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The possible protective mechanism of Hyperbaric oxygen (HBO) in memory impairments induced by $Aß_{25–35}$ in rats

Research Article

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Abstract: Alzheimer's disease is characterized by the accumulation and deposition of Aß peptides in human brains. Aß peptides are toxic to neurons by lots of mechanisms of which Aß induced oxidative stress is one of the hypothesis. The present study aimed to determine the effect of Hyperbaric oxygen (HBO) on $Aß_{25-35}$ induced cognitive deficits and oxidative stress and apoptosis effects in rats. Rats were given an injection of aggregated $Aß_{25-35}$. After treatment with HBO for 20days, the learning and memory ability, hippocampus neuronal apoptosis, the activity of SOD, GSH content and the MDA level and mRNA and proteins expression of Bcl-2 and Bax were detected. Our results demonstrated that HBO could significantly improved the apoptosis hippocampus neuronal induced by $Aß_{25-35}$, involving the improvement of the learning and memory impairment, which accompanied of increasing the gene and protein expression of bcl-2 and enhancing the activity of SOD and GSH content. These findings suggest that treatment of HBO might prevent the $Aß_{25-35}$ induced learning and memory impairment by increasing the gene and protein expression of bcl-2 and enhancing the activity of SOD and GSH content to alleviate the apoptosis hippocampus neuronal. This suggests that HBO may be a potential therapeutic agent for AD.

Keywords: *Alzheimer's disease (AD) • Hyperbaric oxygen (HBO) • Neuroprotective; $Aß_{25-35}$*

1. Introduction

Alzheimer disease (AD) is a progressive and degenerative disease characterized by a progressive cognitive failure and altered behavior, and the classical neuropathological hallmark is the formation of β-amyloid protein (Aβ) and neurofibrillary tangles(NFTs) [1], which plays a central role in the pathogenesis of AD. The deposition of Aβ in the hippocampus and temporal cortex is recognized as an early and critical event in the pathogenesis of AD [2]. Aβ peptide fragment 25–35 ($Aβ_{25–35}$), an active fragment of toxic Aβ, has been shown to be toxic to neurons [3] and have the potential to induce oxidative stress and impairment of spatial learning and memory correlate with cell loss in rat hippocampus [4].

Oxidative stress, caused by deposition of Amyloid β protein(Aβ), may be the main pathogenic mechanism of AD [5], as it causes cell apoptosis and consequent cognitive decline. There is increasing data from experimental models and human brain studies suggest that oxidative stress plays an important role in neuronal degeneration in AD [6]. Some studies found that

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antioxidants such as Vitamins E, melatonin and Ginkgo biloba [7] have protective effect against Aβ-mediated neurotoxicity and treatment with antioxidants may be a promising approach for slowing progression of AD to the extent in AD.

HBO therapy，by exposing patients to barometric pressures 2 atmospheres absolute (ATA) for at least 60 min or 2.4–2.8 ATA for 60–90 min, while they breathe 100% oxygen, is increasing used in a number of areas of medicine practice. Many results indicate that HBO therapy can reduce ischemia–reperfusion injury [8] and develop the neuroprotective effect of traumatic brain injury [9]. Although treatment of HBO may be toxic via stimulating reactive oxygen species (ROS) formation [10], HBO protects against oxidative stress [11] and prevents neuronal apoptosis [12] have been reported. These data suggest that HBO maybe play an important neuroprotective role in alleviating oxidative stress and apoptosis. In this research, We initially explore possible neuroprotective mechanisms of HBO from oxidative stress and apoptosis.

2. Materials and methods

2.1. Animals and Aß preparation

Adult male and female Sprague–Dawley rats (3-4 months old, 200-250g) (Shanghai Laboratory Animal Cente, SLACCAS,China) were housed for at least 1 week before the experiments at 22±1°C with a 12 h light-dark cycle. Food pellets and water were freely available throughout the experiment. Forty-eight rats were randomly divided into four groups: control group (rats with no injection), Sham group (physiological saline injected), model group ($A\beta_{25-35}$ injected) and HBO group ($A\beta_{25-35}$ injected+2ATA HBO treatment). $A\beta_{25-35}$(SIGMA, USA, A4559) was dissolved at a concentration of 1g/L in sterile saline and incubated at 37°C for 7 days before use.

