EFFECTS OF HYPERBARIC OXYGEN PRE-CONDITIONING ON MITOCHONDRIAL PATHWAY OF APOPTOSIS IN NEURONS OF SPINAL CORD INJURED RATS

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ABSTRACT

Aims: This study aimed to investigate effects of hyperbaric oxygen pre-conditioning (HBO-PC) on motor function of rats with spinal cord injury and mitochondrial pathway of apoptosis of neurons of these rats.

Materials and methods: 36 healthy male Wistar rats were randomly assigned into control group, spinal cord injury (SCI) group and HBO-PC group.

Results: Basso, Beattie and Bresnahan (BBB) scores in SCI group and HBO-PC group was significantly lower than those in control group, and BBB score in SCI group was markedly lower than that in HBO-PC group 2 weeks after SCI. Caspase-3 mRNA expression in SCI group increased dramatically when compared with control group 2 weeks after SCI. However, caspase-3 mRNA expression in HBO-PC group was significantly lower than that in SCI group 2 weeks after SCI. CYC mRNA expression in HBO-PC group was significantly lower than those in control group and SCI group. Bak mRNA expression in HBO-PC group and SCI group was 2.15±0.78 and 1.93±0.61, respectively, and significantly higher than that in control group. Bax mRNA expression in HBO-PC group was markedly lower than that in SCI group.

Conclusion: HBO-PC may improve neurofunction of SCI rats, which is ascribed to inhibition of mitochondrion-related apoptosis of neurons.

Key words: hyperbaric oxygen, pre-conditioning, spinal cord injury, neurons, apoptosis.

Received Aug 31, 2014; Accepted April 02, 2015

Introduction

Spinal cord injury (SCI) is a severe trauma to the central nervous system (CNS) and cause high disability and high mortality. Thus, SCI has been a disease threatening the human health and increases the social and family burden. The recovery of the neuron functions after SCI is very difficult, and thus to avoid the secondary neurological injury has been an important focus in the treatment of SCI patients. Several studies have shown that hyperbaric oxygen pre-conditioning (HBO-PC) can attenuate the secondary injury after SCI, protect the residual axons and neurons, and promote the regeneration of neurons and the recovery of neuronal functions, which are ascribed to the reduction of neuronal apoptosis at the spinal cord following ischemia/hypoxia1,2. The apoptosis of neurons in the spinal cord is associated with several signaling pathways in which mitochondrial pathway of apoptosis plays an important role3. On the basis of available findings on SCI, this study was undertaken to investigate the effect of HBO-PC on the motor function of SCI rats with BBB score. In addition, following HBO-PC, factors related to the mitochondrial pathway of apoptosis were detected in the spinal cord (such as cytochrome c [CYC], Bcl-2 homologous antagonist/killer [Bak], B cell lymphoma / lewkmia-2 associated protein x [Bax], Bcl-2-like 1 [Bcl-x], B cell lymphoma / lewkmia-2...
[Bcl-2] and cysteine-containing aspartate-specific proteases [Caspase-3]), aiming to explore the influence of HBO-PC on the mitochondrial pathway of apoptosis of spinal cord neurons in SCI rats.

**Materials and methods**

**Reagents**

A total of 36 healthy male Wistar rats aged 8-12 weeks and weighing 160-180 g were purchased from the Experimental Animal Center of Guangdong Province. Following reagents were used in this study: 5×buffer, Hot Start Taq, c-mmlv (Daangene Biotech Co., Ltd, Guangdong, China), Rnasin, dNTPs (Promega, Beijing, China), SYBRGREEN I (Invitrogene, Shanghai, China), antibodies against CYC, Bak, Bax, Bcl-x, Bcl-2 and Caspase-3 (Santa Cruz, Shanghai, China), horseradish peroxidase conjugated goat anti-rabbit IgG (Beijing Dingguo Biotech Co., Ltd, Beijing, China), DAB/DAB buffer (Guangzhou Linked-Biotech Pathology Co., Ltd, Guangdong, China), and hydrogen peroxide (Guangzhou Chemical Reagent Factory, Guangdong, China). Pressure-cooker (Supor, Guangdong, China), 9700 qualitative thermal cycler and 7500 quantitative thermal cycler (ABI, Beijing, China) were used in this study.

