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## Research Report

# Hyperbaric oxygen preconditioning induces tolerance against brain ischemia–reperfusion injury by upregulation of antioxidant enzymes in rats

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## ABSTRACT

The present study examined the hypothesis that cerebral ischemic tolerance induced by hyperbaric oxygen preconditioning (HBO-PC) is associated with an increase of antioxidant enzyme activity. Male Sprague–Dawley rats (250–280 g,  $n=74$ ) were divided into sham, middle cerebral artery occlusion (MCAO) for 90 min, and MCAO plus HBO-PC groups. HBO-PC was conducted four times by given 100% oxygen at 2.5 atmosphere absolute (ATA), for 1 h at every 12 h interval for 2 days. At 24 h after the last HBO-PC, MCAO was performed and at 24 h after MCAO, neurological function and Nissl Staining were performed to evaluate the effect of HBO-PC. Malondialdehyde (MDA) content, activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) sampled from the hippocampus, ischemic penumbra or core of cortex were measured. HBO-PC decreased mortality rate, improved neurological recovery, lessened neuronal injury, reduced the level of MDA and increased the antioxidant activity of CAT and SOD. These observations demonstrated that an upregulation of the antioxidant enzyme activity by HBO preconditioning plays an important role in the generation of tolerance against brain ischemia–reperfusion injury.

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## 1. Introduction

Cerebral ischemia–reperfusion injury is associated with the production of reactive oxygen species (ROS) (Girn et al., 2007). Hypoxic or ischemic preconditioning episode generates ROS which however could induce ischemic tolerance (Gidday, 2006). Similarly, hyperbaric oxygen preconditioning (HBO-PC) also produces a non-lethal level of ROS which is believed

involved in the tolerance against subsequent severe ischemic attacks (Yogarathnam et al., 2006). Several recent studies reported that HBO-PC produced ischemic tolerance in gerbil hippocampus (Wada et al., 2001) and in spinal cord injury rabbit models (Nie et al., 2006). Our previous study demonstrated that HBO-PC produced ischemic tolerance may be mediated by hypoxia-inducible factor 1- $\alpha$  and erythropoietin (Gu et al., 2008). However, the mechanisms of HBO-PC in focal

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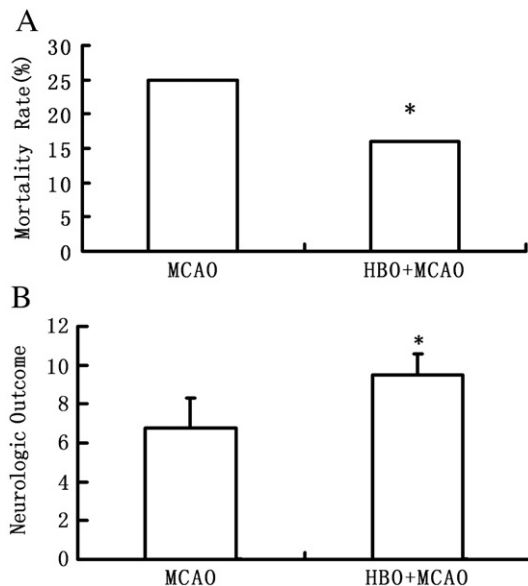
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**Table 1 – Physiologic variables during pre- and post-ischemic state (n=21 in each group)**

	MAP (kPa)	HR (beats/min)	Arterial blood gases			Glucose (mmol/L)	Temperature (°C)
			pH	PaO <sub>2</sub> (kPa)	PaCO <sub>2</sub> (kPa)		
<i>Preischemia</i>							
Sham	11.05±1.21	352±20	7.35±0.02	15.2±0.8	5.0±1.1	4.5±1.2	38.4±0.7
MCAO	11.51±1.53	360±18	7.40±0.08	16.1±0.9	5.2±1.2	5.0±1.1	37.8±0.5
HBO+MCAO	11.94±1.51	358±15	7.40±0.07	15.8±0.9	5.2±0.9	4.7±1.2	38.2±0.4
<i>10 min after the beginning of ischemia</i>							
Sham	11.85±1.2	300±15	7.37±0.06	13.3±1.1	5.9±1.2	4.7±1.1	38.4±0.3
MCAO	11.67±1.24	290±13	7.30±0.05	12.1±1.5	6.2±1.4	4.6±1.1	38.2±0.4
HBO+MCAO	12.23±1.19	295±13	7.32±0.04	12.7±1.2	6.4±1.2	4.8±1.4	38.5±0.2
<i>10 min after the beginning of reperfusion</i>							
Sham	11.76±1.29	339±14	7.30±0.05	12.7±0.7	6.0±1.3	4.9±1.4	38.3±0.6
MCAO	12.59±1.36	345±20	7.25±0.08	11.9±1.2	6.4±1.1	5.4±1.2	37.7±0.5
HBO+MCAO	12.55±1.18	340±16	7.27±0.06	12.01±1.1	6.5±1.3	5.5±1.2	38.4±0.5

