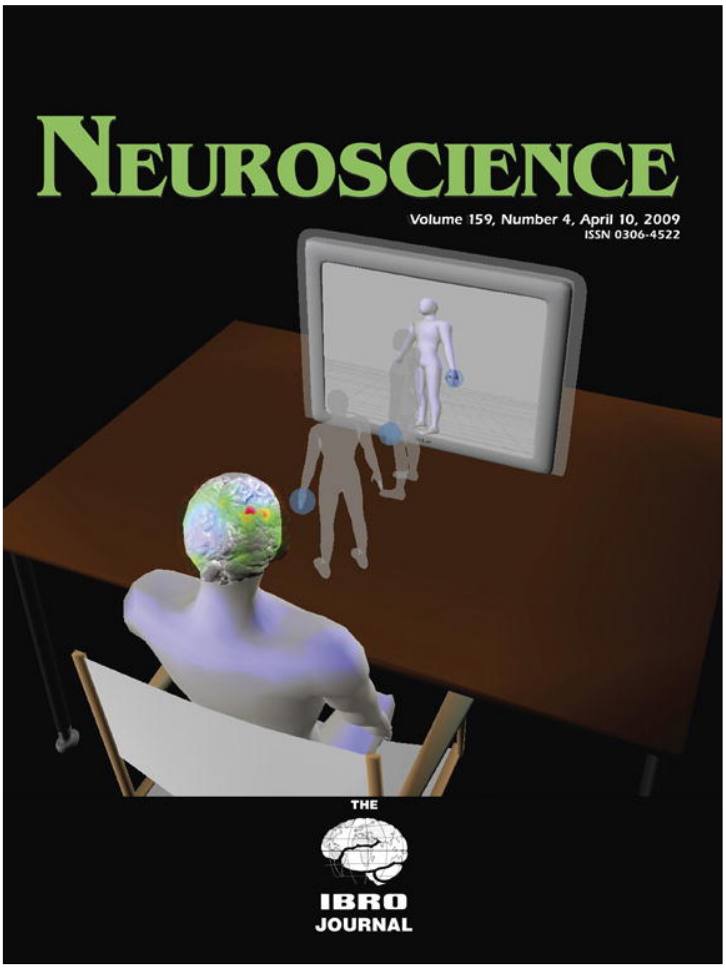

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HYPERBARIC OXYGEN PRECONDITIONING REDUCES ISCHEMIA–REPERFUSION INJURY BY INHIBITION OF APOPTOSIS VIA MITOCHONDRIAL PATHWAY IN RAT BRAIN

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Abstract—This study examined the hypothesis that apoptotic inhibition via mitochondrial pathway was involved in hyperbaric oxygen preconditioning (HBO-PC)–induced neuroprotection on ischemia–reperfusion injury in rat brain. Male Sprague–Dawley rats (250~280 g, *n*=144) were divided into control, middle cerebral artery occlusion (MCAO) for 90 min, and HBO-PC plus MCAO groups. HBO-PC was conducted four times by giving 100% oxygen at 2.5 atm absolute (ATA), for 1 h at 12 h intervals for 2 days. At 24 h after the last HBO-PC, MCAO was performed and at 24 h after MCAO, neurological function, brain water content, infarct volume, and cell death were evaluated. Enzymatic activity of capase-3 and −9, and expression of cytochrome *c*, Bcl-2 and Bax proteins were performed in the samples from hippocampus, ischemic penumbra and core of the brain cortex, respectively. HBO-PC reduced brain edema, decreased infarction volume, and improved neurological recovery. HBO-PC reduced cytoplasm cytochrome c levels, decreased caspase enzyme activity, upregulated the ratio of Bcl-2 and Bax expression, and abated the apoptosis of ischemic tissue. HBO-PC protects brain tissues from ischemia–reperfusion injury by suppressing mitochondrial apoptotic pathways.

Key words: hyperbaric oxygenation, prevention, middle cerebral artery occlusion, apoptosis, ischemic penumbra.

Hypoxic or ischemic preconditioning may have clinical potentials for neuroprotection for neurosurgery patients who undergo temporary clipping of major intracranial vessels during aneurismal or cerebral bypass surgery procedures. The neuroprotective effect of preconditioning has been established in animal models (Rehman et al., 2008; Yu et al., 2008; Shimoda et al., 2007) and observed in clinical cases after multiple transient ischemic attacks (Sitzer et al., 2004). However, the safety concerns and practical feasibility have limited the application of preconditioning in practice.