2.2. Hippocampal injection of Aß

Rats were anaesthetized by chloral hydrate (300 mg/kg, Tianjin, China) and placed in a tereotaxic apparatus (BW-SR-5M, Japan). Two small holes for needle insertion in the skull were drilled with a Dental drill in the parietal bone posterior to bregma on either side of the midline according to the map [13]. In brief, each rat was injected at 3 mm posterior to bregma, 2 mm lateral to the sagittal suture and 3 mm under the scull. The needle was additionally left at the site of injection for 5 min and then was slowly withdrawn. Model group and HBO group were injected with $A\beta_{25-35}$ peptide (10μl) into right and left hippocampus with a microinjector and sham rats were injected with the same volume of physiological saline. Following surgery, all rats were placed into cages, kept warm and received an intramuscular injection of 80,000 unit/0.25 ml of the antibiotic penicillin for 3 days.

2.3. HBO (2ATA) treatment

The rats were placed into the HBO chamber (SHC3200-8500, Shanghai) after three weeks of injection and were exposed to 100% oxygen at a pressure of 2.0 ATA for 60 min including 15 min of compression and decompression. Procedure was continued 2 courses (20 days) with the interval of 3 days between the two courses.

2.4. Morris water maze (MWM) performance

To assess hippocampal dependent spatial learning and memory, rats were trained in Morris water maze task (ZH0065, Zhenghua Bio-equipments, China) on the 3rd day after the treatment. The maze consisted of a black circular pool (1.6cm diameter, 50cm height) filled with water (23± 1°C) to a depth of 50 cm and a circular platform (10 cm diameter) was submerged 2 cm below the surface of the water and hidden from the rat's view. The maze was divided into 4 quadrants (I.II.III.IV), which served as the starting positions for the rat being gently lowered into the water, with its head facing the wall of the tank. The water maze test consisted of two stages: Days 1-6 for hidden platform trials and the 7^{th} day for a probe test. For each trial, the details were recorded by a video camera mounted above the center of the pool (ZH0065, Zhenghua Bio-equipments, China).

Rats were given 2 trials per day with a submerged platform that they could climb onto to escape from the water. The location of the platform was fixed in the middle of the quadrants I and the release points were fixed on the wall of quadrants II and III. At the beginning of each trial, the rat was gently placed into the water facing the wall of the tank at the start location. A trial ended when the rat escaped onto the platform within 90 s and the rat did not found the platform within 90 s was gently guided to the platform and then was allowed to remain on the platform for 20 s.

On the 7^{th} day, a probe trail was conducted in which each rat received a 90s free swim in the pool with the platform removed. The release point was the one of two starting positions in the hidden platform trial (quadrant III).

In each trial, the escape latency and the speed was recorded, while in probe trials, the percentages of time spent in the platform quadrant and the number of

crossings of the exact location where the platform had been located previously were measured.

2.5. TUNEL staining

The rats (n=6) of each group were immediately anesthetized with chloral hydrate at a dose of 300 mg/kg after the Morris maze test and perfused with 0.1 M phosphate buffer (pH 7.4) followed by ice-cold 4% paraformaldehyde through left ventricle. Brains were removed and postfixed in the same fixative solution for 48 h overnight and then were embedded in paraffin.

5μm-thick sections were taken from each brain at the level of anterior hippocampus, approximately -2.0 to -4.0 mm from bregma for TUNEL test. The process was conducted by the manufacturer's instructions of TUNEL kit (Keygen, Jiangsu, China). Briefly，after deparaffinization, the sections were treated proteinase K (20g/ml) for 30 min at 37°C. After treatment with 3% H2O2 in methanol for 10 min to block the endogenous peroxidase activity., the sections were incubated with TUNEL reaction mixture for 60 min at 37°C. Further incubation with Streptavidin-HRP for 30 min at 37°C. The sections were stained with diaminobenzidine (DAB) solution for 5 min at room temperature and then counterstained with hematoxylin. For negative controls, the sections were incubated without terminal deoxynucleotidyl transferase. The cells labeled the brown were the clear apoptotic characteristics(TUNEL positive). The data were represented as the Apoptotic index (AI=apoptosis/total cellular score × 100%) in the CA1 of hippocampus.

