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Research Report

Therapeutic effects of hyperbaric oxygen in a rat model of endothelin-1-induced focal cerebral ischemia

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ABSTRACT

It has been established that hyperbaric oxygen (HBO) treatment reduces brain edema, decreases infarct volume, contributes to neurological functional recovery and suppresses apoptosis in suture-induced focal cerebral ischemic animal models. In the present study, we evaluated the therapeutic effect of HBO in an endothelin-1-induced focal cerebral ischemia in rats and explored the associated mechanisms of HBO-induced brain protection. One hundred twenty male Sprague–Dawley rats (280 to 320 g) were randomly assigned to sham, focal cerebral ischemia and focal cerebral ischemia treated with HBO groups. Brain water content, neurological function, morphology and molecular biological markers were assessed. HBO (100% O_2, 2.5 atmosphere absolute for 2 h) was initiated at 1 h after focal cerebral ischemia. Rats were killed at 24 h to harvest tissues for Western blot or for histology. In HBO-treated animals, an enhanced ratio of Bcl-2 and Bax and a reduced expression of hypoxia-inducible factor-1α (HIF-1α) in the hippocampus after focal cerebral ischemia were observed. These results indicate that HBO provides brain protection that is probably associated with the inhibition of HIF-1α and the elevation of Bcl-2.

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1. Introduction

Since Koizumi et al. (1986) reported the method of middle cerebral artery occlusion by a suture in 1986, most researchers have produced the models of focal cerebral ischemia by filament intraluminal occlusion of the middle cerebral artery (Macrae, 1992; Kuge et al., 1995; Longa et al., 1989; Kawamura et al., 1991). Although this method does not require opening of the skull and has good reproducibility, the suture may directly injure the endothelium. In this study, we used endothelin-1 (ET-1) to induce focal cerebral ischemia, which was first reported by Sharkey et al. (1993). This method is advantageous because the manipulation is simple and tissue damage is limited. This method does not produce surgical complications and therefore decreases surgery-related brain injuries, especially those related to neurological dysfunctions. This model is

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Abbreviations: ANOVA, analysis of variance; ATA, atmosphere absolute; ET, endothelin; ET-1, endothelin-1; HIF-1α, hypoxia-inducible factor-1α; HBO, hyperbaric oxygen; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; PBS, phosphate-buffered saline; PDVF, polyvinylidene; BNIP3, Bcl-2/adenovirus E1B 19-kDa protein-interacting protein 3

[1] Zhen-Xing Huang and Zhi-Min Kang contributed equally to this work.

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closer to mimicking human stroke (Biernaskie et al., 2001) in that it induces stroke when animals are conscious (Sharkey et al., 1993).

The aim of the study was to evaluate the therapeutic effects of HBO on focal cerebral ischemia ET-1 induced. In our study we found HBO treatment can lessen brain edema, decrease infarct volume, contribute to neurologic function recovery and suppress apoptosis. Using this more clinically relevant model, we also revisited hyperbaric oxygen (HBO)-induced brain protection, especially in the areas of apoptosis and hypoxia-inducible factor-1α (HIF-1α). We studied two apoptotic factors Bax and Bcl-2; the former induces the release of cytochrome c (Gogvadze et al., 2001; Sugawara et al., 2004) and the latter prevents the formation of Bax homodimers (Reed et al., 1996), which inhibits the cytosolic accumulation of cytochrome c and caspase-3 activation (Zhao et al., 2003). We also studied the expression of HIF-1α because HIF-1α increases after focal cerebral ischemia (Bergeron et al., 1999) and because HIF-1α induces apoptosis (Carmeliet et al., 1998; Moritz et al., 2002) by the activation of BNIP3 (Ostrowski et al., 2005; Bruick, 2000; Greijer and van der Wall, 2004; Boyd, 1994) or the stabilization of p53 (Greijer and van der Wall, 2004; Chen et al., 2003).

2. Results

2.1. Neurological score

The neurological score was 1.83±0.58 in the sham group versus the minimum of 1 that is obtainable. A significant increase in neurological score (9±0.93) was found in animals 24 h after focal cerebral ischemia (Fig. 1A). Treatment with HBO alleviated the increase in neurological score, even though the level calculated in this group (5.76±0.89) was significantly higher than the control value ($P<0.01$ versus control, ANOVA).

The body weight variance is shown in Fig. 1B. After 24 h, the mean body weight decreased 10.75±3.58 g in the ET group and 8.00±3.77 g in the ET+HBO group ($P<0.05$).

2.2. Brain water content

There was no significant difference in water content of the right hemisphere, brain stem and cerebellum between the three groups. Mean brain water content values of the left hemispheres were 78.76±0.27% in the sham group, 80.08±0.67% in the ET group and 79.27±0.22% in the ET+HBO group (Fig. 2A). The left hemisphere water content of the ET group was significantly higher than the sham ($P<0.01$, ANOVA) and ET+HBO groups ($P<0.05$). There was no obvious difference in brain water content between the sham and ET+HBO groups ($P>0.05$).

2.3. TTC

Infarct volumes derived from postmortem TTC staining at 24 h (Fig. 2C) were 13.34%±2.89% (ET, $n=7$) vs. 5.39%±2.21% (ET+HBO, $n=8$) ($P<0.01$). No cerebral infarction was observed in the sham group ($n=8$). Six sections were cut for each brain. Section 1 represented the most anterior area and section 6 represented the most posterior area. Decreased infarction occurred primarily in sections 1, 2, 3 and 4 (data not shown).

