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Analysis the effect of hyperbaric oxygen preconditioning on neuronal apoptosis, Ca^{2+} concentration and caspases expression after spinal cord injury in rats

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Abstract. – OBJECTIVE: To investigate the effects of hyperbaric oxygen preconditioning (HBO-PC) on neuronal apoptosis, Ca^{2+} concentration, and Caspases expression after spinal cord injury (SCI) in rats.

MATERIALS AND METHODS: A total of 36 rats were randomly divided into control group (CON group), hyperbaric oxygen preconditioning group (HBO-PC group) and spinal cord injury group (SCI group), with 12 rats in each group. Rats in group HBO-PC were given HBO-PC intervention before modeling. SCI model was established by modified Allen method in group HBO-PC and group SCI. Basso-Beattie-Bresnahan (BBB) locomotor rating scale and motor evoked potential (MEP) examination were used to assess the neurological function. The expression of apoptosis gene caspase (3, 7, 8, 12) mRNA was detected by reverse transcription-polymerase chain reaction (RT-PCR). The concentration of Ca^{2+} in spinal cord tissue of each group was detected.

RESULTS: CON group, HBO-PC group, and SCI group were gradually diminishing in BBB score and potential value and amplitude of MEP, respectively. The differences between groups were statistically significant ($p<0.05$). The expressions of Caspase-3 and 7, 8 and 12 mRNA in SCI group were significantly higher than those in CON group and HBO-PC group, respectively ($p<0.05$). There was no significant difference between CON group and HBO-PC group ($p>0.05$). The concentrations of Ca^{2+} in the CON group, HBO-PC group and SCI group were gradually increased; differences between groups were statistically significant ($p<0.05$).

CONCLUSIONS: HBO-PC can reduce the loss of motor function of SCI rats, which may inhibit the activation of endoplasmic reticulum pathway of neural apoptosis, and reduce the calcium overload through inhibiting the expressions of pro-apoptotic proteins (Caspase-3/7/8/12), thus reducing the cell apoptosis and protecting neurons.

Key Words

Spinal cord injury, Hyperbaric oxygen preconditioning, Calcium concentration, Apoptotic gene, Rat.

Introduction

According to epidemiological survey and analysis, there are 2 million patients with spinal cord injury (SCI) in the world with an annual increase of 130 thousand cases[1]. With the increase of traffic accidents, aerial work types and mining[2], the incidence rate of SCI stays at a high level. After SCI, the self-regeneration ability of nerve system will be insufficient, and the axonal regeneration will also be difficult[3,4], so SCI has become a major threat to global health. SCI includes the primary injury and secondary injury; the nerve damage caused by the former is irreversible, while that caused by the latter is reversible and controllable[5]. At present, it is believed that apoptosis is the main mode of cell death after secondary SCI[6]. Scholars[7,8] have shown that Caspase gene family and imbalance of cellular calcium homeostasis play important roles in apoptosis. Hyperbaric oxygen pre-conditioning (HBO-PC) has been used for the treatment of SCI, in which HBO is conducted before SCI, obtaining effects in basic experiments and clinical treatment[9,10]. However, its exact mechanism remains unclear.

The primary purpose of this study was to investigate the effects of HBO-PC on neuronal apoptosis, calcium ion concentration and Caspase expressions in SCI rats and its underlying mechanisms.

Materials and Methods

Experimental Materials

Rat Grouping

Clean-grade Sprague-Dawley (SD) rats aging 4-6 months old (280-320 g), half male and half female, were provided by the Nantong University Experimental Center. Rats were divided into con-

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trol group (CON group, n=12), HBO-PC group (n=12) and SCI group (n=12) using the random number method. Before the experiment, rats were fed and acclimatized in our laboratory for 1 week. This study was approved by Nantong University Animal Ethics Committee, and the experiments were implemented strictly in accordance with the management regulations and methods of experimental animal.

Experimental Methods

Establishment of SCI Rat Model

Rats were anesthetized *via* intraperitoneal injection of 30 mg/kg 1% sodium pentobarbital and received the operation strictly under aseptic conditions according to the modified Allen's weight-drop method. After routine disinfection and draping, the spinal cord under the skin with hair removed was exposed under a prone position. About 2-3 cm-long longitudinal incision was made with T9 spinous process as the center, and T8-10 vertebral plates were separated and removed to expose the spinal canal and dural sac. An impact rod with a diameter of 3 mm and weight of 30 g plummeted from 5 cm above the spinal cord to cause the T10-centered SCI by 150 gcf striking force. Signs of successful impact are as follows: tail-wagging reflex, paralysis of posterior limbs after retraction and flapping of posterior limbs and body. Next, the incision was sutured, followed by postoperative conventional anti-infection, fluid infusion and insulation therapy; rats were assisted in the urination after operation until the establishment of micturition reflex. SCI rat model was made in SCI group and HBO-PC group. All rats in the three groups were fed under the same conditions.