cerebral ischemia remain to be explored. We hypothesize that HBO-PC may be related to enhanced antioxidant activity of brain tissues triggered by ROS.

In the present study, we used an established middle cerebral artery occlusion (MCAO) model described initially by Zea-Longa et al. (1989) with some improvement. ROS production was evaluated by measuring malondialdehyde (MDA) content and endogenous antioxidative enzymes were examined including activity of catalase (CAT), superoxide dismutase (SOD) and Glutathione peroxidase (GSH-px). The neuroprotective effect of HBO-PC was investigated by morphology and neurological functional recovery.



**Fig. 1 – (A)** The bar graph shows the mortality rate of rats at 24 h after MCAO. The mortality rate was decreased in the HBO-PC group (4/25 vs. 7/28; \* $P < 0.05$  vs. MCAO group). **(B)** Beneficial effect of HBO preconditioning on neurobehavioral recovery after transient focal cerebral ischemia. Neurological scores were significantly higher in HBO preconditioned animals as compared with MCAO group after. \* $P < 0.05$  vs. MCAO group.  $n = 21$  in each group.

## 2. Results

### 2.1. Physiologic variables

The physiological parameters for the three groups are provided in Table 1. There was no significant difference in mean arterial blood pressure (MAP), heart rate (HR), arterial pH, arterial oxygen tension, arterial carbon dioxide tension, blood glucose concentration, and pericranial temperature in all groups.

### 2.2. Mortality rate and neurologic outcome

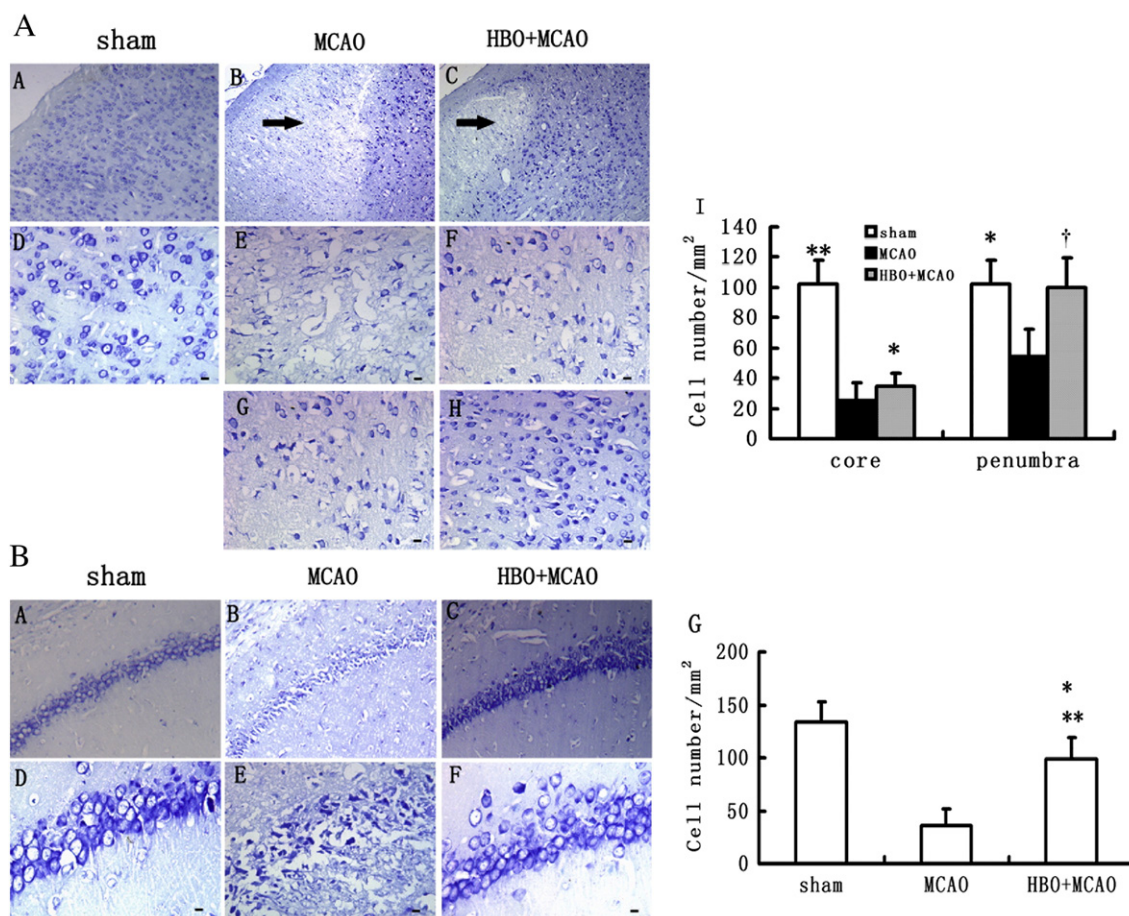
HBO-PC decreased mortality from 25% (7/28) in MCAO alone group to 16% (4/25) (Fig. 1A,  $P < 0.05$ ). Animals subjected to sham surgery showed no neurobehavioral functional deficit. HBO-PC animals showed better neurological functional recovery at 24 h when compared with MCAO alone animals ( $9.5 \pm 1.1$  vs.  $6.8 \pm 1.5$ , Fig. 1B,  $P < 0.05$ ).