2.4. Morphology and Nissl staining

Fig. 3 demonstrates Nissl staining of the cortex (A1–C1) and CA1 sector (A2–C2) in rats at 24 h after focal cerebral ischemia. There were extensive neuronal changes in the CA1 sector of the hippocampus and cortex (Figs. 3B1, B2). Considerable dark, pyknotic neurons were shown. HBO (2.5 ATA, 2 h) applied at 1 h after focal cerebral ischemia reduced the deletion of neuron structure and retained the number of cells in the CA1 and the cortex (C2, C1). All the figures were obtained from the left hemisphere.

2.5. TUNEL staining

The fragmentation of nuclear DNA in cells has been identified extensively with TUNEL staining (Banasiak et al., 2000; Johnston et al., 2001). No detectable TUNEL-positive cells were found in the sham-operated animals (Fig. 4A). In samples collected from the ET group, the damaged cells were characterized by a round and shrunken morphology. The processus disappeared and the neuronal body became rounded with strong TUNEL staining in the nucleus. At higher magnification, the nuclei of cells were clearly stained in the cortex (Fig. 4B). After HBO treatment, the number of positive cells observed in the cortex had decreased dramatically by 24 h (Fig. 4C).

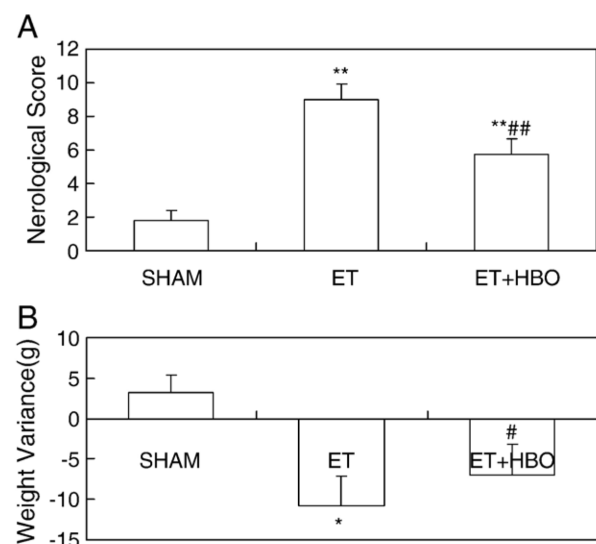

Fig. 1 – (A) The bar graph shows the neurological scores of animals at 23 h after focal cerebral ischemia. The neurological score was profoundly increased in the endothelin (ET) group (9±0.93; $n=26$, $P<0.01$ vs. sham). HBO alleviated the neurological scores (5.76±0.89; $n=26$. $P<0.01$ vs. ET groups) although they remained higher as compared with sham values (1.83±0.58; $n=26$, $P<0.01$ vs. sham). (B) Body weight sharply reduced after focal cerebral ischemia ($P<0.05$ vs. sham), while HBO partially relieved the decrease ($P<0.05$ vs. ET and sham).

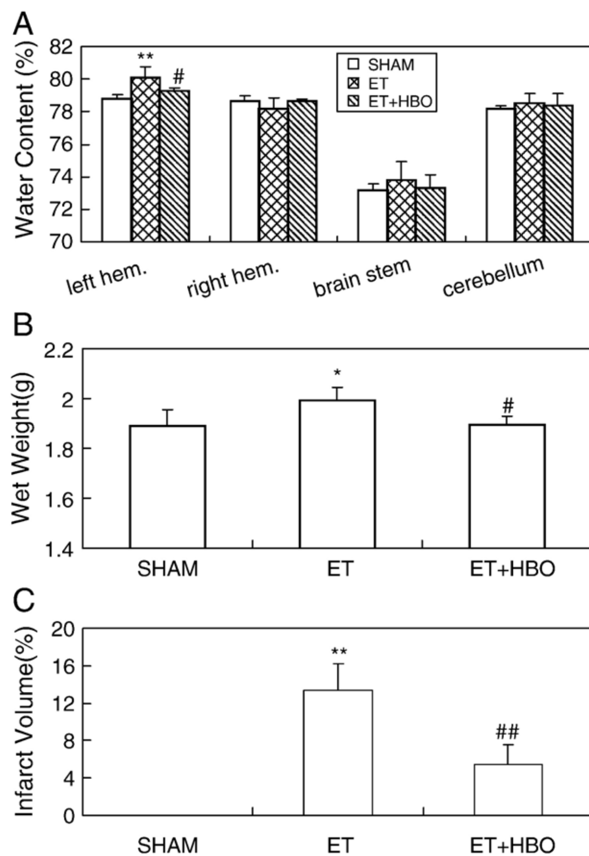

Fig. 2 – (A) Increased brain water content was observed only in the left (ipsilateral) hemisphere after endothelin-1-induced cerebral ischemia ($P<0.01$ vs. sham). HBO reduced brain water content in the left (ipsilateral) hemisphere ($P<0.05$ vs. ET). The water content in HBO group is not significantly different from the sham group ($P>0.05$). (B) Wet brain weight of the ET group was enhanced (1.99±0.05; $n=10$, $P<0.05$ vs. sham). HBO decreased wet brain weight (1.89±0.04; $n=8$, $P<0.05$ vs. ET), which was closer ($P>0.05$ vs. sham) to the value of sham group (1.89±0.07; $n=9$). (C) Marked cerebral infarction was observed in the ET group ($n=8$) and HBO reduced brain infarction ($n=8$, $P<0.01$ vs. ET) partially ($P<0.01$ vs. sham ($n=8$).

