Original Article
Effects of HBO on apoptosis of nerve cells and expression of NF-κB after TBI in neonatal rats

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Abstract: Objective: This study was designed to investigate the protective functions and specific mechanisms of hyperbaric oxygenation (HBO) treatment on traumatic brain injury (TBI) by detecting the effects of HBO treatment on the apoptosis of nerve cells, the expressions of apoptotic genes and apoptosis-related genes in the posterior cerebral cortex and hippocampus, and expression of NF-κB (p-p65) in hippocampus in newborn rats after TBI. Methods: Ninety six healthy 7-day-old newborn Sprague-Dawley (SD) rats were randomly divided into sham operation group (n=32), TBI group (n=32) and TBI+HBO group (n=32). The changes of relevant indexes were observed at four time points (8 h, 16 h, 24 h, and 48 h) after HBO treatment. Theterminal-deoxynucleotidyl transferase mediated dUTP nickendlabeling (TUNEL) method was employed to detect the apoptosis of nerve cells; the real-time fluorescent quantitative PCR (qPCR) method was applied to detect the change rule of apoptosis-related genes (Bcl-2, Bax) and the Western Blot (WB) assay was adopted to detect the expression of NF-κB (p-p65) in hippocampus. Results: In sham operation group, there were no significant differences in the apoptosis of nerve cells and the expressions of apoptosis-related genes among each observation time (P>0.05). Compared with sham operation group, whether in cerebral cortex or hippocampus in TBI group, the number of apoptotic nerve cells was significantly increased (P<0.01), and the Bcl-2/Bax ratio was substantially decreased while this trend was on the rise over time. Compared with TBI group, the apoptosis of nerve cells in cerebral cortex and hippocampus in TBI+HBO group were significantly decreased (P<0.05), and the Bcl-2/Bax ratio was substantially increased (P<0.01). Compared to sham operation group, the expression of NF-κB (p-p65) in TBI group was significantly increased (all P<0.01); and the expression of NF-κB (p-p65) was substantially decreased after HBO treatment (all P<0.05). Conclusion: As a treatment which can effectively relieve the apoptosis of nerve cells in newborn rats after TBI, and weaken the expression of NF-κB (p-p65), HBO has apparently protective effects on brain tissues after TBI.

Keywords: Hyperbaric oxygenation (HBO), newborn rats, traumatic brain injury (TBI), apoptosis of nerve cells, Bcl-2/Bax, NF-κB (p-p65)

Introduction

Traumatic brain injury (TBI) is a very severe brain trauma with clinical features such as high incidence, high mortality and various sequelae [1-3]. TBI can be divided into primary TBI and secondary TBI according to different causes of injury. Primary TBI is caused by the external violence exerted directly on the craniocerebral, which is irreversible. And secondary TBI refers to the changes of structures and functions of brain that happened in the later stage of injury, which mainly includes changes of neurotransmitter and ion level, generation of oxygen free radicals and so on. At present, it has become the main stage for intervention in treatment [4, 5].

In the last few years, a large number of reports have shown that the apoptosis of nerve cells appears obviously in the process of TBI, which can further aggravate the craniocerebral injury in later stage. Therefore, the decrease of apoptosis of nerve cells may have significant effects on the prognosis of TBI [6, 7]. Apoptosis of cells is a kind of programmed cell death which different from the necrosis and it can be induced by a variety of factors. The apoptosis of nerve cells is previously considered to appear in the process of normal growth and development or the lesion of chronic neuron. However, the recent studies have shown that it not only exists in acute craniocerebral injury but also appears throughout the whole process of this kind of disease [6].
Hyperbaric oxygenation (HBO), as a special treatment, has been widely employed in the treatment of clinical diseases. And its therapeutic effects are showed by providing the pure oxygen which higher than the barometric pressure. Clinical and basic researches have shown that HBO treatment has distinct therapeutic effects on the acute cranio-cerebral injury, but its specific mechanisms remain unclear [8].