HBO-PC Treatment

Rats in HBO-PC group were given HBO-PC intervention before modeling in a pure oxygen chamber of experimental animal following the steps below: chamber washing (10 min) → compression (uniform compression for 20 min till 0.2 MPa) → continuous oxygen inhalation (80 min in an oxygen concentration of 80-85%) → decompression (uniform compression for 20 min) → discharge from the chamber. Rats were treated once a day for 10 consecutive days without interruption. At 8 h after the 10th HBO-PC, SCI modeling was performed.

Specimen Collection

Specimens were taken from rats in each group at 2 weeks after SCI modeling. After conventional disinfection and successful anesthesia, about 1.0 cm-long SCI tissues were taken below and above T10, and immediately placed in liquid nitrogen bottle for standby application.

Observation Indexes

Evaluation of Nerve Function

1) Evaluation of neuromotor function: at 2 h and 2 weeks after modeling, rats were placed in an open basin, and the basin wall was tapped to make them crawl; the lower limb walking, trunk movement and coordination were observed. According to the criteria officially published by research workers of Ohio State University in 1995, the lower limb movement is divided into 22 grades. 21 points indicate the complete normality, while 0 points indicate the paralysis of posterior limbs. Basso-Beattie-Bresnahan (BBB) locomotion scores of rats in each group were recorded, and the average score of posterior limbs was taken.

2) Evaluation of nerve conduction function: motor evoked potential (MEP) was often used. Based on the electroencephalogram international 10-20 system electrode placement method, the stimulating electrode and recording electrode were placed on the left central point (C3), right central point (C4) and great thenar of posterior limbs. 35 V stimulus was posed one time without superposition; the stimulus intensity could be increased appropriately if there was no response.

Determination of Caspase Messenger RNA (mRNA)

Specimens were taken from liquid nitrogen, the total RNA was extracted, the first complementary DNA (cDNA) strand was synthesized, and the Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed according to the instructions of TRIzol kit (Invitrogen, Carlsbad, CA, USA), cDNA reagent (Toyobo, Osaka, Japan) and SYBR Green PCR kit (Toyobo, Osaka, Japan), respectively. Amplification system: 12.5 μL SYBR mix, 2 μL cDNA, 0.5 μL forward primer and 0.5 μL reverse primer; finally, double distilled H_2O (ddH_2O) was added until the total volume was 25 μL. Reaction program: 42°C×20 min, 95°C×5 min, (95°C×15 s → 55°C×15 s → 72°C×20 s) ×40, 95°C × 1 min, 55°C×30 s and 95°C×30 s. The experiment was repeated for 3 times to reduce the error and bias, with β-actin gene as the internal reference. Data were analyzed using the built-in software of Real-time detector (ABI-7500, New York, NY, USA), and the relative expression levels of Ca-

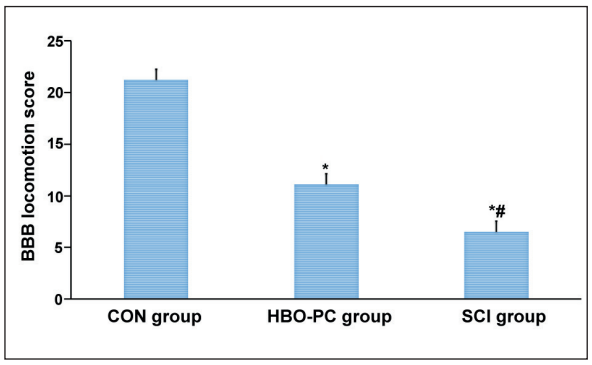

Figure 1. Comparison of BBB locomotion score in each group of rats. Note: compared with CON group, *p<0.05; compared with HBO-PC group, #p<0.05.

spase-3/7/8/12 mRNA in CON group, HBO-PC group and SCI group were presented as $2^{-\Delta\Delta Ct}$.