2.6. Assay the activity of SOD, GSH and MDA level

The rats (n=6) of each group were decapitated after being anesthetized and the brains were rapidly removed on ice. The whole hippocampus was taken out from the brain. The right hippocampus was homogenized in cell lysis buffer (Beyotime, Jiangsu, China). Homogenates were centrifuged at 12,000 g for 5 min and the supernatants were collected for assays the activity of SOD,GSH and MDA (Beyotime, Jiangsu, China). SOD activity was determined with WST-1(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, -disulfophenyl)-2H-tetrazolium,monosodium salt) method, GSH and MDA content was determined with 5,5'-Dithiobis(2-nitrobenzoic acid) and thiobarbituric acid method, respectively.

2.7. RT-PCR analysis

The left hippocampus was used for RT-PCR Total RNA was isolated using Trizol reagent (TAKARA, Japan) and the RNA concentration was determined by measuring the absorbance at 260 nm. The process of the RT-PCR kit (TAKARA, Japan) of each sample according to the manufacturer's protocol. Gene expression was detected by PCR kit (TAKARA, Japan) with the following primers: Bcl-2 (Sense primer: 5′-GGCATCTTCTCCTTCCAG-3′;Aanti- Sense primer: 5′-ATCCCAGCCTCCGTTAT-3′), Bax (Sense primer: 5′-CATCCAGGATCGAGCAGAG-3′; Aanti- Sense primer: 5′-GAGGACTCCAGCCACAAAG-3′), and GAPDH (Sense primer: 5′-TTGCCCTCTTCTACTTTGC-3′;Antisense primer: 5′-AGCCTTGACTGTGCCATT-3′). PCR products were run on a 3% agarose gel in 1x TAE buffer stained with Ethidium Bromide and visualized under UV light.

2.8. Western blot analysis

At the same time, the supernatants of the right hippocampus homogenized in cell lysis buffer were collected for Western blot analysis. Samples of homogenates (15 mg of protein) were subjected to SDS-PAGE (12% or 15% gels) under reducing conditions. Proteins were transferred onto PDVF membranes in transfer buffer 25 mM Tris-HCl (pH 7.4) containing 192 mM glycine and 20% v/v methanol] and further separated at 152mA for 30min to determine the proteins levels. Membrane were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween20 (TBST) at room temperature for 1 h and then incubated overnight at 4°C with anti-bcl-2 (1:1000; CST, USA), anti-Bax 1:1000; CST, USA) and GAPDH (monoclonal antibody, 1:1000; CST, USA). The following day, PDVF membranes were washed three times for 15 min followed by 1h of incubation with HRP conjugated secondary antibody (1:5000) at room temperature. After subsequent washes in 1×TBST, the protein bands were visualized using an ECL detection kit (GE Healthcare, Munich, Germany) and exposure to X-ray films. Relative optical densities and areas of bands were quantified using an image densitometer. The densitometric plots of the results were normalized to the intensity of the GAPDH band.

2.9. Data analysis

The results are expressed as mean ±SD. Differences between groups were analyzed using two-way ANOVA with repeated measures in the Morris water maze test and the other data were analyzed using one-way ANOVA. A level of $P < 0.05$ was accepted as statistically significant. All these analyses were performed using SPSS for Windows version 11.5.

3. Results

3.1. Effects of HBO on spatial learning and memory impairment induced by Aß$_{25-35}$

As shown in Figure 1, the escape latency decreased with the increase in hidden platform trials. Comparing with the model group, the escape latency of HBO group was significantly declined (P<0.05), but no statistically significant difference was observed between HBO group and sham group (P>0.05) (Figure 1A). These effects were not attributable to the presence of motor deficits, because the four groups exhibited similar swimming speeds (Figure 1D).