### 2.3. Nissl staining

Fig. 2 shows Nissl staining of the cortex (a) and CA1 (b) of the hippocampus at 24 h after MCAO. There are more intact neurons in the penumbra than in the core at 24 h in MCAO and HBO-PC+MCAO groups. The intact neuron numbers per mm<sup>2</sup> in the core of HBO-PC+MCAO group were slightly higher than MCAO group, but not significant ( $34.57 \pm 8.62$  vs.  $25.32 \pm 9.53$ ,  $P > 0.05$ ). The intact neuron numbers of the penumbra and the hippocampus were higher in HBO-PC+MCAO group than those of MCAO group ( $99.87 \pm 19.57$  vs.  $54.52 \pm 17.63$ ,  $P < 0.05$  for penumbra;  $98.95 \pm 20.32$  vs.  $35.85 \pm 15.47$ ,  $P < 0.01$  for the hippocampus, respectively).

### 2.4. MDA content

The MDA content of the penumbra (Fig. 3A) and hippocampus (Fig. 3B) in the HBO-PC+MCAO group were significantly lower at 24 h compared with that of MCAO group ( $6.94 \pm 2.21$  vs.  $11.43 \pm 1.18$ ,  $P < 0.05$  for penumbra;  $8.28 \pm 1.89$  vs.  $12.25 \pm 1.65$ ,  $P < 0.05$  for the hippocampus, respectively). No difference was



**Fig. 2** – Nissl staining of the cortex (A) and hippocampus (B). The region directed with arrows were ischemic core, around them were penumbra. Most neurons of the core (A, E, F) both in MCAO and HBO-PC group (A. I HBO-PC+MCAO group vs. MCAO group, \* $P > 0.05$ ; MCAO group vs. sham group, HBO-PC+MCAO group vs. sham group, \*\* $P < 0.01$  for both) and of the CA1 sector in MCAO group (B, B, E) were shrunk with intercellular space enlarged and had deep color staining, even a part of neurons disappeared. The cells with eumorphism of HBO-PC group were significantly better preserved in the penumbra (A, G, H, I † $P < 0.05$  vs. MCAO group; \* $P > 0.05$  vs. sham group) and CA1 sector (B, E, F, G \*\* $P < 0.01$  vs. MCAO group; \* $P > 0.05$  vs. sham group).  $n = 6$  in each group.

observed in malondialdehyde level in the ischemic core (Fig. 3A) between the two groups ( $18.21 \pm 1.98$  vs.  $17.44 \pm 2.53$ ,  $P > 0.05$ ).