2.6. *Bcl-2 immunostaining*

Almost no Bcl-2 staining in the cytosol was found in the sham group (Fig. 5A). Bcl-2 immunoreactive cells were found mostly in the ipsilateral hemisphere, that is, the left hemisphere. HBO treatment significantly elevated the level of Bcl-2 staining in the cerebral cortex and CA1 sector (C1, C2).

2.7. *Bax immunostaining*

Bax expression was increased in the ET and the ET+HBO groups (Fig. 6, versus sham group). The cell shape of most positive staining cells was damaged, and dark, pyknotic nuclei were primarily demonstrated. Although after HBO there was some decrease in the intensity and area of Bax staining, it seemed that HBO did not have a significant effect on Bax.

2.8. *Hypoxia-inducible factor-1α immunostaining*

The cerebral cortex and hippocampus of sham-operated rats showed very weak HIF-1α immunostaining (Figs. 7A1, A2). Brain samples obtained from animals at 24 h after focal cerebral ischemia showed strong HIF-1α staining in the nuclei of neurons as a fine, grain-like substance (B1, B2). HBO treatment reduced the staining of HIF-1α in the CA1 sector and cortex (C2, C1). The decrease in the extent of HIF-1α immunostaining correlated with the presence of well-preserved neurons in the cerebral cortex and in the hippocampus.

2.9. *Western blot of Bcl-2, Bax, hypoxia-inducible factor-1α*

Western blot analysis of brain samples showed that Bcl-2 was not elevated at 24 h after focal cerebral ischemia. After HBO treatment, the expression of Bcl-2 significantly increased to 1.90-fold and 1.95-fold versus sham in the hippocampus (Fig. 8A) and cortex (Fig. 8B), respectively.

Western blot analysis showed that the level of Bax was significantly increased in the brain sample at 24 h after focal cerebral ischemia. The expression increased more than 1.5-fold. Similar levels of Bax protein was observed in the ET+HBO group in the hippocampus (Fig. 9A) and cortex (Fig. 9B).

Accumulation of HIF-1α in the hippocampus (Fig. 10A) and cortex (Fig. 10B) was shown by Western blot analysis. Strong HIF-1α accumulation was observed in the hippocampus (Fig. 10A) at 24 h after focal cerebral ischemia, a 2.23-fold increase ($P<0.01$ versus sham). Treatment with HBO resulted in the diminution of HIF-1α protein content ($P<0.05$ vs. ET). No marked changes of HIF-1α expression were observed in the cortex with or without HBO.

3. Discussion

We observed marked brain injury and cell death in this endothelin-1-induced focal cerebral ischemia rat model. We observed neurological functional disability at 24 h after focal cerebral ischemia, accompanied by brain edema, brain swelling and brain infarct. In addition, molecular changes related to apoptotic cell death were noted in Bcl-2 and HIF-1α. In HBO-treated animals, an elevation of Bcl-2 and a reduction of HIF-1α were observed. This was consistent with some previous reports (Vlodavsky et al., 2005; Wada et al., 2000; Liu et al., 2006; Li et al., 2005). All these observations indicate that HBO may help protect brain tissues if given in the hyper-acute stage of focal cerebral ischemia, which in an animal model resembles human stroke conditions.

The major contribution of the study was that we used an ET-1-induced model which was closer to mimicking human stroke to reveal the therapeutic effects of HBO and the mechanism. One of the advantages of using this endothelin-1-induced focal cerebral ischemia model was to study cerebral edema or swelling. At 24 h after focal cerebral ischemia, the brains showed marked swelling resulting from increases in brain water content that were reflected by increased brain wet weights in this study. HBO reduced both brain water content and brain wet weight. One of the mechanisms of HBO in

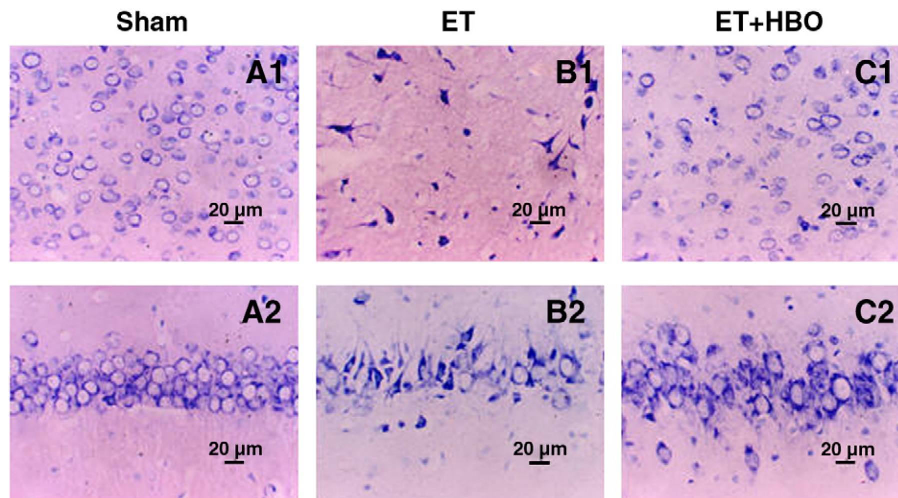

Fig. 3 – In sham group, the structure of most neurons in the cortex and CA1 sector were clear, some Nissl body stained uniformly appeared in the kytoplasm (A1). Most neurons of ET group were shrinked and have deep color staining. At the same time, the intercellular space was enlarged, part of neurons disappeared (B1). In CA1 sector of sham group, the hippocampal pyramidal cells divided into several layers and the structure was compact. The pyramidal cells were big and have abundant cytoplasm and Nissl body (A2). In ET group, pyramidal cells arranged sparsely and the cell outline was fuzzy. The cells with eumorphism were significantly reduced (B2). One single HBO treatment prevented cell loss and preserved the structure of most neurons (C1, C2).

