Protection mechanism of early hyperbaric oxygen therapy in rats with permanent cerebral ischemia

MIN YU1), YIXUE XUE2), WEIDI LIANG1), YUPENG ZHANG1), ZHIQIANG ZHANG1)*

1) Department of Rehabilitation, Shengjing Hospital of China Medical University: No.39, Huaxiang Road, Tie Xi Region, Shenyang City, Shenyang, Liaoning 110022, China
2) Department of Neurobiology, College of Basic Medical Sciences, China Medical University, China

Abstract. [Purpose] The purpose of this study was to investigate whether early hyperbaric oxygen is useful in rats with permanent cerebral ischemia, and whether its mechanism relates to the inhibition of the tumor necrosis factor-alpha-protein kinase C-alpha pathway. [Subjects] Healthy, male Sprague-Dawley rats (N = 108) were the subjects. [Methods] After middle cerebral artery occlusion models were successfully made, rats were randomly divided into sham-operated, cerebral ischemia, and hyperbaric oxygen groups. At 4 and 12 hours after modeling, the volume of cerebral infarction was determined by triphenyltetrazolium chloride staining, and brain water content was measured using the dry and wet method. The expression of tumor necrosis factor-alpha and protein kinase C-alpha in the ischemic penumbra tissue was measured using Western blot analysis. [Results] The data showed that at 4 and 12 hours after modeling, cerebral infarct volume and brain water content decreased in the hyperbaric oxygen group, and expression of tumor necrosis factor-alpha and phospho-protein kinase C-alpha in the ischemic penumbra tissue also decreased. [Conclusion] Our study demonstrates that early hyperbaric oxygen therapy has protective effects on brain tissue after cerebral ischemia, possibly via inhibition of tumor necrosis factor-alpha and phospho-protein kinase C-alpha.

Key words: Hyperbaric oxygen therapy, Cerebrovascular protection, Cerebral ischemic rats

INTRODUCTION

Cerebral ischemia is the third most prevalent disease that threatens human health. It seriously affects the patient’s quality of life and increases the burden on the family. There are few effective therapies for cerebral ischemia except recombinant tissue plasminogen activator which has strict indications for use as medication. The application of hyperbaric oxygen (HBO) in cerebral ischemia has shown to have certain effects on motor function, aphasia, cognition, and post-stroke depression1). HBO can improve tissue hypoxia, maintain cellular energy metabolism, protect the blood-brain barrier, and relieve cerebral edema. Many recent studies have examined HBO treatment of cerebral ischemia, based on experimental models of ischemia and reperfusion. However, in the clinical, cerebral ischemia in humans is most likely permanent ischemia and has an optimal time window for HBO treatment within 2 hours after the ischemic attack2–5). 3–6 hours after the ischemic attack, HBO has no effect on the infarct volume and neurological function6). In this experiment, we created permanent cerebral ischemia in rat model to explore whether HBO has a protective effect on brain tissue, and whether its protective mechanism involves the inhibition of phospho-protein kinase C-alpha (p-PKCa) and tumor necrosis factor-alpha (TNF-α) expression.

SUBJECTS AND METHODS

Adult male Sprague-Dawley rats (weight, 250–300 g) were purchased from the Experimental Animal Center of China Medical University. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the China Medical University. Rats were housed in laboratory cages and maintained on a 12-hour light/dark cycle with free access to food and water throughout the study period.

One hundred and eight healthy Sprague-Dawley rats were randomly divided into six groups of 18: sham-operated (sham) 4-hour and 12-hour groups, cerebral ischemia (CI) 4-hour and 12-hour groups, and HBO 4-hour and 12-hour groups. After middle cerebral artery occlusion (MCAO) models were successfully made, rats in the HBO group were immediately given HBO therapy (2 ATA for 1 hour). Rats in the CI group were not given HBO therapy. Rats in the sham-operated group were only sutured with 1 cm thread without blocking the middle cerebral artery or altering the HBO therapy.

As monitored by laser Doppler blood flow assessments, a rat model of focal CI was made by improved Longa suture...
method). Briefly, rats were anesthetized by being given an intraperitoneal injection of 10% chloral hydrate (360 mg/kg), and their body temperatures (as monitored with a rectal probe) were maintained at 37 ± 0.5 °C, using a heating lamp during the experiment. After successful anesthesia, the rats were fixed in a supine position. Through a ventral midline incision in the neck, the left common carotid artery (CCA), the internal carotid artery (ICA), and the external carotid artery (ECA) were exposed. A nylon filament (diameter 0.286 mm, length 5 cm) with a head-end grinding round and covered with silica gel was inserted into the CCA and gently advanced into the ICA, approximately 18–20 mm from the carotid bifurcation, until meeting slight resistance. The nylon filament was then fixed in the CCA and the skin was sutured.