Hyperbaric oxygen (HBO) has been used for multiple neurological diseases (Lou et al., 2004; Al-Waili et al., 2005; Rosenthal et al., 2003; Ostrowski et al., 2006) and proved a safe treatment modality in all age and gender groups, including neonates (Calvert et al., 2004) and pregnant mothers (Xiao et al., 2006). Hyperbaric oxygen preconditioning (HBO-PC) has been reported to increase ischemic tolerance against neuronal injury in animals (Wada et al., 2001; Nie et al., 2006; Speit et al., 2000; Dong et al., 2002) Recently, we have observed that a single dose of HBO-PC reduced hypoxic–ischemic brain injury in neonatal rats (Freiberger et al., 2006), However, the dosage, the timing of application, and the mechanisms of HBO-PC remain to be determined (Wada et al., 2001; Zhang et al., 2004). Therefore, we examined the neuroprotective effect and mechanisms of a short term HBO-PC in an established middle cerebral artery occlusion (MCAO) rat model.

EXPERIMENTAL PROCEDURES

Experimental groups

The Experimentation Ethics Committee of the Second Military Medical University in Shanghai, China approved the animal protocols of this study. All methods and animal procedures were met or exceeded all federal guidelines for the humane use of animals in research. All efforts were made to minimize the number of animals used and their suffering. A total of 144 male Sprague–Dawley rats weighing 250~280 g (Slaccas, Shanghai, China) were used. They were housed at a temperature of 22–24 °C and 12 h light/dark cycle controlled environment with free access to food and water prior to and following surgery. Rats were randomly assigned to one of the following three groups: control group (*n*=24), MCAO group (*n*=60), and HBO-PC plus MCAO group (*n*=60). All animals were sacrificed at 24 h after MCAO.

HBO-PC

HBO-PC was administered by using 100% oxygen at 2.5 atmosphere absolute for 1 h at 12 h intervals four times in 2 days. The last HBO-PC was performed at 24 h before MCAO. Compression was performed at 1 kg/cm^2/min and decompression was performed at 0.2 kg/cm^2/min. None of the animals had seizures during or after HBO-PC. Chamber temperature was maintained between 22 and 25 °C. Accumulation of CO_2 was prevented by using a small container with calcium carbonate crystals. To minimize the effects of diurnal variation, all exposures were started at 8:00 AM. MCAO rats were placed in the same rodent chamber for 1 h at 12 h interval for 2 days in room air.

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Abbreviations: CCA, common carotid artery; ECA, external carotid artery; HBO, hyperbaric oxygen; HBO-PC, hyperbaric oxygen preconditioning; ICA, internal carotid artery; MCAO, middle cerebral artery occlusion; PBS, phosphate-buffered saline; TTC, 5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

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MCAO procedures

MCAO was produced by the filament model initially reported by Zea-Longa et al. (1989) with some modifications. Briefly, the rats were anesthetized with an i.p. injection of 2% pentobarbital sodium (40 mg/kg) and were allowed to breathe spontaneously. A supplemental anesthetic dose was added if necessary. Via a midline neck incision, the submandibular glands were separated to allow access to the right carotid artery. The common carotid artery (CCA), the external carotid artery (ECA), and the internal carotid artery (ICA) were isolated from connective tissues. The ECA and the proximal end of the CCA stump were cut and a suture loop was put around the distal end of the CCA. A microvascular clip was temporarily put on the ICA. A 0.24 mm diameter carbon fishing-line with a pretreated rounded tip was introduced via the CCA stump into the ICA. After removal of the clip, the filament was advanced further in the ICA until a resistance was felt at approximately 18.0±0.5 mm from the carotid bifurcation. The filament was fastened by tightening the loop around the distal CCA stump and the neck incision was closed. During surgery, a heating blanket was used to maintain the rectal temperature at 37.0±0.5 °C. After 90 min MCAO, blood flow was restored by the withdrawal of the intraluminal suture and then the awaking rats were returned to their cages.

Neurobehavioral functional scoring

The neurological scoring systems proposed by Dean et al. (2003) and Ohlsson et al. (1995) were adopted with modifications as shown in Table 1. All neurological evaluations were done blinded by a researcher without knowledge of the animal groups. The neurological testing was composed by thee sets: Zea-Longa score, beam-walking test, and prehensile traction test. The higher score represents severe deficits.

Table 1. Neurological deficit score for rats

Zea-longa score	
0	The rat has no neurological defect
1	The rat is unable to extend affected forward limb
2	The rat circles while walking
3	The rat tumbles to its side because of hemiplegia while walking
4	The rat is unable to walk and unconsciousness is present
5	The rat is dead
Beam-walking test	
0	The rat crosses the beam with no foot slips
1	The rat crosses the beam with a few foot slips
2	The rat traverses the beam with more than 50% foot slips
3	The rat can traverse the beam, but the affected hind limb does not aid in forward locomotion
4	The rat falls down while walking
5	The rat is unable to traverse the beam but remains sitting across the beam
6	The rat falls down from the beam
Prehensile traction test	
0	The rat hangs on 5 seconds and brings rear limb up to rope
1	The rat hangs on 5 seconds, no third limb up to rope
2	The rat hangs on 3 to 4 seconds
3	The rat hangs on 0 to 2 seconds

Minimum score: 0, namely healthy rats; maximum score: 14, namely death.

