The effects of different hyperbaric oxygen manipulations in rats after traumatic brain injury

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HIGHLIGHTS

- HIF-1α can be an indirect index for the degree of TBI.
- Detected expression of HIF-1α by immunohistochemistry and Western blot.
- Compared effects of HBO after TBI by TUNEL, Western blot, water maze test.

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ABSTRACT

The protective effects of hyperbaric oxygenation following traumatic brain injury have been widely investigated; however, few studies have made systematic comparisons between the different hyperbaric oxygenation manipulations and their corresponding effects. In this study, male Sprague–Dawley rats were observed at 4 h, 15 d and 75 d after traumatic brain injury. The effects of the different hyperbaric oxygenation manipulations on the rats were compared based on morphological, molecular biological and behavioral tests. Our results showed that hyperbaric oxygenation inhibited cell apoptosis in the rat hippocampus and improved their physiological functions. The effects observed in the hyperbaric oxygen-early group were better than the hyperbaric oxygen-delayed group, and the hyperbaric oxygen-early-delayed group demonstrated the best effects among all the groups. Our results showed the hyperbaric oxygenation was recommended early and delayed post-traumatic brain injury and exposure to hyperbaric oxygenation should be prolonged. These findings provide new ideal therapeutic insight for the clinical treatment of traumatic brain injury.

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1. Introduction

Trauma is the leading cause of death in young people worldwide and half of these deaths are due to traumatic brain injury (TBI). In the United States, two million people suffer from TBI each year, half of which require an emergency room visit. Fifty thousand people die each year, making TBI a significant socioeconomic burden.

Many countries contribute a large proportion of energy and funds on TBI research each year, and hyperbaric oxygenation (HBO) is one of the research focuses [1–4]. HBO is oxygen, which is administered to the patient at a pressure greater than atmospheric pressure at sea level. HBO can increase the partial pressure of oxygen within the blood and improve mitochondrial metabolism [5,6]. Previous studies have shown that HBO following TBI provides the following advantages: (1) decreased mortality rates; (2) improved functional outcome; (3) released from hypoxia [7]; (4) improved in microcirculation with a decrease in the intracranial pressure
early-delayed TBI. The study aims to systematically compare different HBO manipulations and determine their effects in the hope of providing new insights for the clinical treatment of TBI using HBO.

2. Experimental procedures

2.1. Animals

A total of 458 male Sprague-Dawley rats weighing 300–350 g were obtained from the Animal Center of Xinxiang Medical University (Xinxiang, China). All of the studies were conducted with the approval of the experimental animal care committee of Xinxiang Medical University.

2.2. Surgical preparation

As previously described [12], the rats were anesthetized with isoflurane (1.5–2.5%) in 100% oxygen, and a 3 mm x 3 mm craniotomy was subsequently performed 1.5 mm posterior to bregma and 8 mm lateral to the midline. A fluid percussion injury cap was fixed on to the bone window prior to connection to the lateral fluid percussion injury (LFP) equipment [13]. In the sham group, the same operating procedure was performed, but with no connection to the LFP equipment.

2.3. Injury induction

The injury induction was performed as previously described [12,13]. The severity of the injury was evaluated by the loss-of-consciousness (LOC) time, and the entire evaluation time was 180 s. A commonly used experimental standard for a severe injury is LOC > 120 s (ICP > 20 mmHg). Eight rats that did not fall within this category of severe injury were excluded, resulting in a total of 450 severely injured rats used in this study.

2.4. HBO treatment

At the designated time points, the rats were subjected to 1.5 ATA 100% oxygen in a Yantai Hongyuan oxygenic limited company chamber. The chamber temperature was maintained at 22 ± 2 °C. The animals were randomly divided into five groups, where each group consisted of 90 rats: sham group, TBI group, hyperbaric oxygen–early group (HBO-E group; HBO treatment began at 6 h after TBI, once a day for 15 days at 90 min each), hyperbaric oxygen–delayed group (HBO-D group; HBO treatment began at 60 days after TBI, once a day for 15 days at 90 min each) and hyperbaric oxygen–early–delayed group (HBO-E-D group; HBO treatment began at 6 h after TBI, once a day for 15 days at 90 min each, and then the HBO treatment was re-administered at 60 days after TBI, once a day for 15 days at 90 min each).