Determination of Ca^{2+} Ion Mass Concentration In Spinal Cord Tissues

Specimens were taken from liquid nitrogen, followed by centrifugation, derivation, weighing, baking, re-weighing, nitrification, inorganic treatment, dilution and centrifugation; the supernatant was taken to be detected. The concentration of Ca^{2+} ion in the solution was determined using an atomic absorption spectrophotometer, and the mass concentration of Ca^{2+} ions was calculated according to the following formula: mass concentration of Ca^{2+} ion (μmol/kg) = concentration of Ca^{2+} ions in the measured solution/specimen mass after drying × dilution ratio.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) were used. Measurement data were presented as mean ± standard deviation ($\bar{x} \pm s$), and one-way analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference) was used for comparison between groups. p<0.05 suggested that the difference was statistically significant.

Results

Comparison of BBB Locomotion Score in Each Group of Rats

BBB locomotion scores in CON group, HBO-PC group and SCI group were (21.23±1.58) points, (11.12±1.75) points and (6.52±1.37) points, respectively. BBB locomotion scores were progressively decreased in CON group, HBO-PC group and SCI group; the differences were statistically significant in the comparisons among groups (p<0.05) (Figure 1).

Comparisons of MEP Value and Amplitude Measured in Each Group of Rats

MEP values in CON group, HBO-PC group and SCI group were (40.12±3.87) ms, (21.03±3.56) ms and (12.94±2.75) ms, respectively. MEP values measured were progressively decreased in CON group, HBO-PC group and SCI group, and the differences were statistically significant in the comparisons among groups (p<0.05) (Figure 2A). The MEP amplitude in CON group, HBO-PC group and SCI group was (1.16±0.24) mV, (0.42±0.06) mV and (0.12±0.05) mV, respectively. The MEP amplitude measured was progressively decreased in CON group, HBO-PC group and SCI group, and the differences were statistically significant in the comparisons among groups (p<0.05) (Figure 2B).

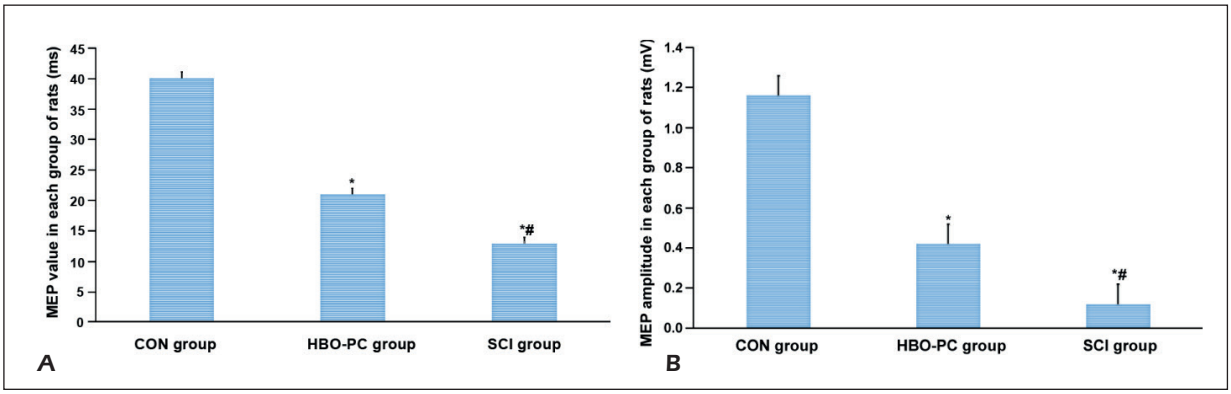

Figure 2. A, Comparison of MEP value in each group of rats. **B**, Comparison of MEP amplitude in each group of rats. Note: compared with CON group, *p<0.05; compared with HBO-PC group, #p<0.05.

Comparisons of Relative Expression Levels of Caspase-3/7/8/12 mRNA in Spinal Cord Tissues of Rats in Each Group

The mRNA expression levels of Caspase-3 in spinal cord tissues in three groups of rats were different. The relative expression levels of Caspase-3 mRNA in CON group, HBO-PC group and SCI group were (1.85±0.31), (1.91±0.28) and (2.43±0.26), respectively. The mRNA expression level of Caspase-3 in SCI group was significantly higher than those in CON group and HBO-PC group (p<0.05). There was no statistically significant difference in the comparison of mRNA expression level of Caspase-3 between CON group and HBO-PC group (p>0.05) (Figure 3A).