TTC staining

Infarct volume was determined by staining with 2,3,5-triphenyltetrazolium chloride (TTC). Briefly, the brains were quickly removed and placed at −20 °C for 15 min, and then cut into five 2 mm coronal slices starting at 1 mm from the frontal pole. After incubation in 1% TTC in 0.2 mol/L phosphate-buffered saline (PBS) at 37 °C for 30 min, the slices were fixed in 4% paraformaldehyde in 0.1 mol/L PBS. After 24 h, the sections were digitally photographed and infarction volumes were analyzed using image analysis system (Image J software (http://www.quickvol.com), 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.

Brain water content

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 an oven at 100 °C for 48 h. After 48 h, the samples were weighed again and the water content was calculated according to the following formula:

$$[(\text{wet weight}-\text{dry weight})/\text{wet weight}]\times 100\%.$$

In situ labeling of DNA fragmentation

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was performed on paraffin-embedded sections according to the manufacturer's instructions (Roche Molecular Biochemicals, Inc., Mannheim, Germany). The sections were dewaxed and rehydrated according to standard protocols, pretreated with proteinase K (20 μg/mL in 0.01 mol/L PBS) for 15 min at room temperature. The slides were rinsed three times with PBS before they were incubated in TUNEL reaction mixture for 1 h at 37 °C. We dried the area around the sample and added converter-AP to samples for 1 h at 37 °C. After rinsing with PBS (5 min, three times), sections were stained in the dark with Nitroblue Tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). For negative controls, the sections were incubated without terminal deoxynucleotidyl transferase. There were two distinct patterns of TUNEL staining. Some cells were densely labeled and showed clear apoptotic characteristics. Other cells were weakly labeled and considered to be necrotic cells. Only the densely labeled cells were counted as TUNEL positive cells. The ischemic core and penumbra of the cerebral cortex and CA1 of hippocampus were photographed (Leica, Germany) in each section. The data were represented as the number of cells per mm^2.

Ischemic core and penumbra dissections

Ischemic core and penumbra were dissected according to well-established protocols in rodent models of unilateral proximal MCAO (Ashwal et al., 1998; Lei et al., 2004). Briefly, each hemisphere was cut longitudinally, from dorsal to ventral at 1.5 mm from the midline to exclude medial brain structures that were supplied primarily by the anterior cerebral artery. A transverse diagonal incision at approximately the 2 o'clock position separated the core from the penumbra.

Caspase activity assay

The activity of caspase-3 and −9 was measured with caspase-3 and caspase-9/CPP32 fluorometric Assay Kit (BioVision, Inc.,

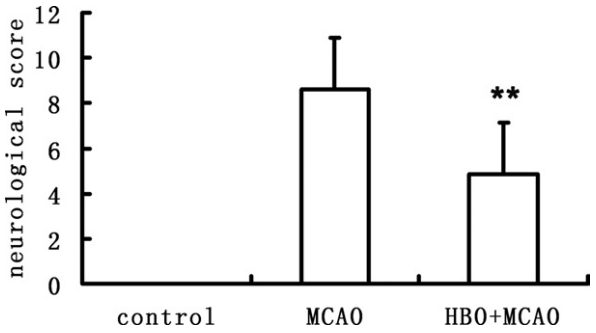

Fig. 1. Beneficial effect of HBO-PC on neurobehavioral recovery after MCAO. Neurological scores were significantly lower (better) in HBO-PC animals as compared with MCAO group (** $P<0.01$, Student's *t*-test). Control $n=24$, MCAO $n=47$, HBO-PC $n=55$.

USA). Briefly, brain samples from the ischemic core and penumbra or hippocampus were homogenized on the ice in ice-cold cell lysis buffer contained in the kit and centrifuged at 12,000 rpm for 15 min at 4 °C, and then the supernatants were assayed for protein concentration by Enhanced BCA Protein Assay Kit. The treated samples were liquated and stored at −80 °C until use. Equal amounts of the protein samples were incubated in a 96-well plate with 50 μl of 2× Reaction buffer. Reactions were initiated by adding 5 μl of the 1 mM DEVD-AFC substrate. After incubation in the dark at 37 °C for 1 h, the plate was read in a fluorometer (Flexstation, Molecular Devices, USA) equipped with a 400-nm excitation filter and 505-nm emission filter. No tissue samples were added for negative controls. The results were expressed as a relative number (fluorescence intensity of sample/fluorescence intensity of negative controls).