reducing brain swelling is the promotion of venous return (Niklas et al., 2004) through the constriction of cerebral vessels since, after focal cerebral ischemia, blood had stagnated in brain tissues due to the dysfunction of autoregulation (Ostrowski et al., 2005). HBO treatment may enhance the opening of capillary vessels, improving microcirculation and collateral circulation, which all help to reduce brain edema (Titovets et al., 2000). In addition, it has been reported that HBO treatment may improve the activity of the Na^+/K^+-ATPase (Mrsic-Pelcic et al., 2004a,b) and reduce BBB damage after focal cerebral ischemia (Mink and Dutka, 1995; Veltkamp et al., 2005).

In this endothelin-1-induced focal cerebral ischemic animal model, we observed that neurons in the hippocampus and cerebral cortex showed cytoplasmic condensation and nuclear pyknosis, similar to those reports using suture occlusion models. Marked neuronal loss was also detected by Nissl staining, especially at CA1 of the hippocampus at 24 h after focal cerebral ischemia. Immunochemistry revealed that strong HIF-1α staining was present in damaged cells. In HBO-treated animals, a partially reduced appearance of dark neurons with cytoplasm shrinkage and nuclear pyknosis was observed. The beneficial effect of HBO was associated at least partially with its anti-apoptotic actions because of the

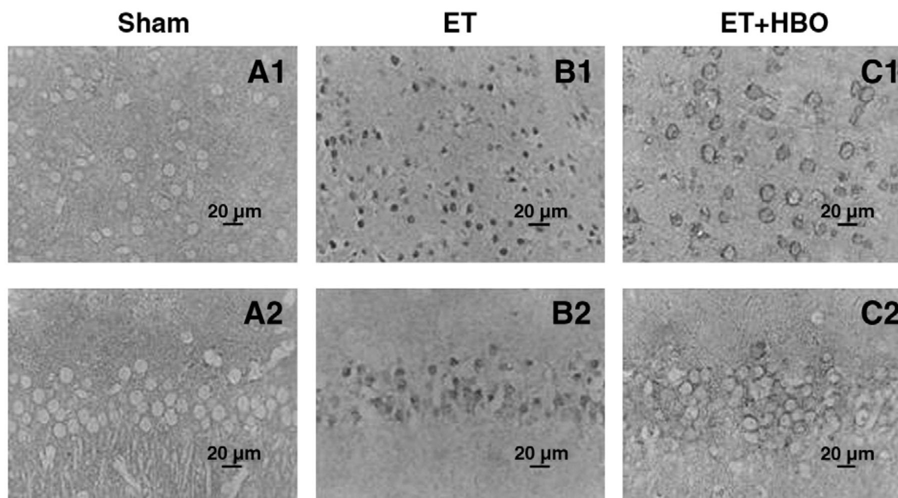

Fig. 4 – Immunohistochemistry showed negative staining of HIF-1α in normal samples (A1, A2). At 24 h after focal cerebral ischemia, positive staining to HIF-1α appeared in the hippocampus (B2) and cortex (B1). HBO treatment reduced HIF-1α expression in the hippocampus and cortex (C2, C1).

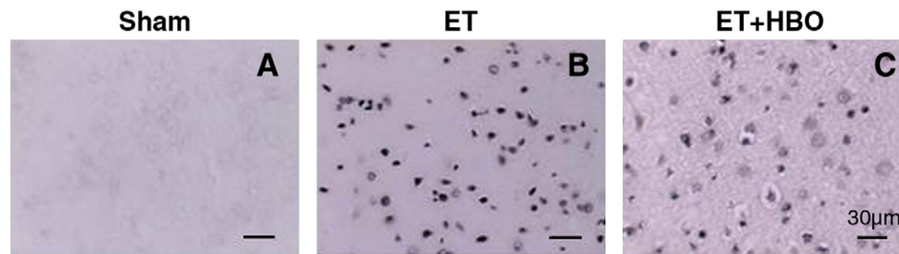

Fig. 5 – TUNEL staining of the cortex at 24 h after focal cerebral ischemia. Staining was performed on paraffin-embedded sections by using the in situ cell death detection kit. No TUNEL-positive cells were found in the cortex of sham animals (A). TUNEL-positive cells were observed in the ipsilateral cortex after cerebral ischemia (B). In the paragraph we can see some cells appeared pyknosis and have deep staining. The chromatin congregated on the cell border, like a half-moon. Some cells formed apoptotic body which have different shape and concentrated kytoplasm. After HBO treatment, trachychromatic cells were significantly reduced, and the cells with apoptosis feature also diminished (C). The scale bar represents 30 μm.

abolition of apoptotic cascades including HIF-1α and the enhanced expression of Bcl-2.