2.5. Tissue preparation for TUNEL and immunohistochemistry

The six rats were deeply anesthetized at 4 h, 15 d and 75 d after TBI and were subsequently sacrificed in each group. The rat brain was perfused, fixed, and then embedded. Serial sections (6 μm) were cut and stored for TUNEL processing and immunohistochemistry.

2.6. TUNEL assay measuring DNA fragmentation

Apoptosis was examined using a TUNEL detection kit (Promega Corporation, USA), which was performed according to the manufacturer’s instructions. For each time point, six sections were examined using a light microscope.

2.7. Immunohistochemistry

According to the instructions, the sections were subjected to antigen retrieval. A rabbit polyclonal primary antibody against HIF-1α (1:100, Millipore Corporation USA) and a biotinylated secondary antibody (1:200, Santa Cruz Corporation USA) were used, followed by the addition of DAB solution. After 10 min, the sections were immediately observed under a microscope and examined for a brown precipitate, which indicated a positive reaction. The sections were re-stained using hematoxylin with 0.1% HCl differentiation and 0.1 M PBS washes were performed to reveal a blue stain.

2.8. Water maze test

The cognitive and memory functions of the rats were tested using a Morris water maze at 4 h, 15 d and 75 d after TBI. For each time point, six rats were used for the water maze test, and the rats were trained twice. For each trial, the rat was placed at the edge of the pool, always facing the same direction. If the rat failed to find the escape platform after 180 s, then the experimenter would place the rat on top of the platform for an additional 10 s. There was a 5 min interval between the two experiments, during which the rats were dried and returned to their cages. Five minutes after the second training session, the test trial was conducted. All of the rats started from the same position opposite the quadrant where the submerged escape platform was positioned and the escape latencies of each rat were measured.

2.9. Brain water content

For each time point, six rats were analyzed for brain water content. The rats were deeply anesthetized and sacrificed at 4 h, 15 d and 75 d after TBI, and their entire brain were weighed immediately after decapitation (wet weight) and after drying at 105 °C for 24 h (dry weight). The percentage of brain water content was calculated using the formula, ([wet weight – dry weight]/wet weight) × 100%.

2.10. EB extravasation

Blood–brain barrier (BBB) damage was assessed by testing for Evans Blue (EB) extravasation at 4 h, 15 d and 75 d after TBI. Six rats were used for EB extravasation studies at each time point. Briefly, EB dye (1%, 2 ml/kg) was injected into the femoral vein and allowed to circulate for 2 h. The rats were deeply anesthetized, and a transcardial perfusion with PBS was performed until the colorless perfusion fluid was observed in the right atrium. The brain were then divided into the right and left hemispheres and separately weighed. The EB dye, which had penetrated into the right hemisphere, was then extracted by incubation in 2 ml/100 mg formamide at 65 °C for 24 h. The concentration of the EB dye was determined by measuring the absorbance at 620 nm. The EB leakage was expressed as μg per g of brain tissue.

2.11. Western blotting analysis

For each designed time point, six rats were used for Western blotting analysis and were sacrificed at 4 h, 15 d and 75 d after TBI. The ipsilateral hippocampus of the LFP was processed for SDS-PAGE, and the blots were probed using primary antibodies against...
HIF-1α (1:200, Millipore Corporation USA) and β-actin (1:10,000; Sigma Corporation USA). The protein bands were scanned using a densitometer, and the quantification was performed using the Multi-Analyst 1.0.2 software.

2.12. Statistics

All of the quantitative data were expressed as the mean ± standard deviation and analyzed by the method of variance (ANOVA) using SPSS 16.0. If the null hypothesis was rejected, then the Tukey’s Multiple Comparison Test was used to determine the specific differences between the group means. A value of \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. HBO reduced TBI-induced apoptosis

In all of the groups, hippocampal apoptosis was observed at 4 h, 15 d and 75 d after TBI. After TBI, apoptosis was enhanced in the TBI group compared to the other groups; however, the HBO-E-D group exhibited the least amount of apoptosis \( (P < 0.05) \) (Fig. 1).