Western blot

The sample proteins were extracted as described above. Equal amounts of the protein samples were loaded per lane and electrophoresed in 12% dodecylsulfate–polyacrylamide gel at 120 V (Mini-Protean III Electrophoresis System, Bio-Rad, USA) for 1 h. Proteins from gels were transferred at 110 mA for 100 min (for Bcl-2 and Bax) or at 70 V for 40 min (for cytochrome *c*) onto a nitrocellulose filter membrane. Membranes were incubated overnight at 4 °C with rabbit anti-Bcl-2 polyclonal antibody (1:1000 dilution, Chemicon International, Inc., USA), rabbit anti-Bax polyclonal antibody (1:1000 dilution, Stressgen Bioreagents, Corp., USA), cytochrome *c* antibody (1:1000 dilution, Cell Signaling Technology, Inc., USA), goat polyclonal β-actin antibody (1:1000 dilution, Santa Cruz, Inc., USA), respectively, and then with horseradish peroxidase–conjugated secondary antibodies diluted at 1:1000 for 1 h at room temperature. The positive bands were revealed using enhanced chemiluminescence detection reagents (Pierce, USA) and autoradiography film. Protein bands were quantified by densitometry (Smartscape, Furi Co. Ltd., Shanghai, China).

Statistical analysis

Data are expressed as mean±SD. Statistical analyses were made using one-way analysis of variance (ANOVA) with Tukey's post hoc inter-group comparisons. A value of $P<0.05$ was considered to denote statistical significance.

RESULTS

Neurological score

A significant increase in neurological score (8.62±2.29) was found in rats at 24 h after MCAO (Fig. 1). HBO-PC alleviated the neurological injury to 4.87±2.29 ($P<0.01$ vs. MCAO group). Animals in the control group showed no neurobehavioral functional deficit.

TTC staining

Fig. 2A shows representative photographs derived from postmortem TTC staining sections at 24 h. The infarct ratio was 0.21±0.04 in the MCAO group and 0.14±0.03 in the HBO-PC group ($P<0.05$, Fig. 2B).

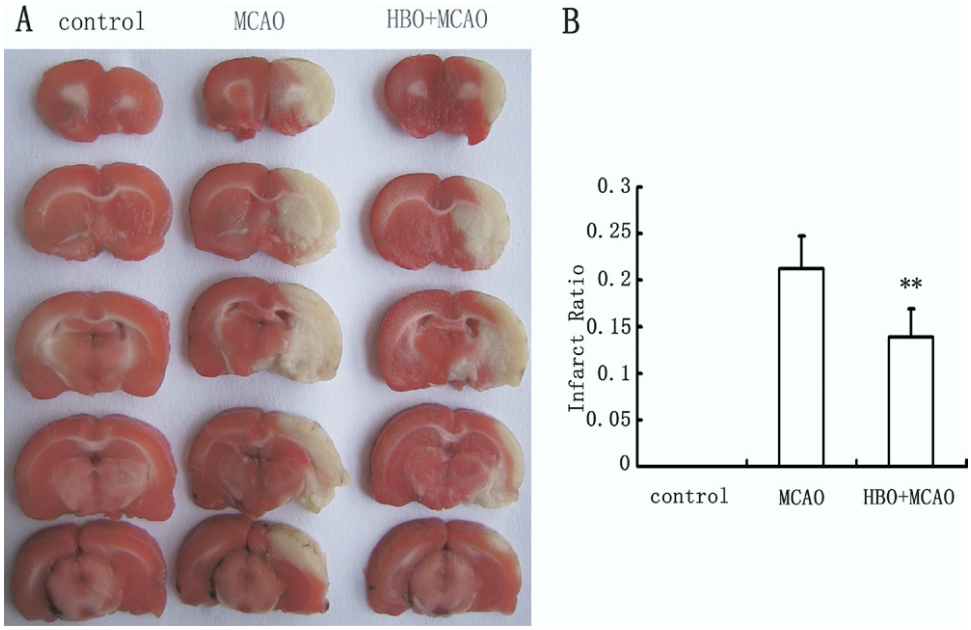

Fig. 2. Effect of HBO-PC on TTC staining at 24 h after MCAO. The infarction volume in the HBO group is significantly decreased as compared with MCAO group (* $P<0.05$, Student's *t*-test). Control $n=4$, MCAO $n=7$, HBO-PC $n=9$.

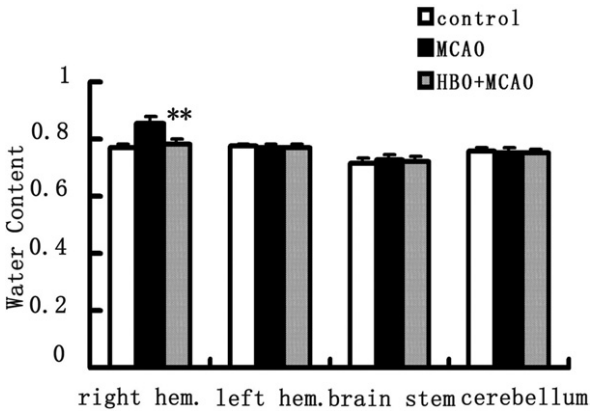

Fig. 3. Effect of HBO-PC on brain water content. HBO-PC reduced significantly brain water content in the right hemisphere (** $P<0.01$ vs. MCAO group). Control $n=4$, MCAO $n=9$, HBO-PC $n=10$.