To confirm proper occlusion of the left middle cerebral artery, a laser Doppler probe (moorLAB, Moor Instruments, UK) was fixed on the skull (1 mm posterior to the bregma and 5 mm from the midline on the left side) to monitor regional cerebral blood flow (rCBF) in the area supplied by the middle cerebral artery. rCBF flux measurements were taken immediately before and after occlusion. The occlusion was considered successful if there was a 30% decrease in local cortical blood flow compared to the baseline pre-occlusion value. The animals that did not comply with the above criteria were excluded from the study.

Cerebral infarct volume was measured using triphenyltetrazolium chloride (TTC) staining. At corresponding time points, rats in each group were sacrificed by giving them an overdose of chloral hydrate. Their brains were rapidly removed, frozen at −20 °C, and sectioned into six coronal slices (2 mm thick) that were immediately immersed in 2% 2,3,5- TTC (Sigma-Aldrich, USA) saline solution at 37 °C for 30 minutes. Normal brain tissue was stained red, whereas infarct areas were not stained and remained white. Stained tissues were then fixed in 4% paraformaldehyde, photographed on both sides, and quantified for infarct area using an image analysis system (NIS-Elements BR 3.0, Nikon Instruments, Melville, NY, USA). The degree of infarct volume was calculated as the percentage of the damaged area of the total area.

Brain water content was determined using a dry and wet method. Rats in each group were sacrificed by decapitation at corresponding time points. After the brains were removed in total, the hemispheres were separated along the interhemispheric plane. Both hemispheres were weighed to assess their wet weights and then dried for 24 hours at 100 °C to determine their dry weights. Water content in both ischemic and nonischemic hemispheres was obtained by the following calculations: Hemispheric water content (%) = (wet weight − dry weight) / wet weight × 100%.

Western blots were used to detect the protein expression levels of TNF-α, p-PKCα, and PKCα. The rats in each group were sacrificed immediately by decapitation at corresponding time points. Brain tissue in ischemic penumbra was obtained immediately. Equal amounts of proteins were separated by size in 6% and 12% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose, and then probed overnight at 4 °C with rabbit polyclonal anti-TNF-α (diluted 1:1000) (Abcam, USA), rabbit polyclonal anti-p-PKCα (diluted 1:1000) (Abcam, USA), and rabbit monoclonal anti-PKCα (diluted 1:2000) (Abcam, USA) antibodies. β-actin (β-actin antibody 1:500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was probed overnight at 4 °C with rabbit polyclonal anti-TNF-α and p-PKCα. Western blot analysis showed that, compared with the sham-operated group, the expression of TNF-α and p-PKCα in the CI group increased significantly. Western blots were used to detect the protein expression levels of TNF-α, p-PKCα, and PKCα. The next day, the membranes were incubated with their respective secondary horseradish peroxidase-conjugated antibodies for 2 hours at room temperature. Protein bands were visualized by chemiluminescence (ECL Kit, Beyotime Inc., China) and scanned with MicroChemi 4.2 (Bio-Imaging Systems Ltd., Jerusalem, Israel). Integrated density values (IDVs) were calculated using a computerized image analysis system (Gel-Pro Analyzer 32) and normalized to that of β-actin (β-actin antibody 1:500, Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

The experiments were repeated at least three times, and all results were presented as mean ± SD. Differences between the two groups were statistically analyzed using t-tests. Differences among multiple groups were statistically analyzed using one-way ANOVA, followed by the Bonferroni test; p<0.05 was considered statistically significant.

**RESULTS**

Early HBO therapy reduced cerebral infarct volume in rats with permanent CI. At 4 and 12 hours, the percentage of infarct volume in the HBO group was reduced significantly compared with the CI group (p<0.05) (Table 1). Values represent means ± SD (n = 6 for each group).

| Group      | Left   | Right  |
|------------|--------|--------|
| Sham-operated | 78.2 ± 0.1% | 78.2 ± 0.2% |
| CI group   | 79.6 ± 0.4%* | 81.7 ± 0.4%* |
| HBO group  | 78.6 ± 0.4% | 80.9 ± 0.4% |