Using this endothelin-1-induced focal cerebral ischemia, we have an opportunity to study the effect of HBO on not only ischemic brain injury, as observed after ischemic stroke, but also ischemic brain injury originating from vascular contractions such as the cerebral vasospasm observed after subarachnoid hemorrhage (Isakov et al., 1985; Kohshi et al., 1993) or after interventional radiology, which may damage arterial endothelial cells (Zhang et al., 1998). In HBO-treated animals, enhanced Bcl-2 expression that may have led to a reduction in apoptotic cell death was observed. The mechanisms for Bcl-2 in reducing apoptosis have been established previously by others. These Bcl-2 mechanisms include the interference of the production of free radicals (Amstad et al., 2001), thereby inhibiting cell death; the prevention of Bax oligomerization in the mitochondrial outer membrane (Mikhailov et al., 2001) or the sequestration of a membrane bound protein X that is needed to drive Bax/Bak activation (Wilson-Annan et al., 2003; Hsu and Youle, 1997; Cory et al., 2003); the inhibition of cytosolic cytochrome c overexpression; and the prevention of caspase-3 activation (Zhao et al., 2003).

The level of Bax in the cytoplasm was not reduced by HBO. This was consistent with some previous reports (Vlodavsky et al., 2005; Zhou et al., 2000). During apoptosis, Bax interacts with the permeability transition pore proteins, adenosine nucleotide translocase or voltage-dependent anion channel and hastens the opening of the pore (Gogvadze et al., 2001) to release cytochrome c. Cytochrome c continues the apoptosis cascade by interacting with Apaf-1 and deoxyadenosine triphosphate, which forms the apoptosome and leads to the activation of caspase-9 (Sugawara et al., 2004).

Although we did not observe a reduction in Bax expression in animals treated with HBO, it did not mean that HBO had no influence on Bax. Because the regulation of Bax on cell apoptosis does not depend on its expression, but chiefly depends on its intracellular translocation (Wolter et al., 1997) and oligomerization (Vladimir Gogvadze et al., 2001). A previous report (Liu et al., 2006) confirmed HBO treatment can reduce the dimers of Bax, thereby alleviate neuronal apoptosis.

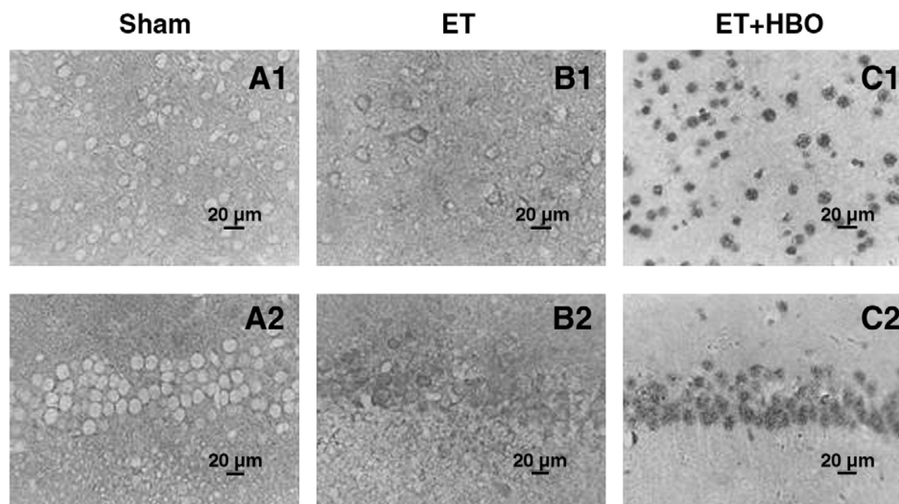

Fig. 6 – Positive immunochemical staining for Bcl-2 was observed both in the CA1 sector and cerebral cortex in ET+HBO group (C). Higher magnifications revealed that the Bcl-2 immunochemical signal showed both nuclear and cytoplasmic localization. The expression of Bcl-2 in the ET+HBO group was notable higher than in the ET group.

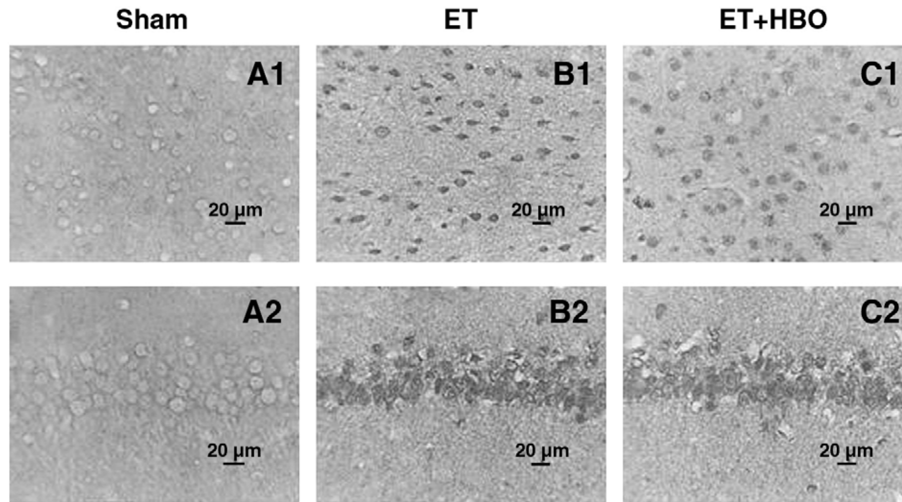

Fig. 7 – Relatively intense staining was observed for Bax (in the CA1 sector and cerebral cortex) after focal cerebral ischemia. The intensity of the staining in the ET group did not differ significantly from that seen in treated animals.