Brain water content

The right hemisphere (infarct side) mean brain water contents were 0.77±0.01, 0.86±0.02, and 0.78±0.01, in the control, MCAO, and HBO-PC groups, respectively (Fig. 3). The water content of the MCAO group was significantly higher than the control ($P<0.01$) and HBO-PC groups ($P<0.01$). No difference in brain water content was found between the control and HBO-PC groups ($P>0.05$).

TUNEL staining

There were more apoptotic neurons in the core (Fig.4aB, C) of HBO-PC rats than MCAO rats (80.43±15.47 vs. 59.23±14.35, $P>0.05$). HBO-PC reduced the number of TUNEL positive cells in the penumbra (84.57±18.75 vs. 145.82±17.02, $P<0.01$, Fig. 4aD, E) and CA1 sector (67.58±18.30 vs. 163.68±20.35, $P<0.01$, Fig. 4bB, C) when compared with MCAO group.

Activities of caspase-3 and −9

At 24 h after MCAO the relative activities of caspase-3 and −9 in the ischemic core were similar for MCAO and HBO-PC, respectively (Fig. 5A, C, $P>0.05$). HBO-PC reduced caspase-3 and −9 activities in the penumbra (Fig. 5A, C, $P<0.05$) and in the hippocampus (Fig. 5B, D, $P<0.05$).