This study aims to further explore the therapeutic effects and specific mechanisms of HBO on the treatment of TBI via establishing TBI model in rats to detect the apoptosis of nerve cells and expression levels of NF-κB (p-p65) before and after the treatment of HBO.

Materials and methods

Experimental animals and grouping

Ninety six healthy 7-day-old newborn Sprague-Dawley (SD) rats weighting 12-17 g were purchased from Nanjing BetterBiotechnology Co. Ltd and were then randomly divided into sham operation group (n=32), TBI group (n=32) and TBI+HBO group (n=32). The changes of relevant indexes were observed at four time points (8 h, 16 h, 24 h, and 48 h) after HBO treatment.

Experimental model

Specific experimental modeling was referred to the previous literatures [9, 10]. At first, the rats were anesthetized using 0.75% pentobarbital (10 µL/g). Then, a right parietal craniotomy (2 mm to the right of center line, 2 mm posterior to the coronal suture, diameter 5 mm) was performed with a dental drill. During this process, the dura mater was confirmed to be intact and the combat intensity was 50 g*15 cm.

HBO treatment

The rats were allowed to recover for 1 h after completing the modeling. The rats in TBI+HBO group were treated for 30 min in a hyperbaric oxygen chamber. First of all, the rats were placed in the hyperbaric oxygen chamber, the pure oxygen was introduced to the chamber for cleaning and the exhaust valve was turned off until the oxygen concentration reached 90%. Then, the oxygen flow rate was adjusted to 6 L/min and the pressure in hyperbaric oxygen chamber was increased slowly until the pressure reached 0.2 Mpa. After that, the exhaust valve was opened, the exhaust flow-volume was adjusted to equal to the intake flow-volume (6 L/min), and the stable pressure in the hyperbaric oxygen chamber and the concentration of oxygen were sustained for 30 min. Finally, the intake valve was turned off, the pressure in the hyperbaric oxygen chamber was reduced to the normal pressure at a constant rate and the rats were moved out of the chamber after standing for 5 min. The HBO treatment was performed again after 1 h, and this treatment was performed twice a day until the end of all experiments.

Test method

TNUEL method: The TNUEL method was employed to detect the apoptosis of nerve cells. And all steps were operated in strict accordance with the kit instruction of Roche Company. Ten views with high magnification (10*40) were randomly selected from each slice, the number of positive cells in each field of view was counted, and the average number was then obtained. Among these nerve cells, positive cells were brown-yellow while negative cells were blue.

Real-time fluorescent quantitative PCR (qPCR) method

The Trizol method was applied to extract the total RNA of brain tissues and the concentration of RNA was then measured. After that, 500 ng of total RNA, 2 ul of 5*PrimeScript RT Master Mix were added into each PCR tube. The nuclease-free water was completed to reach 10 μl and the mixture was then put into the PCR instrument (Applied Biosystem). After reaction, cDNA was obtained from total RNA by reverse transcription and the cDNA obtained was then diluted for 4 times. SYBR® Premix Ex TaqTM kit purchased from TaKaRa Company was applied for the PCR reaction, and the main reagents were detailed as follows: SYBR Premix Ex Taq II (10 ul), upstream and downstream primers (10 um, 0.8 ul each), ROX Reference Dye II (0.4 ul), ddH2O (6 ul), and template which was diluted for 4 times was added finally (2 ul). The reaction systems were as follows: pre-denaturing (at 95°C for 30 s), PCR reaction (at 95°C for 5 s; at 60°C for 34 s, 40 cycles) and dissolution (at 95°C for 30 s; at 60°C for 40 cycles) and dissolution (at 95°C for 15 s; at 60°C for 1 min; at 95°C for 15 s). The quantitative primers (5’-3’) were as follows: β-actin (F): CAACCTCCATCATGAAGTGAC; β-actin (R): CCACACGGAGTACCTTGCCCTC; Bcl-2
Table 1. Detection results of nerve cells apoptosis at each observation time in cerebral cortex and hippocampus (X ±S)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cerebral cortex</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 h</td>
<td>16 h</td>
</tr>
<tr>
<td>Sham operation group</td>
<td>1.93±0.86</td>
<td>1.86±0.73</td>
</tr>
<tr>
<td>TBI group</td>
<td>22.39±5.76**</td>
<td>31.58±7.69**</td>
</tr>
<tr>
<td>TBI+HBO group</td>
<td>15.48±3.37**</td>
<td>22.67±5.36**</td>
</tr>
<tr>
<td>t</td>
<td>9.94**</td>
<td>14.48**</td>
</tr>
<tr>
<td></td>
<td>2.93*</td>
<td>2.69*</td>
</tr>
</tbody>
</table>