3.2. HBO down-regulated TBI-induced HIF-1α over expression in the hippocampus

The expression of HIF-1α in hippocampus was examined using immunohistochemistry. The expression of HIF-1α was observed in the hippocampus at 15 d after TBI in TBI group and HBO-E group (Fig. 2).

3.3. HBO prevented TBI-induced memory loss

The cognitive and memory functions of the rats after TBI were assessed by their performance in the water maze test. The time to find the platform in five groups of rats is shown (Fig. 3A).

3.4. HBO decreased TBI-induced increased brain water content

Brain water content is a marker of the degree of brain damage. With HBO treatment, there was a dramatic decrease in the brain water content in several of the HBO groups compared to the TBI group \( (P < 0.05) \) at 15 d after TBI. However, the outcome analysis at 75 d did not show a significant difference between the TBI and HBO groups (Fig. 3B).

3.5. HBO reduced EB (Evans Blue) extravasation induced by TBI

An additional effect of TBI is BBB destruction, which may be measured by EB extravasation. There was no significant difference in the level of EB extravasation in the sham group at all time points. After HBO treatment, the EB extravasation levels were significantly reduced in the HBO groups compared to the TBI group \( (P < 0.05) \). The outcome analysis at 75 d did not reveal a significant difference between the TBI and HBO groups (Fig. 3C).

3.6. HBO significantly reduced HIF-1α protein expression in the injured brain

In this experiment, we examined the expression of the 120-kDa HIF-1α protein using Western blotting. HIF-1α protein expression was detected in the rat brain at 4 h, 15 d and 75 d post-TBI. A significant difference in the level of HIF-1α protein expression after the treatment of HBO in all of the groups was observed \( (P < 0.05) \) (Fig. 4).

4. Discussion

Studies of HBO in experimental models of TBI have shown a variety of neuroprotective effects [1,6]. Previous studies using animal models with severe brain trauma suggest immediate administration of HBO treatment in patients [14]. In addition, these studies have demonstrated that HBO can significantly improve the recovery of nervous system function when it is delayed post-TBI [15]. This study was motivated by the lack of comparison on the
pathological, molecular biological and behavioral effects of different HBO manipulations post-TBI.

In contrast, the time and pressure of HBO treatment is not the bigger the better. The lung is an organ that is most commonly damaged by hyperoxia because the O$_2$ tension in the lungs is substantially higher compared with other tissues [16]. Until now, mechanisms of oxygen toxicity have not been fully understood; however, it is known that HBO induces oxidative DNA damage in the blood cells of healthy subjects. This DNA damage was only found after the first treatment, but not after the second or subsequent HBO treatments, indicating rapid repair by DNA repair mechanisms [17]. This study's treatment schedule was 100% oxygen at 1.5 ATA for 90 min daily for 15 days. However, in this study, we did not observe any oxygen-induced convulsions.

There are some papers comparing neuroprotection between normobaric oxygen (NBO) and hyperbaric air (HBA). Breathing of 100% oxygen already results in a 100% saturation of hemoglobin, and increases the physically transported O$_2$ portion. Under hyperbaric conditions, there is a massive increase of the physically

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Fig. 3. (A) Water maze test(s), (B) brain water content (%), (C) EB content [μg/g]. The values are given as the mean ±SD. n=6. *P<0.05 as compared with TBI group.
transported O₂ portion, also the inadequate transport and delivery of oxygen to the tissue can be compensated by high-pO₂ gradients. Hyperbaric reduction in blood–brain barrier damage preventing inflammatory processes and inhibition of neutrophil adhesion to the endothelium are also factors which may contribute in neuronal protection.

LFP injury is a commonly used TBI model and can accurately reflect the pathophysiological changes that occur in the brain after TBI. Furthermore, a standard STBI model may be examined by testing the LOC post-LFP.