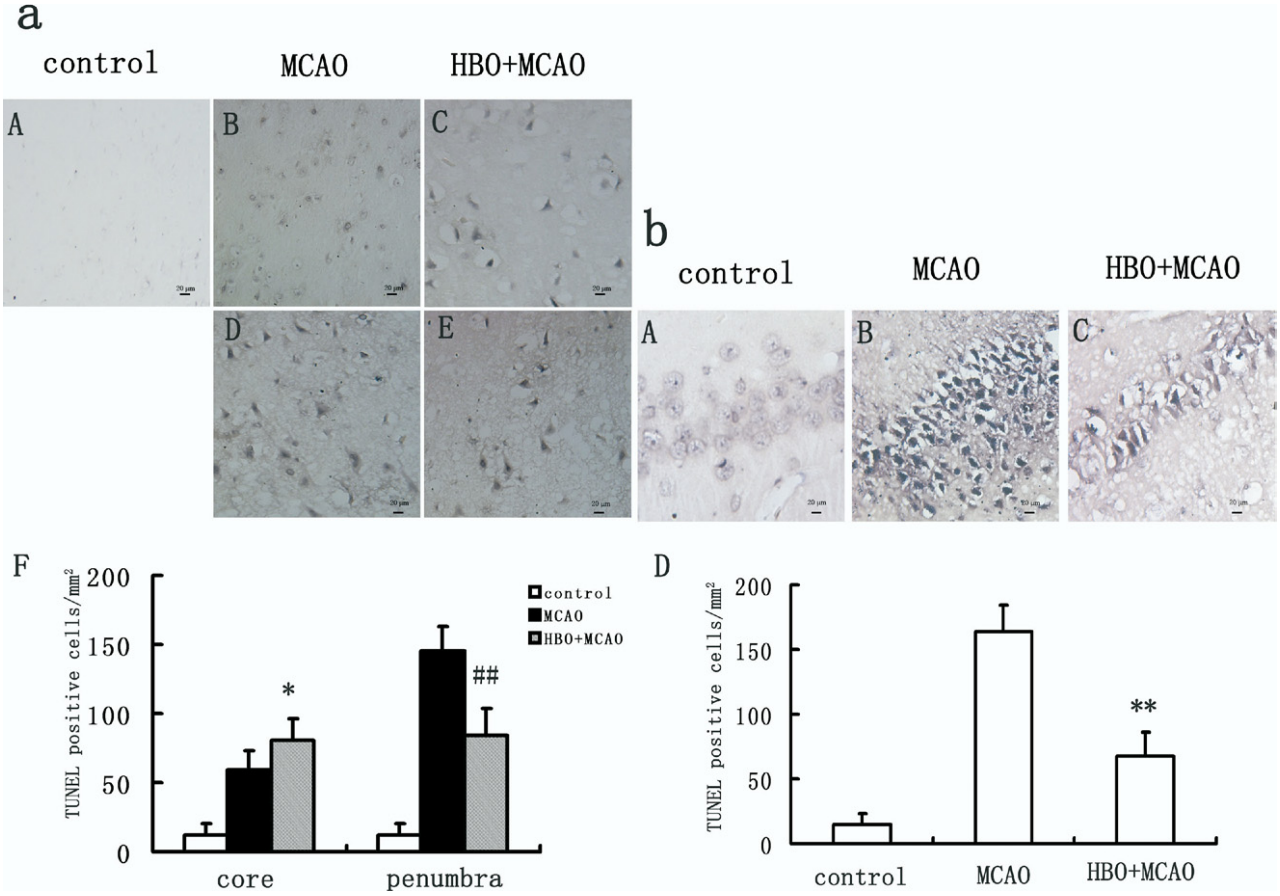

Fig. 4. (a) TUNEL staining of the core (B, C) and penumbra (D, E) of the cortex. (b) TUNEL staining of CA1 sector (A–C). Only the densely stained cells were counted as TUNEL positive cells. There were no TUNEL positive cells in the cortex and CA1 sector in control animals (a A, b A). TUNEL positive cells were observed in the ipsilateral cortex and hippocampus after MCAO. The chromatin congregated on the cell border and some cells formed apoptotic bodies which have different shapes and concentrated cytoplasm with characteristic triangles. The penumbra ($^{\#\#}$ $P<0.01$ vs. MCAO group) and CA1 sector (** $P<0.01$ vs. MCAO group) of the HBO-PC group had fewer apoptotic cells; however, the core had more than the MCAO group (* $P>0.05$). $n=4$ In the control group, $n=8$ in the MCAO group, $n=10$ in the HBO-PC group.

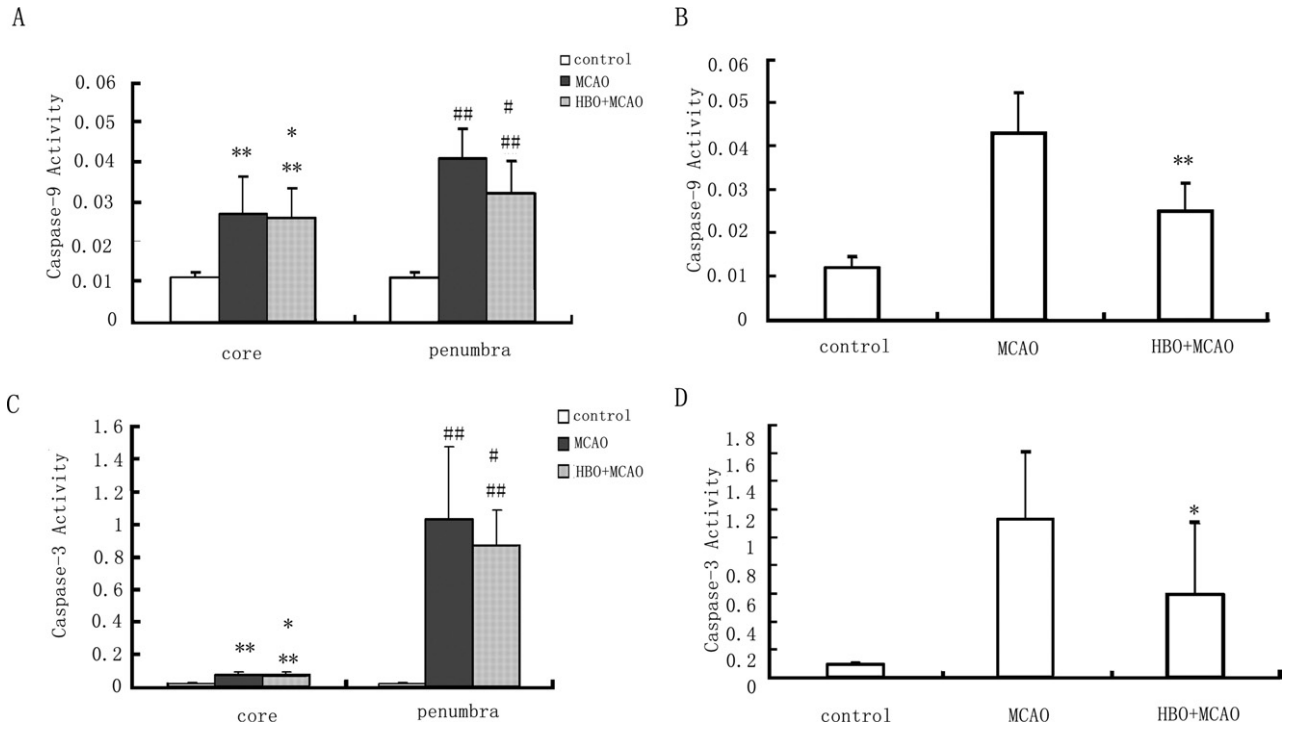

Fig. 5. The enzymatic activity of caspase-9 (A, B) and caspase-3 (C, D). The enzymatic activity of caspase-3 or caspase-9 was reduced in the ischemic core (* $P>0.05$, HBO-PC group vs. MCAO group; ** $P<0.01$ HBO-PC group vs. control group, MCAO group vs. control group) than in the penumbra ($^{\#}$ $P<0.05$ HBO-PC group vs. MCAO group; $^{\#\#}$ $P<0.01$ HBO-PC group vs. control group, MCAO group vs. control group). The caspase activity is more pronounced in the hippocampus in MCAO group than the HBO-PC group (* $P<0.05$, ** $P<0.01$). $n=4$ In the control group, $n=7$ in the MCAO group, $n=9$ in the HBO-PC group.