Rats were allowed to accommodate themselves to the environment for 1 week, and then were randomly assigned into control group (n=12), SCI group (n=12) and HBO-PC group (n=12). Each group has 12 rats. Rats were fed individually and given ad libitum access to water and food at a 12:12 light/dark cycle.

**Preparation of SCI animal model**

Modified Allen’s method was used to prepare the SCI animal model in rats. In brief, rats were intraperitoneally anesthetized with amobarbital sodium at 30 mg/kg. Hair on the back was removed, and sterilization was done. A midline incision was made at the back taking the T8 spinous process as the center (about 2-3 cm). Soft tissues were separated and the laminas were exposed. The lesioned spinal cord (about 0.5 cm) was collected into liquid nitrogen.

**Therapies**

In HBO-PC group, HBO-PC was performed before introduction of SCI. In brief, animals were placed in an oxygen chamber which was flushed with 1.5m2 pure oxygen for 10 min. Then, the chamber was pressurized to 0.2 MPa (2 ATA) within 20 min. The chamber oxygen concentration was maintained at 80-85%, and HBO-PC was done for 80 min once daily for consecutive 7 days. Decompression was performed at an uniform rate within 20 min. At 8 h after last HBO-PC, SCI was induced.

**Sample collection**

2 weeks after SCI, samples were collected. Rats were anesthetized as above mentioned, and sterilization of the back at T7-9 was done with 75% ethanol and a midline incision was done taking the T8 spinous process as the center (about 2-3 cm). The soft tissues were separated and the laminas were exposed. The lesioned spinal cord (about 0.5 cm) was collected into liquid nitrogen.

**Motor function evaluation**

Basso, Beattie and Bresnahan (BBB) scoring was performed before SCI and at 2 weeks after SCI as previously reported. Scoring of motor function was done at 8:00 am, and BBB scoring system is classified as 3 domains: first domain (0-7) was used to evaluate the activity of joints of hind limbs; second domain (8-13) was used to evaluate the gait and coordination; third domain (14-21) was used to evaluate the fine movement of paws. The total score was 21. Evaluation was done individually by two investigators and an average was obtained.

**Real-time PCR**

Sequences of Caspase-3, CYC, Bak, Bax, Bcl-x and Bcl-2 were obtained from GeneBank, and primers were designed with Primer premier 5.0 as follows: caspase-3: TGACGACAGGGTGCTACGA (forward), ATTTGAGGCTGCTGCTACGA (reverse), Bcl-2: TCGGGGCGCTGCTGCTACGA (forward), ATTTGAGGCTGCTGCTACGA (reverse).
(reverse); CYC: TGCCCTTTCAAGTCCACCACA (forward), CAGCCATAGTATAGCAGCGTCTC (reverse); Bak: ATGCCTACGAACTCTTCACCA (forward), CAGGAAGCCAGTCAAACCAC (reverse); Bax: GGTGCTCAAGGCCCTGTG (forward), GGAGAGGAGGCCTTCCCA (reverse); Bcl-x: GGAGCTGGTGGTTGACTTTC (forward), CTCCCTTTCTGGTTCAGTTTCT (reverse); Bcl-2: ACGGTGTTGAGGAACCTTCT (forward), GGGTGACATCTCCCTGTTGAC (reverse). β-actin served as an internal reference.

RNA was extracted by lysis with guanidinium and adsorption with silica particles. A 50-ul mixture was prepared with 2.5 U of c-mmlv, 20 U of Rnasin, 250 uM of dNTPs, 1x reverse transcription buffer and 0.1 P each primer. Reverse transcription was done at 50°C for 1 h and 95°C for 10 min. Then, 48-ul mixture was prepared with 5U Hot-start Taq, 1× SYBR Green I (Invitrogen, Shanghai, China), dNTPs (promega, Beijing, China), 1x quantification buffer, 10 P of each primer and 2 ul of cDNA. SYBR Green-none fluorescence was used. PCR was done at 95°C at 15 min, 94°C for 15 sec and 55°C for 45 sec for a total of 40 cycles. Melt curve was obtained for further analysis. The mRNA expression of each gene was normalized to that of β-actin, and average and standard deviation (SD) were obtained. Data with SD higher than 1 were not used for further analysis. 2-△△Ct method was employed to calculate 2-△△Ct and data with deviation of μ±1.65σ≤X≤μ+1.65σ were not included for further analysis10. Data were used to calculate the average and SD for further analysis.