Unlike Bcl-2 or Bax, HIF-1α produces apoptosis by the activation of BNIP3 and NIX and by the stabilization of p53 (Ostrowski et al., 2005; Bruick, 2000; Greijer and van der Wall, 2004; Boyd, 1994). Therefore, a major neuroprotective effect of HBO may be associated with the inhibition of HIF-1α expression as shown in this study. A likely mechanism of HIF-1α reduction possibly involves an increased oxygen supply to hypoxic tissues in the hyperbaric environment because exposure to HBO is associated with a several-fold increase in the saturation values of the blood and peripheral tissues (Veltkamp et al., 2000). This unique observation indicates that HBO has the potential to prevent or reduce apoptosis through mechanisms other than the Bcl-2 family, especially in the absence of inhibitors of BNIP3 and NIX.

In conclusion, HBO reduced brain injury after endothelin-1-induced focal cerebral ischemia, probably by increasing the ratio of Bcl-2/Bax and reducing HIF-1α, factors related to ischemic cell change and apoptosis. These associated changes may underlie HBO neuroprotection, resulting in reduced infarct volumes and improved neurological function after focal cerebral ischemia.

4. Experimental procedures

4.1. *Experimental groups*

All surgical procedures were approved by the Ethics Committee for Animal Experimentation and were conducted according to the Guidelines for Animal Experimentation of our institutes.

One hundred twenty male Sprague–Dawley rats weighing 280 to 320 g were used in this study. The animals were housed in an air-conditioned room (at a temperature of 22–24 °C) with

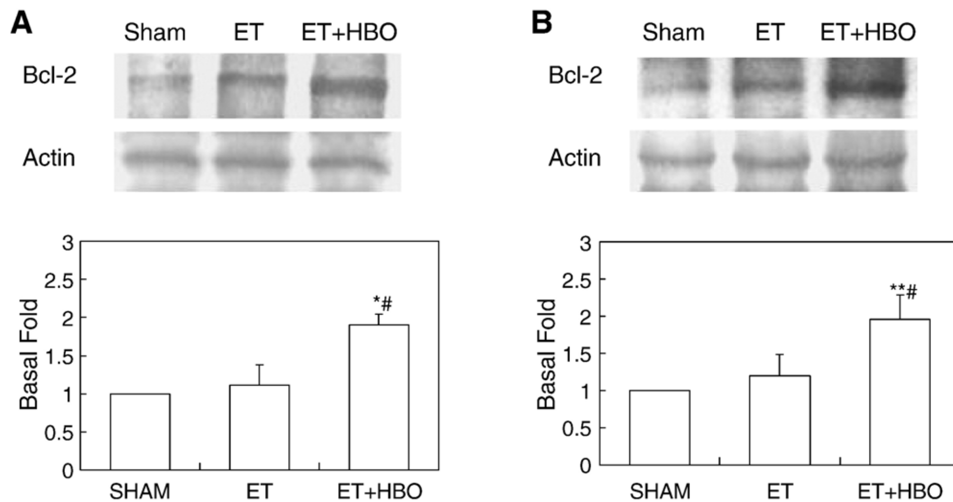

Fig. 8 – Western blot shows Bcl-2 protein bands and corresponding β-actin bands (top panels). HBO treatment can enhance the expression of Bcl-2 notably in the ipsilateral hippocampus (A) and cortex (B) tissues ($P<0.05$ vs. sham and ET groups, $n=4$ in each group), thereby provided the neuroprotection. In ET group, Bcl-2 protein expression rose insignificantly.

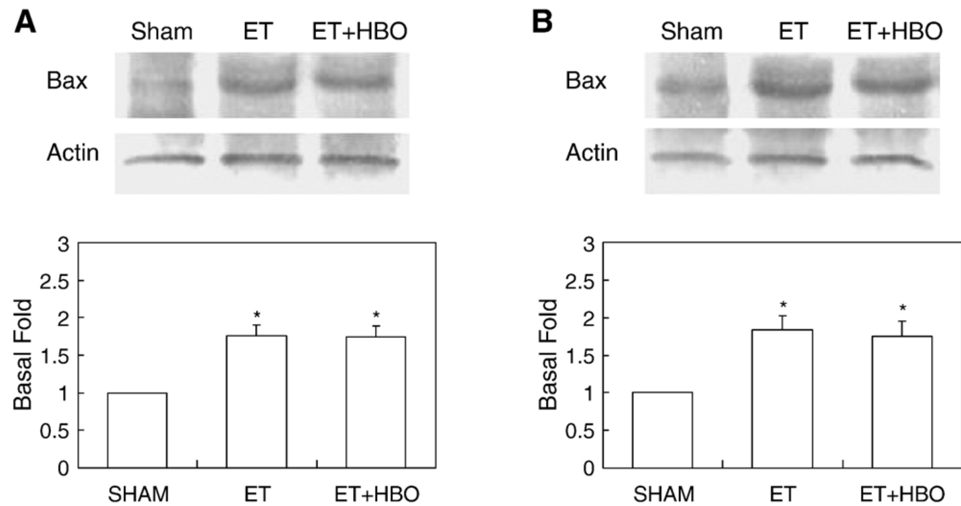

Fig. 9 – Bax expression was enhanced ($P<0.05$ vs. sham) in the ipsilateral hippocampus (A) and cerebral cortex (B) at 24 h after cerebral ischemia. Bax expression increased more than 1.5-fold at 24 h in the brain. HBO treatment seemed to have no significant effect on Bax ($P<0.05$ vs. sham, $P>0.05$ vs. ET, $n=4$ in each group). After HBO treatment, the concentration of Bax was still much higher than the control level, and the decrease was trifling. The same experiment was performed three times with similar results.

free access to food and water. The animals were randomly assigned to the following groups: sham, endothelin (ET) and ET+HBO. All brain samples were obtained at 24 h after surgery.