*p<0.05
CI: cerebral ischemia; HBO: hyperbaric oxygen

In contrast, the expression of TNF-α and p-PKCα decreased.
CI: cerebral ischemia; HBO: hyperbaric oxygen; protein kinase C-alpha; p-PKCα: phospho-protein kinase C-alpha; TNF-α: tumor necrosis factor-alpha; β-actin: beta-actin; PKCα: protein kinase C-alpha; p-PKCα: phospho-protein kinase C-alpha; CI: cerebral ischemia; HBO: hyperbaric oxygen

Fig. 1. Western blot-detected expression of TNF-α, β-actin, PKCα, and p-PKCα in each group

Representative photographs of Western blot bands in each group. TNF-α: tumor necrosis factor-alpha; β-actin: beta-actin; PKCα: protein kinase C-alpha; p-PKCα: phospho-protein kinase C-alpha; CI: cerebral ischemia; HBO: hyperbaric oxygen

significantly in the HBO group compared with the CI group. An analysis of TNF-α to β-actin IDVs and p-PKCα to PKCα IDVs revealed that the expression of TNF-α and p-PKCα in ischemic penumbra tissue in CI group rats increased significantly compared with the sham and HBO groups (p<0.05) (Tables 3 and 4).

**DISCUSSION**

HBO refers to breathing pure oxygen in an environment in which the atmospheric pressure is higher than normal; the method of inhaling HBO in disease treatment is called HBO therapy. HBO can significantly increase the uptake and utilization of oxygen in organisms to increase their blood oxygen content, increase oxygen partial pressure, and enhance oxygen-diffusing capacity; thus, HBO is an effective means of treating various hypoxic diseases. Tissue hypoxia after focal CI is one of the main causes of cell injury. HBO can improve tissue oxygen supply, maintain cellular energy metabolism, protect the blood-brain barrier, and relieve cerebral edema; therefore, it is considered to be a promising method for treating CI. It is well known that the ischemic penumbra surrounding the ischemic core can turn into normal area with blood and oxygen supplies, rather that turning into an area of infarct. Consequently, improving the supply of oxygen in the ischemic penumbra as soon as possible is the theoretical reason for using HBO in the treatment of CI. In this experiment, HBO therapy was given immediately after modeling, thereby maintaining oxygen and blood supplies in ischemic brain tissue during the formation of CI, and consequently reducing the ischemic penumbra. At 4 and 12 hours after modeling, TTC (a marker of cellular respiration) staining was detected. It was found that cerebral infarct volumes in rats in the HBO group were obviously reduced. Similar studies showed that HBO therapy should be performed in the early stages of CI. Sunami et al. found that infarct volume decreased 18% in rats with permanent CI following administration of HBO 10 minutes after ischemia. Similarly, Schäbitz et al. confirmed on magnetic resonance imaging (DWI, PI, T2) that HBO therapy was effective in reducing the volume of cerebral infarction and symptoms. Prior research showed that increased endothelial permeability due to ischemia and inflammatory factors was PKCα-dependent. One previous study showed that the activation of TNF-α could cause phosphorylation.

Table 3. The ratios of TNF-α to β-actin integrated density values in each group

| Group          | 4 hour       | 12 hour      |
|----------------|--------------|--------------|
| Sham-operated  | 0.12 ± 0.02  | 0.12 ± 0.02  |
| CI group       | 0.31 ± 0.03* | 0.09 ± 0.02  |
| HBO group      | 0.14 ± 0.12  | 0.13 ± 0.018 |

*p-Compared with the sham and HBO groups, the ratios of TNF-α to β-actin integrated density value in the CI group significantly increased (p<0.05). Values represent means±SD (n=6 for each group).

Table 4. The ratios of p-PKCα to PKCα integrated density values in each group

| Group          | 4 hour       | 12 hour      |
|----------------|--------------|--------------|
| Sham-operated  | 0.09 ± 0.03  | 0.10 ± 0.02  |
| CI group       | 0.45 ± 0.018*| 0.37 ± 0.022*|
| HBO group      | 0.18 ± 0.025 | 0.15 ± 0.028 |

*p-Compared with the sham and HBO groups, the ratios of p-PKCα to PKCα increased significantly (p<0.05). Values represent means±SD (n=6 for each group).
and translocation of downstream PKCα(19). Our Western blot analysis results likewise showed that the expression of TNF-α and p-PKCα both increased during CI. Under the therapeutic protection of HBO, the expression of TNF-α and p-PKCα decreased significantly, verifying that TNF-α and PKCα signaling might be one of the mechanisms through which HBO protects brain tissue.

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