The mRNA expression levels of Caspase-7 in spinal cord tissues in three groups of rats were different. The relative expression levels of Caspase-7 mRNA in CON group, HBO-PC group and SCI group were (0.63±0.12), (0.71±0.13) and (1.13±0.09), respectively; the mRNA expression level of Caspase-7 in SCI group was significantly higher than those in CON group and HBO-PC group (p<0.05). There was no statistically significant difference in the comparison of mRNA expression level of Caspase-7 between CON group and HBO-PC group (p>0.05) (Figure 3B).

The mRNA expression levels of Caspase-8 in spinal cord tissues in three groups of rats were different. The relative expression levels of Caspase-8 mRNA in CON group, HBO-PC group and SCI group were (0.84±0.23), (1.09±0.17) and (1.52±0.18), respectively; the mRNA expression level of Caspase-8 in SCI group was significantly higher than those in CON group and HBO-PC group (p<0.05); there was no statistically significant difference in the comparison of mRNA expression level of Caspase-8 between CON group and HBO-PC group (p>0.05) (Figure 3C).

The mRNA expression levels of Caspase-12 in spinal cord tissues in three groups of rats were different. The relative expression levels of Caspase-12 mRNA in CON group, HBO-PC group and SCI group were (0.76±0.19), (0.82±0.21) and (1.18±0.16), respectively; the mRNA expression level of Caspase-12 in SCI group was significantly higher than those in CON group and HBO-PC group (p<0.05). There was no statistically significant difference in the comparison of mRNA expression level of Caspase-12 between CON group and HBO-PC group (p>0.05) (Figure 3D).

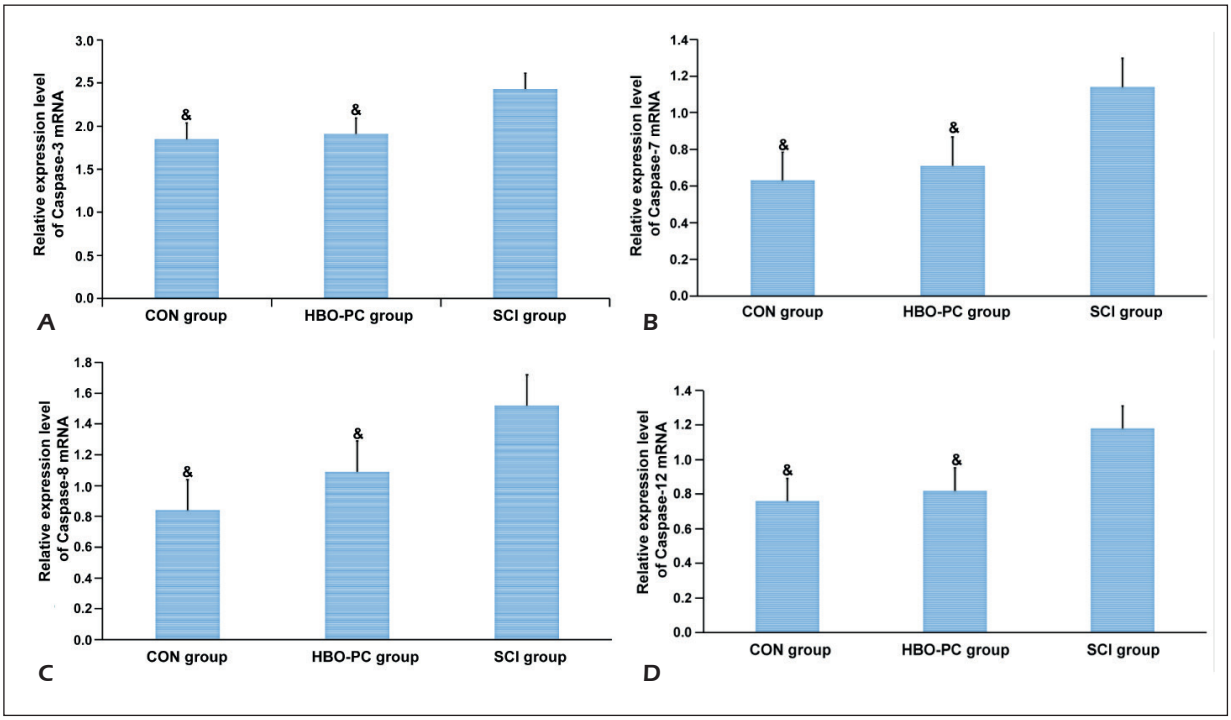

Figure 3. **A**, Relative expression level of Caspase-3 mRNA in each group of rats. **B**, Relative expression level of Caspase-7 mRNA in each group of rats. **C**, Relative expression level of Caspase-8 mRNA in each group of rats. **D**, Relative expression level of Caspase-12 mRNA in each group of rats. Note: compared with SCI group, &p<0.05.