Note: 1. In sham operation group, there were no significant differences in the apoptosis of nerve cells at each observation time in cerebral cortex (F=0.06, P>0.05); there were no significant differences in the apoptosis of nerve cells at each observation time in hippocampus (F=0.03, P>0.05); 2. Comparison between the apoptosis of nerve cells at each observation time in cerebral cortex in TBI group (F=16.70, P<0.01); comparison between apoptosis of nerve cells at each observation time in hippocampus in TBI group (F=21.80, P<0.01); 3. Comparison between the apoptosis of nerve cells at each observation time in cerebral cortex in TBI+HBO group (F=11.75, P<0.01), comparison between the apoptosis of nerve cells at each observation time in hippocampus in TBI+HBO group (F=19.51, P<0.01); 4. Compared TBI group and sham operation group, there were obvious differences in the apoptosis of nerve cells at each observation time in cerebral cortex and hippocampus (**P<0.01); 5. Compared TBI+HBO group and TBI group, there were significant differences in the apoptosis of nerve cells at each observation time in cerebral cortex and hippocampus (**P<0.01, **P<0.01).

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Discussion

With the development of economy and society, the incidence of TBI is increasing year by year. And TBI has caused great mental pressure and financial burden to the patients’ families for its features of high incidence and high mortality [6, 11, 12]. A lot of studies have shown that obvious shrink can appear in arteries of the brain and the brain blood flow and oxygen supply are significantly decreased after TBI. The HBO treatment is helpful to make up the reduction of brain blood flow, improve the blood oxygen diffusion capacity, enhance the blood oxygen-tension in brain tissue and reduce the brain edema by increasing the blood oxygen content. Moveover, the HBO treatment can protect the cell membranes and blood brain barrier, which can reduce the production of toxicants and then lessen the damage to nerve cells [13, 14]. However, its molecular mechanisms of specific neuroprotective effects have not yet been clarified.

In this study, the specific molecular mechanisms of HBO for the protective effects of TBI were investigated. After TBI, the apoptosis of nerve cells appeared significant in cerebral cortex and hippocampus, but the apoptosis of nerve cells was reduced substantially after the HBO treatment (Table 1). The expression of antiapoptosis gene (Bcl-2) in TBI group was increased while that in TBI+HBO group was distinctly increased. And the expression of pro-apoptosis gene (Bax) in TBI group was significantly raised while that in TBI+HBO group was obviously fewer. Therefore, the Bcl-2/Bax ratio in TBI group was substantially decreased while that in TBI+HBO group was apparently increased (Figures 1, 2). Likewise, the expression of NF-κB (p-p65) was significantly increased after TBI and obviously decreased after the treatment of HBO (Figure 3).
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Bcl-2 protein family is closely related to the apoptosis, among which the antiapoptosis gene (Bcl-2) and proapoptosis gene (Bax) are the most important ones. With the continuous development of knowledge and the increasing innovation of ideas, people’s knowledge about apoptosis has changed from the nucleus-centric regulatory mode to the mitochondrion-centric regulatory mode [15-17]. This regulatory mode can be influenced by multiple ways, one of which is mitochondrial permeability transition pore (mPTP). As a kind of non-selective and high conductive channel, mPTP crosses between the inner and outer membranes of mitochondrion with two states (open and close). When it is opened, it can promote the mitochondrion to release cytochrome C and apoptosis-inducing factor (AIF), which can lead to the consumption of transmembrane potential and apoptosis of cells. And when it is turned into the close state, the apoptosis of cells can also be inhibited. The Bcl-2 protein family can control the open and close states of mPTP and then influence the apoptosis of cells [18-22].