Western blot of cytochrome c, Bcl-2 and Bax

Western blot analysis of brain samples at 24 h after MCAO showed similar levels of cytoplasmic cytochrome *c* in the ischemic core (Fig. 6A) in MCAO and HBO-PC groups ($P>0.05$). HBO-PC reduced cytochrome *c* in the penumbra and in the hippocampus (Fig. 6A) ($P<0.05$). HBO-PC enhanced Bcl-2 levels in the ischemic core, penumbra and hippocampus (Fig. 6B). The level of Bax remains constant in all groups after MCAO (Fig. 6C).

DISCUSSION

HBO-PC was neuroprotective in previous studies but a 10 days protocol was used to give HBO every other day for five treatments (Wada et al., 2001) which is difficult to apply in clinical practice. In this study we observed that a condensed application of HBO-PC in 2 days produced a marked brain protection in an established focal cerebral ischemia model. Four applications of HBO-PC in 2 days suppressed brain edema, decreased cerebral infarction and improved neurological function at 24 h after MCAO. These outcomes that cytochrome c release was suppressed, caspase-3 and -9 activity reduced, and apoptotic cell decreased were observed at cellular level and might be responsible for neuroprotection. These results are consistent with previous HBO-PC in other animal models that required 5 to 10 days' preconditioning (Xiong et al., 2000; Dean et al., 2003).

We chose an MCAO rat model for this study because similar major cerebral arteries may be temporarily clipped during aneurismal surgery or cerebral bypass. In addition, an MCAO rat model provides a clear ischemic core and penumbra (Kaufmann et al., 1999) and the neuronal injury in the ischemic penumbra is reversible and may be salvaged by proper interventions. Temporary clipping of major cerebral arteries during the abovementioned neurosurgery procedures might result in a penumbra type of neuronal injury. Indeed, we have observed neuroprotective effect in the penumbra by HBO-PC in this study. Therefore, this 2-day HBO-PC protocol may have clinical potentials for patients undergoing major aneurismal surgery, spinal cord surgery or even for cardiac bypass or transplantation procedures.

One of the major cell death patterns in the ischemic penumbra is apoptosis especially mediated by mitochondrial pathways (Graham et al., 2001). In this study, we have observed a higher number of TUNEL-positive cells in the ischemic core in the HBO-PC group than in the MCAO group. This might indicate that more cells survived from necrosis in the ischemic core region after HBO-PC. However, it is the penumbra where HBO-PC exerted major neuroprotection. The penumbral cortex was mostly spared from infarction in the HBO-PC group. Since the blood supply of the hippocampus comes from many arteries, especially the anterior choroidal artery and posterior cerebral artery, the hippocampus represents a penumbra type of injury in this MCAO rat model. Similar neuroprotection by HBO-PC was observed in the hippocampus in TUNEL studies. Furthermore, not only the numbers of TUNEL positive cells decreased in the penumbra in HBO-PC