HIF-1, which consists of HIF-1α and HIF-1β, is a transcription factor that regulates gene expression in response to changes in intracellular oxygen concentration [18]. As an oxygen-regulating protein, HIF-1α is undetectable under normal oxygen concentration. During hypoxia, degradation of HIF-1α is decreased and a large amount of HIF-1α is transferred to the cytolymph to mediate the body’s hypoxic response, enabling its detection. Furthermore, HIF-1α is associated with the survival and apoptosis of neural cells. A series of experiments have demonstrated that cerebral ischemia plays a key role in TBI, which results in an increase in HIF-1α [19]. Because the expression of HIF is specifically associated with oxygen content, HIF-1α may serve as an indirect index for the degree of brain injury.

It is well known that the limbic system is closely related with the cognitive and memory functions of animals. TBI results in massive apoptosis in the hippocampus, which may be assessed by measuring the number of DNA breaks using TUNEL staining. Thus, the reduced apoptosis observed in our animal model may be linked to a decreased production of free radicals via improved membrane integrity, reduced calcium entry, improved energy balance or enhanced antioxidant production [20]. Under hyperbaric conditions, the oxygen concentration in the cell vicinity was markedly
increased due to an improved diffusion gradient [21]. Another potential explanation for the anti-apoptotic effects of HBO in head injury is the redistribution of cerebral blood flow (CBF) [22].

The BBB is a specialized structure that safeguards the inner environment of the central nervous system, prevents harmful substances from damaging the brain tissues and allows the entry of essential metabolic factors. It has been reported that hypoxia, hypertension and ionizing radiation improve BBB permeability. In this study, the changes in BBB permeability post-exposure to HBO were analyzed using EB and by measuring the brain water content. HBO caused cerebral vasoconstriction and decreased CBF, which reduced cerebral edema [23]. Moreover, there was increased availability of O2 at the cellular level, improved microcirculation, and accelerated BBB recovery. Izawa et al. [24] previously showed that vasoconstriction was limited to areas with intact auto-regulation, while the arterial vasodilatation in ischemic areas was not affected. HBO compensation or neutralization of severe oxygen deficiency resulted from the drastic decrease in CBF [25] and acute respiratory dysfunctions [26,27]. Our study showed that early exposure to HBO post-TBI could significantly decrease BBB permeability; however, delayed exposure to HBO post-TBI did not have any obvious effects. Cerebral edema represents a challenge for medical doctors and current treatments for cerebral edema are limited and have largely been unsuccessful. For example, osmotherapy involves the administration of hypertonic mannitol or hypertonic saline; however, its beneficial effects are limited because osmotherapy, which withdraws water, shrinks the healthy parts of the brain in addition to the damaged area. Moreover, glucocorticoids have also not been very successful in the treatment of most forms of edema. Thus, these conventional treatments of cerebral edema require improvement [11]. Effective control of cerebral edema is essential for neuroprotection following injury. Oxygen at 1.5-2 ATA reduces cerebral edema by vasoconstriction and prevents cerebral ischemia via high oxygen tension. HBO modifies several pathophysiological changes in TBI and reduces intracranial pressure.

Most medical physicians have performed delayed HBO in patient post-TBI as a method to improve functional recovery. However, the results of this study showed that early exposure of HBO post-TBI enhances outcomes.

Finally, this study showed that both the HBO-E and HBO-D groups exhibited obvious effects. The HBO-E group demonstrated more enhanced effects compared to the HBO-D group. Thus, HBO is recommended early, and delayed HBO post-TBI is suggested.

In comparison with the present therapeutic method for TBI treatment, HBO exhibits the following advantages: (1) HBO is a non-invasive therapy, which is more acceptable in patients; (2) HBO is an inexpensive therapy and may be widely used in clinical practices; and (3) because HBO does not affect the effects of other therapeutic treatments, a combined medication is preferable in TBI patients. Taken together, these results show that HBO should be widely used in TBI treatment.

References