The probe trial was employed to determine memory of the rats after treatment. On the 7th days, the percentage of time in the target quadrant was significantly increased in the HBO group (P<0.05) compared with the model group (Figure 1B). And the number that the rats of HBO group crossed over the position where the platform located was more than that of model group (P < 0.05), but revealed no significant difference compared to that of the control and sham groups (P>0.05) (Figure 1C)

3.2. The results of TUNEL staining

We employed TUNEL assay and examined effects of HBO on the Aβ25-35 induced apoptosis in rat hippocampal neurons (Figure 2). The results showed that TUNEL-positive cells (refer to the arrows) were largely observed in the hippocampus of the model group (Figure 2C), but were also seen in the group treated with HBO (Figure 2D). Almost no TUNEL-positive cells were found in the normal and sham groups (Figure 2A-B). The apoptotic index showed the significant differences in the HBO as compared with the model group (*P < 0.01).

3.3. Effects of HBO on the activity of SOD, GSH content and MDA in the hippocampus tissue

Oxidative stress and its production may be pathologically important in Alzheimer's disease (AD) and we detected the activity of SOD, GSH content and the level of MDA in the four groups to research the possible association. We found that the activity of SOD and GSH content in the hippocampus of model group was reduced significantly compared with the other groups (P<0.05) (Figure 3A,B). And the SOD activity and GSH content of HBO group

Figure 1. Effects of HBO on hidden platform trials and the probe test of MWM performance.(A) Comparison of escape Latency among groups in the hidden platform trials.(B)Percentage of time spent in the target quadrant area relative to the total in the pool in the probe trial. (C) The number of crosses over the exact location of the hidden platform in the probe trial. (D) The average speed of rats in the hidden platform training (* P<0.05 vs. Model group).

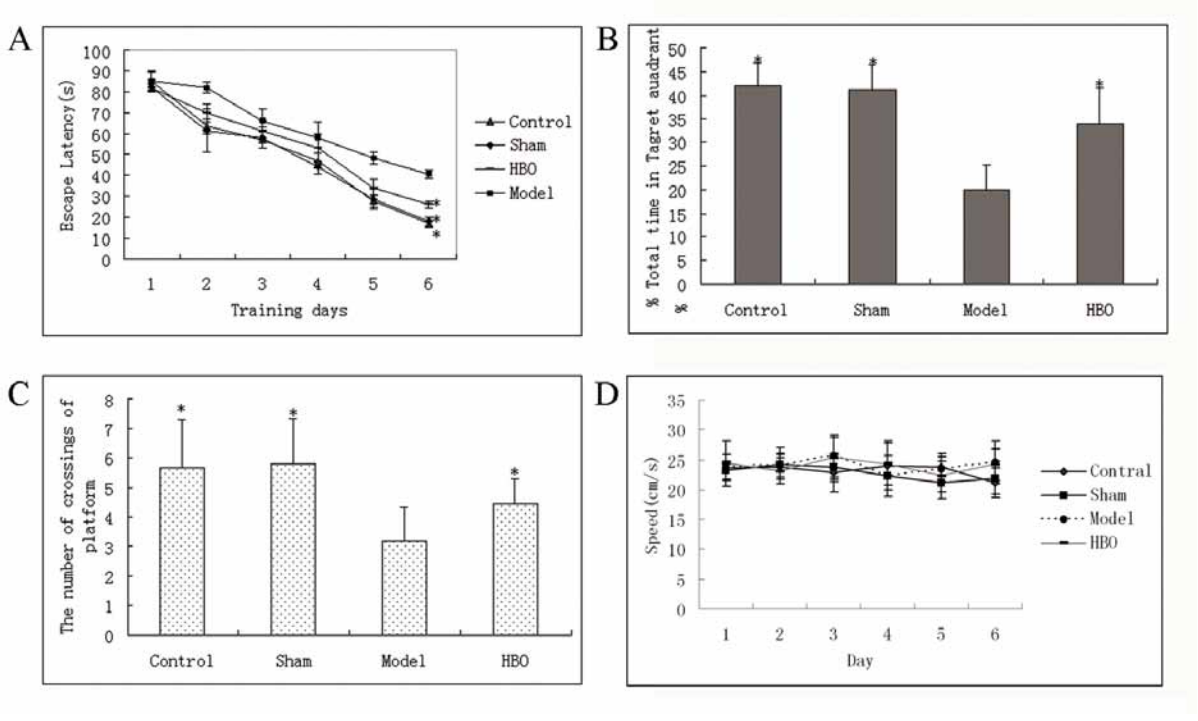
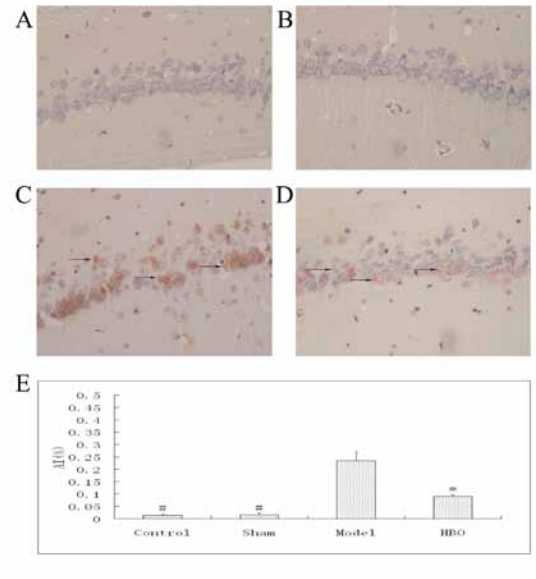