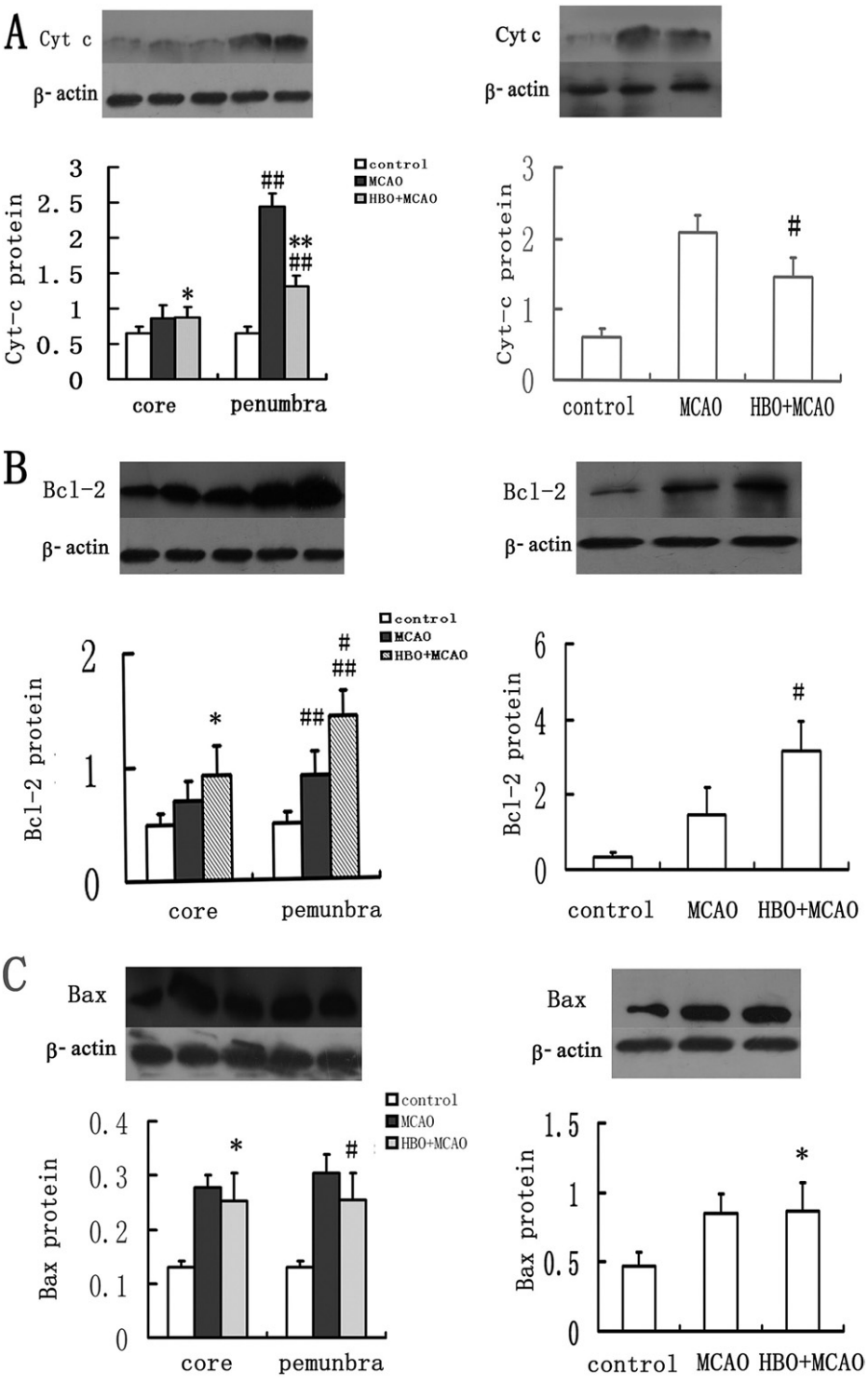

Fig. 6. Western blot analysis of cytochrome *c* (A), Bcl-2 (B) and Bax (C) protein. (A) Pretreatment with HBO decreased the expression of cytoplasmic Cytochrome *c* in the penumbra (** $P<0.01$ vs. MCAO group; ## $P<0.01$ vs. control group) and hippocampus (# $P<0.05$ vs. MCAO group), but not the ischemic core (* $P>0.05$ vs. MCAO group). (B) Bcl-2 increased in the ischemic penumbra and hippocampus in the HBO-PC group (# $P<0.05$, vs. MCAO group; ## $P<0.01$ vs. control group). but not the ischemic core (* $P>0.05$ vs. MCAO group). (C) Bax expression was enhanced after MCAO compared with control rats but no difference was found between MCAO and HBO-PC groups (* $P>0.05$, # $P>0.05$ vs. MCAO group). $n=4$ In the control group, $n=9$ in the MCAO group, $n=9$ in the HBO-PC group.

group, but also the molecular events related to mitochondrial apoptotic pathways that were altered by HBO-PC.

During the process of apoptosis, the release of cytochrome *c* from the mitochondria into the cytosol has been

proposed as a trigger for the development of neuronal apoptosis (Green et al., 1998). The cytochrome *c* release can activate caspase-3 by activating apoptotic protease activating factor 1 and complexes of procaspase-9. Caspase-3 then causes degradation of cytoskeleton, DNA fragmentation, and eventually cell death. Our observation made in this study indicates that HBO-PC reduced cytochrome *c* release, and markedly inhibited caspase activity in the penumbra and hippocampus. Two upstream apoptosis factors Bcl-2 and Bax in the mitochondrial pathways were examined in this study. Upregulation of Bcl-2 or downregulation of Bax (Adams et al., 2007) has been found to attenuate apoptotic cell death previously. We have observed in this study that HBO-PC enhanced the Bcl-2 but did not change Bax expression in the penumbra and hippocampus, increasing Bcl-2/Bax ratio.

CONCLUSION

In conclusion, 2 days' HBO-PC decreased neuronal injury in the ischemic penumbra after MCAO possibly mediated by reduction of apoptosis. This short term HBO-PC may be clinically feasible for major intracranial surgery for neuroprotection.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.neuroscience.2009.01.011.

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