4.2. *Endothelin-1-induced focal cerebral ischemia rat model*

Briefly, the rats were anesthetized with an intraperitoneal injection of 2% pentobarbital sodium (40 mg/kg) and were allowed to breathe spontaneously. A supplemental anesthetic dose was added if necessary. Then the animals were placed in the stereotaxic frame in the prone position. The skin of the calotte was incised in midline, and the skull was exposed. Using a microdrill, a hole was made at the site localized at 0.9 mm anterior and 5.2 mm leftward to the bregma. When the microsample injector reached 8.7 mm in depth, 3 μl ET-1 was injected at a rate of 0.6 μl/min. After waiting for 5 min, the needle was withdrawn slowly and the wound was sutured. During surgery, a heating blanket was used to maintain the rectal temperature at 37.0 °C±0.5 °C. All rats were closely monitored for 1 h after surgery and then returned to their cages or transferred to an HBO chamber.

4.3. *HBO treatment*

Rats were placed into an HBO chamber (2.5 atmospheres absolute, ATA) for 2 h at 1 h after surgery. After ventilation for

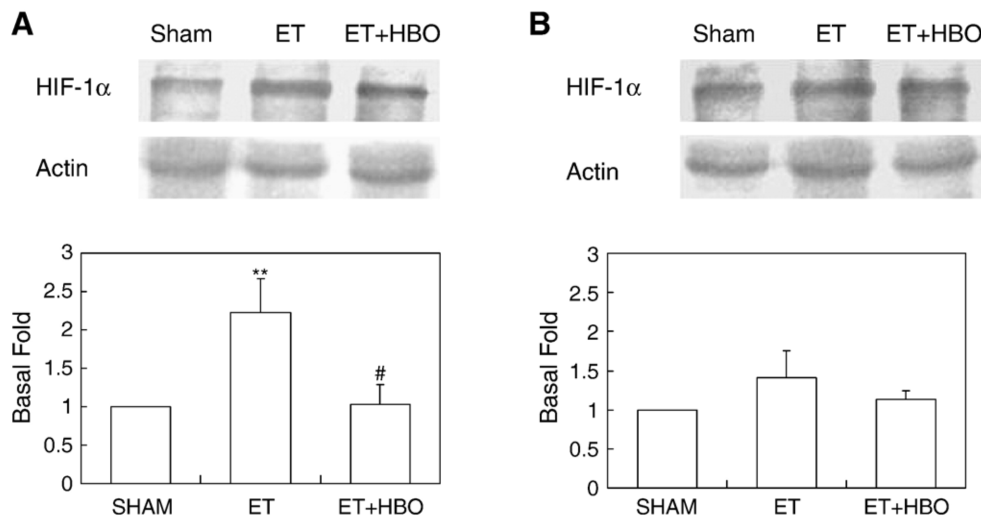

Fig. 10 – Western blot analysis shows an elevated expression of HIF-1α in the ipsilateral hippocampus (A) ($P<0.01$ vs. sham) but not in the cortex (B) ($P>0.05$ vs. sham) at 24 h after cerebral ischemia. HBO treatment abolished the elevation of HIF-1α in the hippocampus ($P<0.05$ vs. ET, $P>0.05$ vs. sham) without affecting the expression of HIF-1α in the cortex. β-Actin was used as control. Four to six animals were used in each group.

10 min with 100% oxygen, compression was started at a rate of 0.5 ATA/min. Upon reaching the desired pressure, the flow of oxygen was reduced to maintain constant pressure while allowing a flow out of the chamber. Accumulation of CO_2 was prevented by adding a small container with calcium carbonate crystals to the chamber. In order to avoid oxygen toxicity and its complications, a pause of 10 min was allowed after 1 h of HBO treatment. After HBO treatment, decompression was conducted for 10 min. Then the rats were returned to their cages until time of sacrifice.

4.4. *Neurological score*

The animals' behavior was neurologically assessed at 23 h after surgery. We used a neurological scoring system proposed by B. B. Johansson (Johansson, 1995) and Julio H. Garcia (Garcia et al., 1995) and modified it in our laboratory. The neurological testing was composed by four small tests: symmetry in the movement of four limbs, forepaw outstretching, beam-walking test and limb-placement test. All the tests were divide into some grades. The higher score represents the more serious symptom. Meanwhile, body weight was traced before surgery and before sacrifice since any differences can reveal the effect of HBO on feeding behavior.

4.5. *Brain water content*

At 24 h after surgery, the brains were harvested and quickly separated to the left and right hemispheres, cerebellum and the brain stem. Brain samples were weighed on a precise electronic balance and placed in the oven at a temperature of 105 °C for 48 h (Schwab et al., 1997). After 48 h, the samples were weighed again and the water content was calculated according to the following formula: [(wet weight − dry weight)/ wet weight] × 100%.