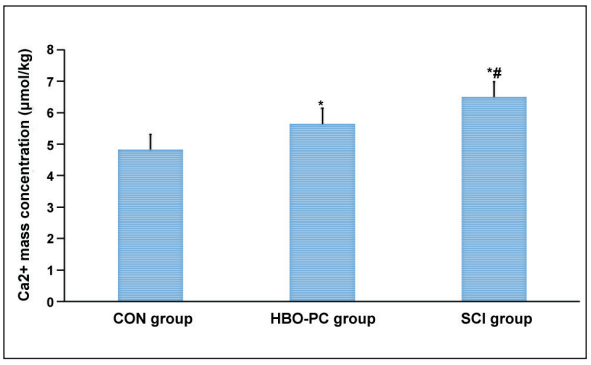

Figure 4. Comparison of Ca^{2+} mass concentration in spinal cord tissues of rats in each group. Note: compared with CON group, *p<0.05; compared with HBO-PC group, #p<0.05.

Comparison of Ca^{2+} Mass Concentration in Spinal Cord Tissues of Rats in Each Group

The Ca^{2+} mass concentrations in spinal cord tissues of rats in three groups were different. The Ca^{2+} mass concentrations in CON group, HBO-PC group and SCI group were (4.83±0.18) μmol/kg, (5.65±0.24) μmol/kg and (6.51±0.46) μmol/kg, respectively. The Ca^{2+} mass concentrations were progressively decreased in CON group, HBO-PC group and SCI group, and the differences were statistically significant in the comparisons among groups (p<0.05) (Figure 4).

Discussion

SCI refers to the complete or incomplete spinal cord motor, sensory, sphincter and autonomic nerve function disorders due to the violent impact on spinal cord, resulting in permanent disability and even death of patients[11]. SCI animal model is a basis for the study on SCI, and currently SCI animal models include the spinal cord impact, pinching, compression and cutting injury models. In 1911, Allen established the vertical attack-induced SCI model for the first time, commonly known as Allen's weight-drop method[12]. T10 segment is the most common injured part in clinical practice, which is easy to be identified and positioned with less vascular distribution, and can avoid the blood loss of experimental animals, so T10 was selected as the so-called injured area in this investigation to ensure the consistency of experiment. The modified Allen's weight-drop method is characterized by fixed point, fixed height, constant weight, convenience, stable modeling and similar injury mechanism to that of clinical SCI patients, so currently it is a recognized method with the best repeatability and correlation[13]. Therefore, Allen's weight-drop method was used in this experiment to make the SCI rat model. In this study, the success rate of modeling was 97.30% (36/37). The standardized and normalized establishment of model laid a foundation for subsequent experiments and improved the comparability among groups. SCI is a serious disease of the central nervous system, becoming one of the most difficult medical problems nowadays. A series of researches[14-17] confirmed that HBO has a significant repairing effect on injured neurons, which can prevent or reverse the secondary pathological changes to SCI from many aspects. This work proved that the BBB locomotion scores were progressively decreased in CON group, HBO-PC group and SCI group, and the differences were statistically significant in the comparisons among groups (p<0.05); MEP values and amplitude measured were progressively decreased in CON group, HBO-PC group and SCI group, and the differences were statistically significant in the comparisons among groups (p<0.05), suggesting that HBO is effective in reducing the local neuron apoptosis and alleviating the secondary injury of spinal cord tissues.

Apoptosis, also known as programmed cell death, is another mode of cell death, which is different from the physiological death of cells, necrosis. The mechanism of HBO-PC in protecting the nerve function of SCI is related to the fact that HBO-PC can reduce the apoptosis of neuronal cells after SCI. This study showed that the mRNA expression of Caspases in SCI group was significantly higher than those in CON group and HBO-PC group (p<0.05), but there were no statistically significant differences between CON group and HBO-PC group (p>0.05), indicating that after SCI, the apoptosis *via* Caspase pathway in rats is activated, leading to apoptosis of rat spinal cord neuronal cells. This may also be an important reason for the secondary SCI, and its mechanism is related to the fact that HBO-PC can alleviate the apoptosis of spinal cord neuronal cells *via* Caspase pathway in SCI rats. The Caspase family plays a crucial role in regulating the apoptotic pathway of spinal cord neuronal cells. According to the different positions into apoptotic pathway, Caspases can be divided into two subtypes: 1) promoter Caspases, including Caspase-1/2/4/5/8/9/10; 2) effector Caspases, including Caspase-3/6/7/14[18]. Under the action of apoptotic signals, promoter Caspases are