**Statistical analysis**

Quantitative data were expressed as mean ± standard deviation (S), and SPSS version 13.0 was used for statistical analysis. BBB score and mRNA expression of Caspase-3, CYC, Bak, Bax, Bcl-x and Bcl-2 were compared with one way analysis of variance among groups. A value of P<0.05 was considered statistically significant.

**Results**

**Comparisons of motor function of rats**

2 weeks after SCI, the BBB score was 21.00±0.00 which was significantly higher than that in SCI group (6.15±1.24) and HBO-PC group (10.80±2.16) (P<0.05). Moreover, the BBB score in SCI group was markedly lower than that in HBO-PC group (P<0.05).

**mRNA expression of caspase-3 and cyc in the spinal cord of rats**

2 weeks after SCI, the mRNA expression of caspase-3 and cyc was detected (Table 1). As shown in Table 1, the mRNA expression of caspase-3 increased markedly in SCI group when compared with control group, but the caspase-3 mRNA expression in HBO-PC group was slightly higher than that in control group (P>0.05). When compared with SCI group, the caspase-3 mRNA expression reduced dramatically in HBO-PC group (P<0.05). Following HBO-PC, the mRNA expression was significantly lower than that in control group and SCI group (P<0.05). Moreover, CYC mRNA expression in SCI group was significantly higher than that in control group (P<0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Caspase-3</th>
<th>CYC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>1.86±0.53</td>
<td>1.98±0.62</td>
</tr>
<tr>
<td>SCI</td>
<td>12</td>
<td>2.46±0.60*</td>
<td>2.69±0.50*</td>
</tr>
<tr>
<td>HBO-PC</td>
<td>12</td>
<td>1.97±0.28b</td>
<td>1.38±0.22a</td>
</tr>
</tbody>
</table>

Table 1: mRNA expression of caspase-3 and CYC in the spinal cord of rats of different groups at 2 weeks after SCI (x±s).

Footnotes: * P<0.05 vs. control group; b P<0.05 vs. SCI group

**mRNA expression of Bak, Bax, Bcl-x and Bcl-2 in the spinal cord of SCI rats**

2 weeks after SCI, the mRNA expression of Bak, Bax, Bcl-x and Bcl-2 in the spinal cord was detected (Table 2). As shown in Table 2, Bak mRNA expression in HBO-PC group and SCI group was significantly higher than that in control group (P<0.05). When compared with control group, Bax mRNA expression reduced in HBO-PC group and increased in SCI group without significant difference (P>0.05). In SCI group, Bax mRNA expression in SCI group was markedly higher than that in control group and HBO-PC group (P<0.05). There was no significant difference in the mRNA expression of Bcl-2 and Bcl-x among three groups (P>0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Bak</th>
<th>Bax</th>
<th>Bcl-x</th>
<th>Bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12</td>
<td>1.21±0.46</td>
<td>0.92±0.30</td>
<td>1.29±0.40</td>
<td>0.59±0.16</td>
</tr>
<tr>
<td>SCI</td>
<td>12</td>
<td>1.93±0.16a</td>
<td>1.06±0.16a</td>
<td>1.35±0.40</td>
<td>0.64±0.19</td>
</tr>
<tr>
<td>HBO-PC</td>
<td>12</td>
<td>2.15±0.78a</td>
<td>0.98±0.08b</td>
<td>1.25±0.12</td>
<td>0.68±0.15</td>
</tr>
</tbody>
</table>

Table 2: mRNA expression of Bak, Bax, Bcl-x and Bcl-2 in the spinal cord of rats of different groups at 2 weeks after SCI (x±s).