### 2.5. Antioxidant enzyme activities

At 24 h after ischemia–reperfusion injury, activity of SOD in the penumbra and hippocampus in HBO-PC+MCAO group were higher than that of MCAO group ( $162.55 \pm 14.44$  vs.  $124.47 \pm 13.86$  for penumbra;  $146.75 \pm 11.48$  vs.  $105.29 \pm 10.55$  for the hippocampus,  $P < 0.01$ , Fig. 4), so was activity of CAT ( $22.47 \pm 2.89$  vs.  $9.69 \pm 3.27$ ;  $19.66 \pm 3.98$  vs.  $7.62 \pm 3.77$ ,  $P < 0.01$ ). In the ischemic core no significant difference in activity of SOD were observed between the two groups ( $120.51 \pm 15.24$  vs.  $90.62 \pm 16.56$ ,  $P > 0.05$ ), but activity of CAT in HBO-PC+MCAO was higher than in MCAO group ( $14.62 \pm 4.51$  vs.  $6.28 \pm 3.88$ ,  $P < 0.05$ ).

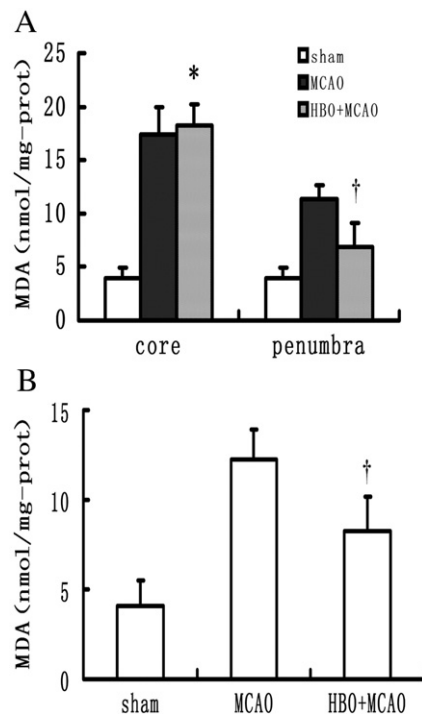
As for GSH-px activity, no difference was found either in the penumbra or in the core of the cortex or in the hippocampus between HBO-PC+MCAO group and MCAO group at 24 h ( $20.54 \pm 3.58$  vs.  $19.38 \pm 4.11$ ;  $15.69 \pm 4.01$  vs.  $14.22 \pm 3.62$ ;

$20.85 \pm 4.51$  vs.  $21.67 \pm 3.22$ , respectively,  $P > 0.05$ ). Compared with sham group, GSH-px activity in the other two groups decreased significantly ( $P < 0.05$ ) except that in HBO-PC+MCAO group in the hippocampus ( $P > 0.05$ ).

## 3. Discussion

In this study we utilized HBO-PC as an oxidative stress stimulus (Nemoto and Betterman, 2007) to induce enhanced activities of endogenous antioxidant enzymes. A two-day short term HBO-PC was used in this study which is more clinically feasible than a previous five-day HBO-PC protocol (Wada et al., 2001). We found this short term HBO-PC protocol decreased mortality rate, improved neurobehavioral function, protected the neurons in the cerebral cortex and in the hippocampus, decreased cerebral lipid peroxidation, and increased the activity of antioxidant enzymes. However, we did not directly compare the effect of our short term HBO-PC with the once daily five-day HBO-PC protocol (Wada et al.,





**Fig. 3 – The content of MDA in the cortex (A) and hippocampus (B). MDA content of the penumbra and hippocampus in the HBO-PC + MCAO group were significantly lower than in the MCAO group ( $^{\dagger}P < 0.05$ ). But in the core there was no difference between the two groups ( $^*P > 0.05$ ).**

2001) in this study and we are not clear if this short term HBO-PC is as effective as the five-day HBO-PC reported by others (Wada et al., 2001).

Focal cerebral ischemia is known to result in an ischemic core and an ischemic penumbra (Memezawa et al., 1992; Kaufmann et al., 1999). Many neurons in the penumbra die of apoptosis, while most neurons in the core die of necrosis. The neuronal injury in the ischemic penumbra is reversible and may be salvaged by early and effective treatments (Hossmann, 2006). Due to the different cell injury patterns in the core and penumbra after ischemia-reperfusion insult, we studied the ischemic core and penumbra separately and found the ischemic core was less sensitivity to HBO-PC than the penumbra. Therefore, HBO-PC may be effective in the preservation of neurons in the penumbra regions.