Figure 2. Effects of HBO on the hippocampus neuron in the CA1 induced by $A\beta_{25-35}$ by TUNEL staining.

Apoptosis hippocampus neurons were often seen in the hippocampus of model group(C) and almost no apoptosis cells in the control and sham animals (A,B) and HBO group showed several apoptosis hippocampus neurons (D). The apoptotic index of HBO group showed the significantly decreased compared with the model group (*P < 0.05 vs. Model group), and the control and sham groups show the apoptotic index approaches zero (# P < 0.001 vs. Model group)



was found to be significantly increased compared to the model group (*P*<0.05).

MDA is the last product of lipid peroxidation and is toxic to cells and cell membranes. Our researchers found that MDA levels of the groups showed the adverse results compared with the SOD and GSH (Figure 3C). There was a significant reduction in the level of MDA of HBO (2ATA) group as compared to control group (*P*<0.05).

3.4. Expression of mRNA of bcl-2 and Bax of hippocampus

Bcl-2 and Bax are very important in the apoptosis and to explore the possible relationship between the HBO and the Bcl-2 and Bax, the effect of HBO on the genes expression of Bcl-2 and Bax was examined.

As shown in Figure 4, the genes expression of Bcl-2 in the HBO group increased significantly (P < 0.05) (Figure 4C) compared with model group, while the genes expression of Bax shown the contrary results (P<0.05) (Figure 4D).

Figure 3. The measurement of SOD activity and level of MDA. (A) SOD activities were significantly decreased in model group compared with HBO group; and the MDA levels in each group showed the opposite tendency with the SOD activity (B) (*P<0.05 vs. Model group).