Footnotes: * P<0.05 vs. control group; a P<0.05 vs. SCI group
Available studies reveal that there are two mechanisms underlying the pathogenesis of SCI: primary injury and secondary injury. On the basis of pathogenesis of SCI, clinical therapies are mainly classified as three categories: 1) to eliminate the continuous effects of exogenous factors on the spinal cord may avoid the expansion of injury area, and therapies include surgical decompression and therapy with dehydrating agents which are basis of therapies for SCI. 2) to protect the residual neurons may promote the functional reconstruction of neurons and these therapies include therapy with neurotrophic drugs, cell transplantation, gene therapy and hyperbaric oxygen therapy; 3) to block or limit the secondary pathology to protect the residual axons and neurons against further injury.

These therapies include pharmacotherapy with methylprednisolone, calcium channel antagonists or naloxone, focal hypothermia protection and artificial high-pressure perfusion. In recent years, HBO-PC has been used to attenuate spinal cord ischemia / reperfusion injury and protect the neurofunction of the spinal cord. Increasing studies focus on the application of HBO in SCI. Several studies have shown that the protective effects of HBO-PC on the neurofunction following SCI is related to the attenuation of neuronal apoptosis. However, few studies are conducted to investigate the mechanisms underlying the HBO-PC induced inhibition of neuronal apoptosis following SCI. It has been reported that HBO-PC can regulate the expression of superoxide dismutase, catalase, Bcl-2, Caspase-3 and Caspase-9 to inhibit the neuronal apoptosis and exert neuroprotection.

There is evidence showing that mitochondrial pathway of apoptosis play an important role in the cell apoptosis of vertebrates. Following injury, CYC is released from the mitochondria to activate the precursors of caspases and then activate caspase-3, which induce caspases cascade resulting in cell apoptosis. In the mitochondrial pathway of apoptosis, Bcl-2 family is crucial for the cell apoptosis. Members of Bcl-2 family can be classified as 2 categories: anti-apoptotic proteins and pro-apoptotic proteins. Bak, Bax and Bcl-x are representative pro-apoptotic proteins in Bcl-2 family. When cells receive death signals, Bak, Bax and Bcl-x are released from the cytoplasm into the membranes. Then, these proteins interact with anti-apoptotic proteins on membranes and in cells, which may block the anti-apoptotic protein induced inhibition of apoptosis, resulting in cell apoptosis. However, Bcl-2 in the Bcl-2 family may inhibit above processes in the cell apoptosis.

Our results showed the mRNA expression of CYC, Bak and Bax in the spinal cord of SCI increased significantly when compared with control group, but there was no difference in the mRNA expression of Bcl-x and Bcl-2 between SCI group and control group. This indicates that the mitochondrial CYC is released and activate the mitochondrial pathway of apoptosis following SCI, which results in caspase cascade and subsequent apoptosis of neurons in the spinal cord. In this process, the activation of Bak and Bax promotes the apoptosis of neurons in the spinal cord. Of note, there was marked difference in the mRNA expression of Bcl-2 and Bcl-2 among groups (P>0.05). This suggests that the regulatory effects of HBO-PC on neuronal apoptosis in SCI rats are not associated with Bcl-x and Bcl-2. In addition, our findings also revealed that the mRNA expression of CYC and Bax in HBO-PC group reduced significantly when compared with SCI group. This suggests that HBO-PC can attenuate the activation of mitochondrial apoptosis pathway which is associated with reduced Bax mRNA expression, compromised reduction of CYC release, decreased caspase-3 mRNA expression and subsequent attenuation of caspase cascade.

Both of our results showed that HBO-PC could improve the motor function of SCI rats and reduce the caspase-3 expression in the spinal cord. These indicate that HBO-PC induced re-training of neurofunctions in SCI rats is related to the compromised caspase cascade and the inhibition of caspase-3 related apoptosis following SCI in rats. HBO-PC inhibits the caspase-3 related apoptosis in SCI rats in following ways: HBO-PC reduces the Bax expression in the spinal cord and inhibits the release of CYC to attenuate the activation of mitochondrial apoptosis pathway in the spinal cord of SCI rats.
References


Acknowledgment

The study was supported by Science and Technology Project of Guangdong Province (2010B031600264); Guangzhou City Special Funds for the Returnees from Oversea (20120717).

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