activated, leading to the cascade reaction of Caspases; the specific substrates are lysed by effector Caspases, leading to the apoptosis. Caspases-8 is a promoter in the cascade reaction of Caspases, while Caspases-3 is an effector during the process. Caspase-8 participates in almost all Caspase activation-related pathways, and can activate almost all Caspases; it is in the peak of apoptosis cascade reaction, and is a marker of the initiation and progression of Caspase apoptotic pathway. Caspase-3 is the most representative effector Caspase, which exerts its effect in the downstream of cells, holds a key position and acts as a characteristic sign of apoptosis[19, 20]. The conduction pathways of Caspase-induced apoptosis include the endoplasmic reticulum pathway, death receptor pathway and mitochondrial pathway. Caspase-12 is located in the endoplasmic reticulum, which is necessary for the endoplasmic reticulum stress-induced apoptosis. Endoplasmic reticulum stress induces the expression of Caspase-12, and also makes Caspase-7 in the cytoplasm transfer onto the surface of endoplasmic reticulum. Caspase-7 activates Caspase-12, and cleaves Caspase-3 in turn, inducing the apoptosis[21]. In this work, it was found that the expressions of Caspase-12 and Caspase-7 in spinal cord tissues of SCI rats were significantly increased compared with those in CON group and HBO-PC group, but there were no statistically significant differences in the Caspase-12 and Caspase-7 expressions in spinal cord tissues of rats between CON group and HBO-PC group, which, combined with the results of Caspase-8 and Caspase-3 in this study, suggested that the endoplasmic reticulum apoptotic signaling pathway of neuronal cells in rats is activated after SCI. At the same time, the above results also indicated that the protective effect of HBO-PC on neuronal cells is related to the fact that HBO-PC can block the endoplasmic reticulum signaling pathway of neuronal cells to a certain extent, thus decreasing the expression of downstream Caspases and reducing the apoptosis degree of neuronal cells. Moreover, HBO-PC can protect the motor function of SCI rats to a certain extent and reduce the degree of nerve injury, and its mechanism is related to the fact that HBO-PC can reduce the endoplasmic reticulum apoptotic pathway of neuronal cells in rat after SCI; specifically, HBO-PC can inhibit the expressions of Caspase-12/7/8/3, thus reducing the activation degree of endoplasmic reticulum apoptotic pathway of neuronal cells in rat and alleviating the apoptosis. Whether it is related to the apoptosis conduction pathways (mitochondrial pathway and neuronal cell death signaling pathway) remains to be further studied deeply.

Scholars[22] have shown that intracellular Ca^{2+} within the normal concentration range can maintain the neuronal excitatory calcium overload and reduce the neuronal excitation threshold, leading to neuronal apoptosis. Calreticulin is a main molecular chaperone binding to Ca^{2+} in the endoplasmic reticulum, which regulates the dynamic equilibrium of intracellular Ca^{2+}. The imbalance of cellular calcium homeostasis is one of the important mechanisms of apoptosis. Meanwhile, it is considered that Ca^{2+} plays a crucial role in the regulation of apoptosis in SCI[23]. Our results showed that the Ca^{2+} mass concentrations were progressively decreased in CON group, HBO-PC group and SCI group, and the differences were statistically significant in the comparisons among groups ($p<0.05$). Those results suggested that the intracytoplasmic Ca^{2+} concentration is significantly increased in apoptosis, and the calcium overload causes varying degrees of damage or loss of brain neurons; at the same time, a variety of abnormal enzymatic reactions are thus triggered, leading to the irreversible cell death[24].

Conclusions

We found that HBO-PC can alleviate the loss of motor function in SCI rats, which may inhibit the activation of endoplasmic reticulum pathway of neural apoptosis, and reduce the calcium overload through inhibiting the expressions of pro-apoptotic proteins (Caspase-3/7/8/12), thus reducing the cell apoptosis and protecting neurons.

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Conflict of interest

The authors declare they have no conflict of interest.

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