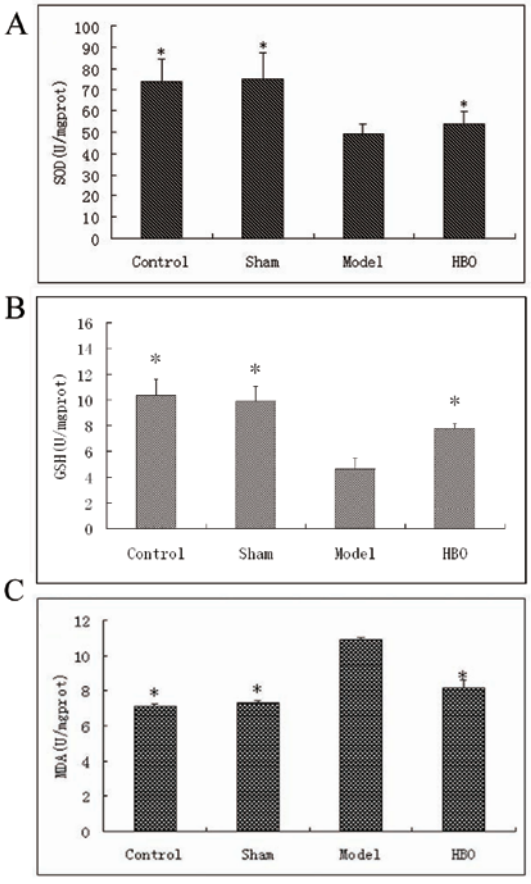
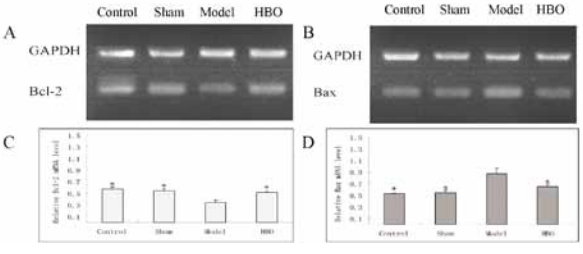


Figure 4. RT-PCR for Bcl-2 and Bax mRNA expression in the hippocampus. (C.D)The bar graphs show semiquantitative results for relative levels of mRNA for Bcl-2 and Bax. The mRNA expression in Bcl-2 and Bax in the HBO group is compared with that the model group (* P< 0.05 vs. Model group).



3.5. Expression of proteins of bcl-2 and Bax of hippocampus

To further explore the possible mechanism of protective of HBO, we examine the proteins of Bcl-2 and Bax. The results indicated that the HBO (2ATA) could significantly increased the expression of Bcl-2 comparing with the

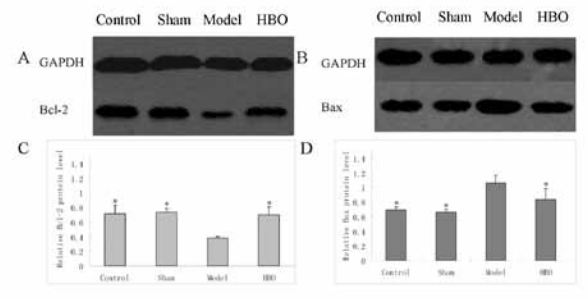


Figure 5. Western blot analysis of Bcl-2 and Bax levels in hippocampus. GAPDH was used as a reaction standard. Comparison of Bcl-2 and Bax levels in hippocampus among groups (* P<0.05 vs. Model group).

model group (P<0.05) (Figure 5C), and the proteins of Bax was decreased comparing with the model group (P<0.05) (Figure 5D).

4. Comment

As the most common form of neurodegenerative disorder with dementia, Alzheimer's disease (AD) has become a serious growing public-health problem around the world. Though the key pathogenic mechanism of AD has improved in the past few decades, they are still not completely understood. Growing data from animals models [14] and human brainstudies [15] suggests that oxidative stress are involved in the neurotoxicity of Aβ. In addition, several studies have concluded that $Aβ_{25-35}$ administration increase ROS and MDA levels in the hippocampus [16], which suggest that oxidative stress may result in learning and memory deficits. In the pathogenesis of AD, oxidative stress maybe damage the cellular components results in alteration of the membrane properties such as fluidity, enzyme activities, and protein cross-linking, and eventually results in neuronal cell apoptosis or death [17], inducing the learning and memory deficits.

In the present study, the behavioral data demonstrated that injection of aggregated $Aβ_{25-35}$ induced marked amnesic effects in rats. These results suggest that the accumulation of neurotoxic Aβ fragments $Aβ_{25-35}$ impairs spatial reference memory in rats, which is consistent with previous reports [18]. And the HBO improved the learning and memory impairment of AD rats could be seen (Figure 1). The escape latency in searching for the hidden underwater platform decreased with the increasing in training days in the four groups and the escape latency of HBO group was significantly shortened than that of the model group. In the probe trials, we found that the HBO group spends more time in the platform quadrant and the times of crossing the platform showed that the HBO group was more than that of the model group. These behavioral observations indicated that HBO can relieve the learning and memory impairment induced by injection of by $Aβ_{25-35}$.