In our experiment, repeated HBO-PC induced an increase in the activity of SOD and CAT in the brain tissue but not the activity of GSH-px. In mammals, three isoforms of SODs are known: cytosolic copper-zinc SOD, mitochondrial manganese SOD (Mn-SOD), and extracellular SOD, which are products of distinct genes but catalyze the same reaction. Among them Mn-SOD localizes in mitochondria and is considered to be a first line of defense against oxidative stress (Finkel, 2003). It has been proved that the 5' flank region of the Mn-SOD gene contains a putative binding site for NF- $\kappa$ B which is activated by ROS (Bubici et al., 2006) and this suggests that Mn-SOD expression can be regulated by ROS through NF- $\kappa$ B. Even though we measured total SOD activity, it is likely Mn-SOD is involved in HBO-PC. It has been demonstrated that CAT plays an

important role in the induction of ischemic tolerance by HBO-PC (Kim et al., 2001; Nie et al., 2006). Moreno et al. (1995) studied immunocytochemical localization of CAT in the central nervous system of the rat and found that intensely stained CAT-immunoreactive neurons were resistant to ischemia-reperfusion injury, whereas weakly stained neurons which were more susceptible to ischemia. Thus, the activity of CAT may be critical for a protective effect against oxidative stress under pathological conditions, such as ischemia-reperfusion injury. Our study demonstrates that HBO-PC leads to the induction of catalase in the region of ischemia-reperfusion injury, even in the ischemic core. Since HBO-PC stimulates ROS production, it is plausible that increased levels of ROS stimulate the induction of the catalase gene. As for GSH-px activity, no significant difference was observed between MCAO group and HBO-PC + MCAO group at 24 h after ischemia-reperfusion injury in the present study. There are two possible explanations: firstly, GSH-px enzyme in the brain is insensitivity to HBO-PC; secondly, the level of GSH-px didn't change obviously at the time points examined.

The content of MDA, an indicator of lipid peroxidation, reflected the damage caused by ROS. The present results showed HBO-PC could lower the level of MDA in the ischemic penumbra and hippocampus. One of the possible explanations is that elevated activities of CAT and SOD scavenged excessive ROS and attenuated the lipid peroxidation.

The present findings imply that HBO-PC may be clinically useful for preventing ischemia-reperfusion injury in special surgical procedures such as cardio bypass or heart transplantation. The neuroprotective effect or ischemic tolerance produced by HBO-PC may be mediated by an upregulation of endogenous antioxidative enzymes such as SOD or CAT as shown in this study. Other mechanisms may be involved including hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) (Gu et al., 2008). Future clinical studies are warranted.

## 4. Experimental procedures

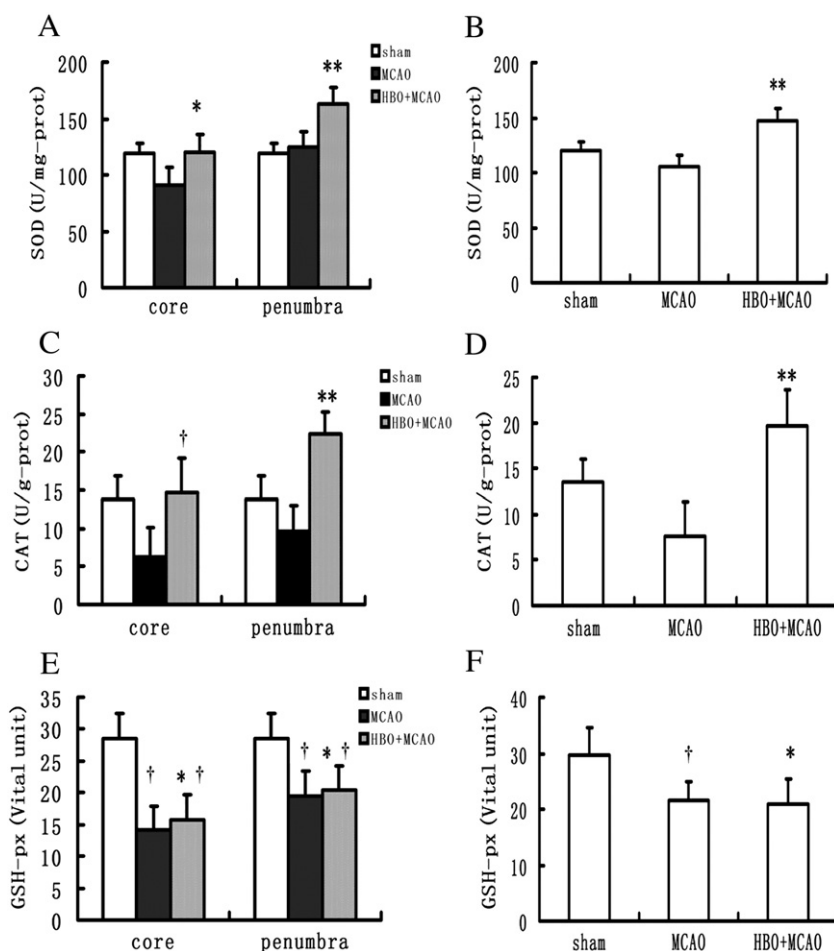
All surgical procedures were approved by the Ethics Committee for Animal Experimentation and conducted according to the Guidelines for Animal Experimentation of our institutes. All efforts were made to minimize the number of animals used in this study and every effort was taken to reduce animal suffering.

