

Introduction

Hypoxic ischemic brain damage (HIBD) is a common and severe complication resulting from reduced supply of oxygen in brain [1]. The neonatal HIBD may cause metal impairment, seizures, motor deficits, cognize and learning disability [2, 3]. Besides, HI brain damage is always associated with high rate of morbidity and mortality in newborns [4]. The HIBD especially in newborns is a heath concern highlighted all over the world.

Hyperbaric oxygenation (HBO) has been applied in neonatal disease treatment for many years [5]. Recently, there is an issue on debate whether hyperbaric oxygenation (HBO) is an effective therapy for HIBD treatment. Some studies reported that HBO stimulated the proliferation and migration of neural stem cells (NSCs) in brain tissues, which contributed to central nervous system recovery and repaired the brain damage [6]. While others are anxious about the safety and validity of HBO treatment due to the oxygen toxicity, which may increase the infract area [7]. However, there is no confirming verdict about this issue.

The HIBD model was successfully constructed with 7-day-postnatal rats and widely used to investigate the mechanism underlying HIBD development in neonates [8]. In the present study, we constructed the HIBD model with Sprague-Dawley rats. Combined with BrdU (5-bromo-2’-deoxyuridine) labeling technology, we assessed the Nestin and BrdU positive cells to trace the proliferation of NSCs. The learning
and memory disability of HBO treatment rats were evaluated by Morris water maze (MWM) test. The purpose of this work was to explore the therapeutic effect of HBO on HIBD neonates.

Materials and methods

Animals and group

Unsexed 7-day-postnatal Sprague-Dawley (SD) rats (purchased from the Animal Department of the Third Military Medical University, Chongqing, China) were randomly divided into 3 groups: sham group (underwent surgery but no carotid ligation and no hypoxia) (n = 40), HI control group (carotid ligation and hypoxia, no intervention) (n = 40) and HI-HBO group (carotid ligation and hypoxia, HBO treatment) (n = 40). In each group, the pups were confirmed to be from each litter for parity. After surgery for 15-30 min, the pups in HI-HBO group were placed in the baby HBO chamber (YLC0.5/1A, Wuhan, China) and treated with HBO. The HBO treatment was performed daily as previously described [9]. All the pups were maintained with food and water ad libitum normally throughout the study. At postnatal day 30, 8 pups from each group underwent Morris water maze behavior measurement and same numbers of rats were used at different time points.
for hematoxylin-eosin (H-E) and immunohistochemical staining.

**Hypoxia-ischemia (HI) animal model construction**

The protocol was approved by the local Animal and Ethics Review Committee. The 7-day-postnatal (SD) rats were subjected to the construction of HIBO animal model based on the modified Rice-Vannucci procedure [8]. Briefly, neonatal rats were administrated with ether inhalationally. At a temperature of 37°C, the left common carotid artery of the pups were exposed and double ligatured with 0 surgical sutures. The pups were placed in self-controlled hypoxic device perfused with a mixed gas of 8% O₂ + 92% N₂ for 2 h. After hypoxia exposure, the pups were returned to their dams with food and water available ad libitum.

**BrdU (5-bromo-2'-deoxyuridine) labeling**

All the rats were administrated with BrdU (Sigma, USA) (50 µg/g) intradermally at one day before being killed per 4 h for 3 times. At 12 h after the last injection, the rats were put to death for further study.

**Tissue preparation for microscopy and H-E staining**

Animals (n = 8) were death by lethal injection with 4% paraformaldehyde at 4 sequential time

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**Figure 2.** Immunohistochemical staining demonstrated Nestin expression in the hippocampus of each group (× 400).
Table 1. Numbers of Nestin positive cells in hippocampal DG region at different time points

<table>
<thead>
<tr>
<th>Groups</th>
<th>4 d</th>
<th>7 d</th>
<th>14 d</th>
<th>21 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>30.50 ± 2.45</td>
<td>20.38 ± 1.69</td>
<td>8.63 ± 1.60</td>
<td>2.88 ± 1.25</td>
</tr>
<tr>
<td>HI control</td>
<td>40.00 ± 4.81</td>
<td>29.50 ± 2.45</td>
<td>16.63 ± 1.69</td>
<td>4.75 ± 1.28</td>
</tr>
<tr>
<td>HI-HBO</td>
<td>51.25 ± 6.04</td>
<td>40.00 ± 4.90</td>
<td>24.75 ± 3.11</td>
<td>8.00 ± 1.31</td>
</tr>
</tbody>
</table>

*P < 0.01 compared with sham group; *P < 0.01 compared with HI group; DG: dentate gyrus.

Morris water maze (MWM) test

In order to assess the spatial learning and memory of the experimental animals, we performed the MWM test. The maze was composed of a circular tank (130 cm diameter, 45 m depth) and a computerized tracking system (MT-200, Chengdou Taimeng, China). The tank with white interior was filled with milk powder solution with a temperature of 23-25°C. The rats were labeled with picric acid at the head. The tank and surrounding environment remained the same throughout the MWM test.