Hippocampus is a brain region playing a key role in the learning and memory processes [19]. Hippocampus neuronal apoptosis is among one of the most important causes of amnesia in human disorders accompanied by neurodegeneration and the neuronal alteration in hippocampus plays an important role in AD like cognitive deficits [20]. In the present study, we detected the neuronal apoptosis in the hippocampal CA1 subregion of rats in the different groups through TUNEL staining. We found that the apoptosis pyramidal neurons of CA1 subfield of the hippocampus in the HBO group were significantly decreased comparing to the model group, which suggested the apoptosis hippocampus neuronal in the CA1 subfield may be at least partially responsible for the learning and memory deficits and HBO may be able to improve the apoptosis hippocampus neuronal to increase the ability of spatial learning and memory in AD rats.

To research the possible protective mechanism of HBO, we detected the activity of SOD, GSH content and MDA levels of hippocampus. Oxidative stress is an imbalance of the antioxidative defense and oxidative system, which in AD is the result of increasing production of Aβ. MDA is the last product of lipid peroxidation and is toxic to cells and cell membranes. SOD and GSH are the most important physiological antioxidants against free radicals and can prevent subsequent lipid peroxidation. SOD and GSH could catalyzes the disproportionation of superoxide to molecular oxygen and peroxide, and is critical for protecting cells against the toxic products of oxidative stress. Our results indicate that HBO could significantly increase the activity of SOD and GSH content and decrease the production of MDA, which may depress the hippocampus neuronal apoptosis from oxidative stress. Although the treatment of HBO may be toxic via stimulus of ROS formation [21], our results and some previous researches indicated that HBO could protect against oxidative stress and protect hippocampus neuronal [22,23].

In the process of protection hippocampus neuronal, the effects of anti-apoptosis of HBO maybe play an important role except for improving the activity of SOD and GSH content. Many studies have demonstrated that reactive oxygen species (ROS) and the resulting oxidative stress play a pivotal role in apoptosis. Taken together ROS and the resulting cellular redox change can be part of signal transduction pathway during apoptosis, which maybe damaged hippocampus neuronal resulting in apoptosis and the following the learning and memory impairments. In our study, we explored the

expression of genes and proteins of Bcl-2 and Bax. Bcl-2, the first mammalian regulator gene, was identified to have anti-apoptotic potential in a variety of cell systems. It is the location of Bcl-2 on the cytoplasmic face of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope currently that to be believed this strategic localization allows it to register and counterbalance the oxidative damage done to these compartments. Bax, one of the Bcl-2 family members work in a manner opposite to that of Bcl-2.The results indicate that HBO could increase the gene and proteins expression of Bcl-2 and depress the gene and proteins expression of Bax, which suggested that HBO could increase anti-apoptosis gene expression, which was consistent with some studies [24], to inhibit the apoptosis of hippocampus neuronal induced by oxidative stress. Though our study suggest that HBO could raised the gene and proteins expression of Bcl-2 and to improve the ability of anti-apoptosis, the detail of mechanism should be further studied in the following research.

In the present day, the mechanism of Aβ-induced neurotoxicity and cognitive impairments remains unclear. Many studies suggest that reactive oxygen species and nitric oxide productions, decreased membrane fluidity, alteration of the cytoskeleton and nucleus are probably involved in the mechanisms of Aβ-induced neurotoxicity [25]. The new neuroprotective agents targeted on these potential AD pathogenesis are under investigation.

Our study indicated that oxidative stress-induced damage or apoptosis may be a major feature in the pathophysiology of Alzheimer's disease (AD) and the treatment of anti-oxidative stress could be a potential neuroprotective agent. We suggest that, except the physical role of increasing the oxygen content of blood and penetrating into ischemic areas more deeply [26], HBO may play its neuroprotective effects through anti-oxidative stress and anti-apoptosis in molecular level and HBO may be a potential therapeutic agent for specific neurodegenerative diseases. The protection of HBO on spatial learning and memory impairments in our study is on the surface, however. Further study will be required to work out the details of protective mechanism.

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