### 4.1. Animals and groups

A total of 74 male Sprague-Dawley rats weighing 250–280 g were used for the experiment. The rats were housed at 22–24 °C with light from 08:00 a.m. to 08:00 p.m. and free access to water. All animals were fed a commercial diet before and during the experiment. Animals were randomly distributed into three groups as follows: sham group, MCAO group, and HBO-PC plus MCAO group.

### 4.2. HBO preconditioning

A special animal hyperbaric chamber was used for HBO-PC. The HBO-PC procedure was conducted by consecutive four



**Fig. 4 – Activity of superoxide dismutase (SOD) (A, B), catalase (CAT) (C, D), glutathione peroxidase (GSH-px) (E, F). Activity of SOD and CAT in the penumbra and hippocampus in HBO-PC+MCAO group were higher than that in the MCAO group (\*\* $P < 0.01$ ). In the ischemic core no significant difference in activity of SOD between the two groups were observed ( $P > 0.05$ ), but activity of CAT in HBO-PC+MCAO group was higher than in MCAO group ( $P < 0.05$ ). No difference in GSH-px activity was found either in the cortex or the hippocampus between HBO-PC+MCAO group and MCAO group ( $P > 0.05$ ). Compared with sham group, GSH-px activity in the other two groups decreased significantly ( $P < 0.05$ ) except that in HBO-PC+MCAO group in the hippocampus ( $P > 0.05$ ).  $n = 15$  in each group.**

times of 1 h HBO exposure (2.5 ATA, 100%  $O_2$ ) at an interval of 12 h. The sham group was not exposed to HBO in order to determine the basal levels of antioxidant enzymes. Compression was performed at 1  $kg/cm^2/min$ , and decompression was performed at 0.2  $kg/cm^2/min$ . Accumulation of  $CO_2$  was prevented by using a small container with calcium carbonate crystals. Chamber temperature was maintained in the range 22–25 °C. No seizures were observed in any animals during all HBO-PC procedures. MCAO rats were placed in the same rodent chamber for 1 h at 12 h interval for 2 days in room air without increased pressure. All HBO-PC administrations were started at the same hour in the morning (08:00 p.m.) to prevent biological rhythm changes.

#### 4.3. MCAO procedures

MCAO models were performed at 24 h after the last HBO-PC and produced by the filament modeling initially reported by

Zea-Longa et al. (1989) with some modifications. After an overnight fast with unrestricted access to water, 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. A temperature probe was inserted beneath the temporalis muscle for continuous monitoring of pericranial temperature. During surgery, pericranial temperature was held between 37.0 and 38.7 °C using a heating blanket. The tail artery was cannulated for continuous blood pressure recording and to provide samples for glucose and blood gas measurements. 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. After 90 min middle cerebral artery occlusion, blood flow was restored by the withdrawal of the intraluminal suture and then the awakened rats were returned to their cages.

#### 4.4. Neurologic functional scoring

A neurological scoring system proposed initially by Johansson (1995) and Garcia et al. (1995) and modified in our laboratory was used by an independent observer. The neurologic testing was composed of four small tests (Table 2): symmetry in the movement of four limbs, forepaw outstretching, beam-walking test and limb-placement test. The lower score represents the more serious symptom.