4.6. *TTC*

Infarct volume was determined by staining with 2,3,5-triphenyltetrazolium chloride (TTC) as described (Joshi et al., 2004). After decapitation, the brains were quickly isolated and placed in cold phosphate-buffered saline (PBS) for 10 min, and then coronal sections (2 mm thick) of the brain were cut. After incubation in 0.1% TTC at 37 °C for 30 min, the slices were fixed in 4% formaldehyde in PBS.

After 5 h, the sections were digitally imaged and analyzed by an image analysis system (ImageJ, a public domain image analysis program developed at the National Institutes of Health). The percentage of infarction (infarct ratio) was calculated by dividing the infarct volume by the total volume of the slices.

4.7. *Nissl staining*

Animals were killed in deep anesthesia by perfusion through the left ventricle with 200 mL of ice-cold 0.1 mol/L phosphate-buffered saline (PBS) followed by 400 mL of 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4). Brains were postfixed in the same fixative overnight. After fixation, brains were sectioned into three 4-mm-thick coronal slices. After dehydration in the graded ethanol and xylene, the brain sections were embedded in the paraffin. Coronal sections of the brain were cut at a thickness of 6 μm and prepared for Nissl staining.

We dewaxed and rehydrated tissue section according to standard protocols. Then sections were hydrated in 1% toluidine blue at 50 °C for 5 min. After rinsing with double distilled water they were dehydrated in increasing concentrations of ethanol and cleared in xylenes, then mounted with Permount, coverslipped and observed under a light microscope.

4.8. *TUNEL staining*

The In Situ Cell Death Detection Kit, AP (11684809910) (Roche) was employed to demonstrate apoptotic cells in ischemic brain tissue. The tissue sections were dewaxed and rehydrated according to standard protocols. Then coronal sections were digested with proteinase K (20 mg/mL; Bio-Light) in 0.01 mol/L PBS at room temperature for 15 min. To interrupt the digestion, the sections were dipped in 4% formaldehyde in 0.01 mol/L PBS for 5 min. After rinsing with PBS (5 min, 3 times), the sections were immersed in TUNEL reaction mixture for 1 h at 37 °C. In order to remove background staining caused by non-specific binding of anti-fluorescein-AP, the sections were treated with 1% BSA in PBS for 30 min at 37 °C. After rinsing with PBS (5 min, 3 times), the sections were colorated in the dark with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

4.9. *Immunohistochemistry*

Brain sections were hydrated with 0.01 mol/L PBS with Tween 20 and subsequently treated with 2% hydrogen peroxide for 30 min to prevent reaction with endogenous peroxidases. After incubating in 10% goat serum in a humidified chamber for 30 min at 37 °C, the sections were incubated with primary antibodies overnight at 4 °C. The following antibodies were used: monoclonal mouse anti-Bcl-2 (B9804) (Sigma), polyclonal rabbit anti-Bax (554106) (BD Biosciences) and polyclonal rabbit anti-HIF-1α (H206) (Santa Cruz Inc.), at a concentration 1:100 each. After rinsing with PBS (5 min, 3 times), ABC staining system (Sino-American Biotechnology Co.) was used.

Sections were incubated with secondary antibody (1:50) for 1 h at 37 °C and were placed in an avidin–peroxidase complex solution for 40 min at 37 °C. Peroxidase activity was revealed by dipping the sections for 5 min in a mixture containing DAB and H_2O_2 at room temperature. The application of PBS, instead of the primary antibody, on other sections of the same brain samples provided a negative control for each staining.

4.10. *Western blot*

We used nuclear extract buffer (40010) (Active Motif) to extract nuclear protein. Briefly, brain samples were homogenized in nuclear extract buffer according to the manufacturer's recommended protocol. After immersion in ice-cold water for 15 min, brain samples were centrifuged (centrifuge 5810R, Eppendorf AG) at 850×*g* for 10 min at 4 °C to pellet the nuclei. The supernatant was poured off and hypotonic buffer was added to the pellet. The resulting homogenate was incubated on ice for 15 min. After adding detergent, a spin at 14000×*g* for

1 min at 4 °C was performed. The supernatant was removed. Then the pellet was resuspended in complete lysis buffer and incubated for 30 min on ice to extract the nuclear fraction. The suspension was centrifuged at 14000×*g* for 10 min at 4 °C to remove nuclear debris.

Protein samples denatured at 100 °C for 4 min. Fifty micrograms of protein was loaded per lane and electrophoresed in 10% (for HIF-1α) or 12% (for Bcl-2 and Bax) dodecyl sulfate-polyacrylamide gel at 150V (Mini-PROTEAN III Electrophoresis System, BioRad) for 1 h. Proteins from gels were transferred at 180 mA for 105 min onto polyvinylidene difluoride (PVDF) membrane previously blocked with 5% dry nonfat milk in Tween–TBS (TBST). Membranes were incubated overnight at 4 °C with the same primary antibodies as for immunohistochemistry, diluted at 1:200 with 5% nonfat milk in TBST. Probing the same membranes with goat anti-β-actin antibody 1:1000 (Santa Cruz, Inc.) served as loading control. After washing with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies diluted at 1:2000 for 50 min at 37 °C. Protein bands were quantified by densitometry (Smartscape).

4.11. *Data analysis*

Data are expressed as mean ± SD. Statistical significance was verified by analysis of variance performed in one-way ANOVA followed by the Tukey test for multiple comparisons. Significance of differences in neurological scores was analyzed by Kruskal–Wallis one-way ANOVA followed by multiple comparison procedures by Dunn's method.

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