Sensitive degree of cells in body towards apoptosis is decided by the expression levels of Bcl-2 and Bax, and the forms of competitive dimers. When the Bcl-2 expression is in high grade, the body can mainly produce Bcl-2/Bcl-2 homodimer and Bcl-2/Bax heterodimer, both of which can inhibit the apoptosis of cells and control it via limiting the activity of endogenous endonuclease; when the Bax expression is in high grade, it can mainly produce the Bcl-2/Bcl-2 homodimer, which can promote the apoptosis of cells [19, 23, 24]. In this study, the Bax expression was mainly in high grade at each observation time in TBI group, and the Bcl-2/Bax ratio was obviously decreased. At that moment, the obvious apoptosis was appeared in nerve cells. In TBI+HBO group, however, the expression of Bcl-2 was significantly increased while that of Bax was distinctly decreased, the Bcl-2/Bax ratio was significantly increased. At that moment, the apoptosis was mainly inhibited (Figures 1, 2).

TBI is accompanied by some degree of inflammatory response, which can damage the nerve
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cells [25-27]. In this study, we paid more attention to the inflammatory response mediated via NF-κB (p65) pathway. NF-κB is a multifunctional nuclear transcription factor protein family with a wide range of biological activities. And it plays a key role in mediating the transcription and regulation of cell signal. After being activated, it can mediate the transcription of gene such as cytokines related to inflammatory, chemokines and so on [28-30]. The dimer formed by the subunit of NF-κB can combine with the special sequence of target gene and then regulate the gene transcription. And the heterodimer formed by P50 and P65 is the most common one and P65 has transcriptional activation area. In the resting state, NF-κB dimer can combine with the inhibitory unit (IκB) via ankyrinrepetitive sequence and exist in cytoplasmic with

Figure 3. Expressions of NF-κB (p65) at each observation time (8 h, 16 h, 24 h, 48 h) in hippocampus. The expression levels of NF-κB (p-p65) were determined at different observation time (8 h (A), 16 h (B), 24 h (C) and 48 h (D) in hippocampus). *P<0.05; **P<0.01; ***P<0.001.
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When there appear external stimuli, I-κB kinase (IKK) can be activated, thereby causing the phosphorylation and ubiquitination of I-κB and finally degrading the I-κB protein. The active site of NF-κB dimer is exposed after being degraded and separated. And the released NF-κB is further activated and transferred quickly into the nucleus through various modifications and then it combine with the target gene to promote the transcription of target gene, thereby promoting the release of inflammatory factors [31, 32]. The experimental results in this study showed that the number of activated NF-κB (p65) was constantly increased over time in TBI group and the inflammation was obviously increased to aggravate the apoptosis of nerve cells. After the HBO treatment, however, the activation of NF-κB was significantly decreased (Figure 3).

However, this study still has some deficiencies although a good result can be obtained. First of all, detection about the long term therapeutic effects of HBO on TBI is still not enough. Meanwhile, the caspase family which is related to the apoptosis is not detected in this study. HBO treatment can obviously accelerate the rehabilitation and improve the prognosis for patients with TBI, which is a very effective treatment and can profoundly influence the medical science in the future.

In conclusion, HBO treatment can release the apoptosis of nerve cells after TBI, reduce the expressions of apoptosis-related genes (Bcl-2, Bax), and decrease the activation of NF-κB, thereby substantially protecting the nerve cells. HBO treatment is a promising therapy worthy of clinical promotion.

Disclosure of conflict of interest

None.

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