In the spatial navigation test, the animals were released in the water from 4 different initial points: N (north), E (east), S (south), W (west) to locate the hidden platform (11.5 cm diameter, 30 cm depth, 2 cm below the water surface) in the NE (Northeast) of the tank. Before the test, the rats were allowed to swim freely in the circular tank for 2 min to be familiar with the maze environment. Every rat was subjected to 4 swimming trials in morning and afternoon time period for 5 consecutive days. The rats started the test randomly from four positions and the escape latency time (from starting point to platform) was recorded for each trial. If the rats failed to reach the hidden platform within 120 s, they were guided to the platform and the escape latency time was recorded as 120 s for each rat in this trial. The time interval was set as 60 s between two trails.

On day 6 of the MWM test, the spatial probe trial was carried out to evaluate the spatial memory. The platform was removed before the rats were released to the water from random starting points at the same time. The number of times that one rat crossed the former platform area within 2 min was recorded as the platform crossing frequencies. In the test process, the computerized tracking system was applied for automatic video and data acquisition.

Data analysis

All the data were displayed as mean ± standard deviation (SD) and analyzed by SPSS 16.0 software. The immunohistochemical data was examined by one-way ANOVA combined with Fisher’s Least Significant Difference (LSD) test.
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Differences among groups at different time points in the escape latency time and platform crossing frequencies were evaluated by the two-way ANOVA with repeated measures and one-way ANOVA, respectively. $P < 0.05$ was considered as significant.

After the model construction, 5 rats became weak for infection and other factors. Although these animals did not die immediately, all the 5 rats were dead within 4 days after surgery, among which, 2 ones died from bites of the mother rat and 3 ones for disability to suck. The dead animals were replenished to maintain the number of rats as 40. But during the experimental period, no animal died unexpectedly.

Figure 3. Immunohistochemical staining demonstrated BrdU labeled cells in the hippocampus of each group (x 400).

Table 2. Numbers of BrdU labeled cells in hippocampal DG region at different time points

<table>
<thead>
<tr>
<th>Group</th>
<th>4 d</th>
<th>7 d</th>
<th>14 d</th>
<th>21 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>50.00 ± 4.54</td>
<td>34.50 ± 2.45</td>
<td>15.13 ± 2.03</td>
<td>2.13 ± 0.64</td>
</tr>
<tr>
<td>HI control</td>
<td>64.88 ± 4.67$^*$</td>
<td>46.00 ± 4.07$^*$</td>
<td>24.50 ± 3.34$^*$</td>
<td>6.75 ± 1.28$^*$</td>
</tr>
<tr>
<td>HI-HBO</td>
<td>80.00 ± 4.34$^*$,#</td>
<td>56.88 ± 4.29$^*$,#</td>
<td>35.00 ± 3.12$^*$,#</td>
<td>12.75 ± 1.83$^*$,#</td>
</tr>
</tbody>
</table>

Note: $^*P < 0.01$ compared with sham group; $^#P < 0.01$ compared with HI control group; DG: dentate gyrus.
Table 4. Changes in platform crossing frequencies among three groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham</th>
<th>HI-HBO</th>
<th>HI control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequencies</td>
<td>8.13 ± 0.64</td>
<td>7.38 ± 1.06</td>
<td>5.00 ± 0.76</td>
</tr>
</tbody>
</table>

*p < 0.01 compared with Sham group; *p < 0.01 compared with HBO group.

Expression of BrdU in hippocampal DG region

The positive-BrdU cells mainly distributed in the polymorphous cell layer and adopt round shape, fusiform shape and pole shape (Figure 3). The BrdU positive cells peaked 4 days after surgery and began to decrease at 7 d after surgery in all of the three groups and there was significant difference in the number of BrdU positive cells between different time points (P < 0.01). A significant difference of the number of BrdU positive cells was observed at different time points compared the sham group with HI group. And there were significant increases of BrdU positive cells at different time points between HI group and HI-HBO group (P < 0.01) (Table 2).

MWM test analysis

The learning and memory abilities of the rats were manifested by delayed escape latency time and platform crossing frequencies. In the spatial navigation test, all the animals were likely to find the platform more quickly after the first trial constructed. Among the three groups, rats in HI group spent the statistically longest time to search for the platform hidden in water (P < 0.05). Compared with HI-HBO group and sham group, difference was not observed in the escape latency time of the rats (P > 0.05) (Table 3). In the spatial probe trial, rats exposed to the HBO showed significant higher platform crossing frequencies compared with HI rats (P < 0.05). And sham-surgery rats showed similar memory ability with HBO-treated rats (P > 0.05) (Table 4).

Discussion

Currently, an important understanding of HIBD pathogenesis is the strong activation of neuronal apoptosis in brain [13]. NSCs have potential of self-renewal, migration and multiple different...
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Hyperbaric oxygenation can improve the hippocampal function and declined the learning and memory ability. Our paper designed MWM test for pups at 30 days after surgery to test HBO effect on the learning and memory abilities by delayed escape latency time and platform crossing frequencies. The MWM test showed that the HIBD significantly induced the cognitive dysfunction evidenced by long escape latency time and less platform crossing frequencies. The rats exposed to HBO retained similar learning and memory function compared with those in sham group. Therefore, HBO therapy can improve the learning and memory ability following HIBD.

In summary, HBO treatment promoted the repair and regeneration of the nervous system and contributed to the self-recovery and protection of the damaged brain. The improvements in learning and memory function were observed by HBO treatment. HBO may be a promising therapy for HIBD in newborn based on the appropriate treatment time and pressure. However, the optimum treatment condition needs to be further studies.

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Disclosure of conflict of interest

None.

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