#### 4.5. Nissl staining

Animals were anesthetized with 2% pentobarbital sodium (50 mg/kg) and then perfused 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). The brains were removed and immersed in the same fixative for 2 days. After dehydration in the graded ethanol and xylene, the brain sections were embedded in paraffin. Paraffin sections 4 µm in thickness were dewaxed and rehydrated according to standard protocols, and then stained in 1% toluidine blue at 50 °C for 5 min. After rinsing with double distilled water, the sections were dehydrated in increasing concentrations of ethanol and cleared in xylene, then mounted with Permount cover slip and observed under a light microscope. Cells that contained Nissl substance in the cytoplasm, loose chromatin and prominent nucleoli were considered to be normal neurons, and damaged neurons were identified by the loss of Nissl substance, cavitation around nucleus and by the presence of pyknotic homogenous nuclei. Ischemic core and penumbra of the cerebral cortex and CA1 of hippocampus were photographed in each section. The data were represented as the number of cells per mm<sup>2</sup>.

#### 4.6. Ischemic core and penumbra dissections

The ischemic core and penumbra of the cortex 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.

#### 4.7. Measurement of antioxidant enzyme activity and malondialdehyde content

The samples were homogenized in cold saline with a weight-to-volume ratio of 1:9. The homogenate was centrifuged at 2000 rpm for 10 min at 4 °C and the supernatant was assayed for protein concentration by Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, China). The treated samples were removed to a cuvette for the next assay. Both the measurements of antioxidant enzyme activities and malondialdehyde (MDA) content in tissue homogenate were performed according to the technical manual of the detection kits (Jianchen Biological Institute, China). Superoxide dismutase (SOD) activity was measured following the reduction of nitrite by a xanthine-xanthine oxidase system, which is a superoxide anion generator. One unit of SOD is defined as the amount that shows 50% inhibition. The activity was expressed as U/mg-protein. Catalase (CAT) activity was assayed by measuring absorbance at 240 nm using an ultraviolet light spectrophotometer and was expressed as U/g-protein. The definition of its activity was based on the hydrogen peroxide decomposition rate at 240 nm in the reactive mixture, of which the absorbance was between 0.50 and 0.55. Glutathione peroxidase (GSH-px) activity was measured by measuring the absorbance at 412 nm. The GSH-px in 1 mg protein that led to the decrease of 1 µmol/L GSH in the reactive system per minute was defined as one vital unit. GSH-px activity was presented as U/mg-protein. Malondialdehyde (MDA) content was measured with the thiobarbituric acid (TBA) reaction. The method was used to obtain a

**Table 2 – Neurologic functional scoring**

Symmetry in the movement of four limbs	0	Forelimb on the left side did not move at all.
	1	Limbs on the left side showed minimal movement.
	2	Limbs on the left side extend less or more slowly than those on the right.
	3	All four limbs extended symmetrically.
Forepaw outstretching	0	Left forelimb did not move.
	1	Left forelimb moved minimally.
	2	Left side outstretched less than the right, and forepaw walking was impaired.
	3	Both forelimbs were outstretched, and the rat walked symmetrically on forepaws.
Beam-walking test	0	The rat falls down.
	1	The rat is unable to traverse the beam but remains sitting across the beam.
	2	The rat falls down while walking
	3	The rat can traverse the beam, but the affected hindlimb does not aid in forward locomotion.
	4	The rat traverses the beam with more the 50% footslips.
	5	The rat crosses the beam with a few footslips.
	6	The rat crosses the beam with no footslips.
The limb-placement test	0	No placing.
	1	Incomplete and/or delay (>2 s).
	2	Immediate and correct placing.

Maximum possible score, 15, namely healthy rat.

spectrophotometric measurement of the color produced during the reaction of TBA with MDA at 535 nm; estimated MDA level was expressed as nmol/mg-protein.

#### 4.8. Statistical analyses

Data are expressed as mean  $\pm$  SD. Statistical analyses were made using one-way ANOVA, followed by a Student–Newman–Keuls (SNK) test for multiple comparisons. A *P*-value of less than 0.05 was considered to be statistically significant.

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### Glossary

- HBO-PC: hyperbaric oxygen preconditioning  
MCAO: middle cerebral artery occlusion  
ATA: atmosphere absolute  
MDA: Malondialdehyde  
CAT: catalase  
SOD: superoxide dismutase  
GSH-px: Glutathione peroxidase  
ROS: reactive oxygen species  
CCA: common carotid artery  
ECA: external carotid artery  
ICA: internal carotid artery  
TBA: thiobarbituric acid  
ANOVA: analysis of variance