Hyperbaric Oxygen Preconditioning Attenuates Early Apoptosis after Spinal Cord Ischemia in Rats

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Abstract

This study tested the hypothesis that spinal cord ischemic tolerance induced by hyperbaric oxygen preconditioning (HBO-PC) is mediated by inhibition of early apoptosis. Male Sprague-Dawley rats were preconditioned with consecutive 4 cycles of 1-h HBO exposures (2.5 atmospheres absolute [ATA], 100% O_2) at a 12-h interval. At 24 h after the last HBO pretreatment, rats underwent 9 min of spinal cord ischemia induced by occlusion of the descending thoracic aorta in combination with systemic hypotension (40 mmHg). Spinal cord ischemia produced marked neuronal death and neurological dysfunction in animals. HBO-PC enhanced activities of Mn-superoxide dismutase (Mn-SOD) and catalase, as well as the expression of Bcl-2 in the mitochondria in the normal spinal cord at 24 h after the last pretreatment (before spinal cord ischemia), and retained higher levels throughout the early reperfusion in the ischemic spinal cord. In parallel, superoxide and hydrogen peroxide levels in mitochondria were decreased, cytochrome c release into the cytosol was reduced at 1 h after reperfusion, and activation of caspase-3 and −9 was subsequently attenuated. HBO-PC improved neurobehavioral scores and reduced neuronal apoptosis in the anterior, intermediate, and dorsal gray matter of lumbar segment at 24 h after spinal cord ischemia. HBO-PC increased nitric oxide (NO) production. L-nitroarginine-methyl-ester (L-NAME; 10 mg/kg), a nonselective NO synthase (NOS) inhibitor, applied before each HBO-PC protocol abolished these beneficial effects of HBO-PC. We conclude that HBO-PC reduced spinal cord ischemia-reperfusion injury by increasing Mn-SOD, catalase, and Bcl-2, and by suppressing mitochondrial apoptosis pathway. NO may be involved in this neuroprotection.

Key words: antioxidant enzymes; hyperbaric oxygen preconditioning; mitochondria; neuronal apoptosis; nitric oxide

Introduction

S PINAL CORD ISCHEMIA, which could result in paraplegia and paralysis, remains a devastating complication after repairing of thoracoabdominal aortic aneurysms. A variety of preconditioning stimuli have been used to render spinal cord protection, such as brief periods of ischemia (Abraham et al., 2000), transient episodes of electroconvulsion (Kakinohana et al., 2005), and exposure to anesthetic inhalants (Park et al., 2005). However, the utility of the preconditioning effects induced by these stimuli in clinical practice is questionable because of potential risks for patients. Hyperbaric oxygen preconditioning (HBO-PC) has been reported as a safe procedure for patients and has been tested in different animal models. Repeated exposure to moderate pressure of HBO has been shown to provide protection against subsequent injury in spinal cord (Dong et al., 2002).

The mechanisms of HBO-PC that generate ischemic tolerance of neurons in the spinal cord remain unclear. It is established that mitochondria play a trigger and pivotal role in neuronal death following ischemia and reperfusion (Duchen, 2004). High levels of oxygen-free radical production in mitochondria during the period of reperfusion (Solenski et al., 2002) can induce the mitochondrial permeability transition by oxidizing thiol groups of the adenine nucleotide translocase (ANT) of the mitochondrial inner membrane (Kanno et al., 2004), release cytochrome c into the cytosol, and trigger apoptosis. Indeed, an early study using a rabbit model of spinal

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cord injury showed that HBO-PC upregulated activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, and ameliorated the spinal cord injury (Nie et al., 2006). However, the potential effect of HBO-PC on apoptotic cell death in spinal cord injury was not investigated.

In this study, we examined the activities of antioxidant enzymes and expression of antiapoptotic protein in the mitochondria that prevented neurons from apoptosis by inhibition of mitochondrial dysfunction as well as apoptotic factors after HBO-PC in a rat model of transient spinal cord ischemia.

Materials and Methods

The experimental protocol used in this study was approved by the Ethics Committee for the Animal Experimentation and was conducted according to the Guideline for the Animal Experimentation of the Second Military Medical University in Shanghai, China. Male Sprague-Dawley rats weighing between 300 and 350 g were acquired from the university vivarium and were housed in individual cages in a temperature-controlled and light-dark-cycle–controlled environment with free access to food and water.

HBO preconditioning and animal groups

We applied preconditioning regimen of four episodes of HBO pretreatments at every 12 h, each of which lasted for 1 h with 100% oxygen at 2.5 atmospheres absolute (2.5 ATA). Rats were pressurized in a research hyperbaric chamber (NGT50B; Ningbo, Beijing, China) with an oxygen flow of 20 L/min. Compression and decompression were maintained at a rate of 5 psi/min.

A total of 336 rats were randomly assigned into three groups ($n = 112$ for each group): the control, HBO, and HBO + NAME groups. Animals in the control group underwent 9 min of spinal cord ischemia without any treatment. Animals in the HBO group underwent HBO-PC and 9 min of spinal cord ischemia at 24 h after the last HBO pretreatment. Animals in the HBO + NAME group were injected intraperitoneally 10 mg/kg nonselective nitric oxide synthase (NOS) inhibitor, L-nitroarginine-methyl-ester (L-NAME; Beyotime Institute of Biotechnology, Haimen, China) at 20 min before each HBO-PC, and then underwent 9 min of spinal cord ischemia at 24 h after the last HBO pretreatment.

Induction of spinal cord ischemia and measurement of SCBF

Rat transient spinal cord ischemia model was conducted as described previously (Taira and Marsala, 1996). The rats were anesthetized with chloral hydrate (400 mg/kg, intraperitoneally) and were allowed to breathe spontaneously in an air–oxygen mixture (1:1) by an inhalation mask. Rectal temperatures were maintained at 37–38.5°C using a heating lamp. To monitor the mean distal arterial pressure (MDAP), a 24-gauge catheter (B. Braun Medical, Irvine, CA) was inserted into the tail artery. The left common carotid artery was cannulated with a 24-gauge catheter to monitor the mean proximal arterial blood pressure (MPAP) and control the MPAP at 40 mmHg during the period of aortic occlusion, and then the catheter was connected to an external blood reservoir that contained lactated Ringer solution (37.5°C). MPAP and MDAP were monitored

continuously by a calibrated pressure transducer connected to an invasive pressure monitor (PowerLab data acquisition system; ADInstruments, Castle Hill, Australia). After administration of 400 U/kg heparin through left common carotid artery, a midline laparotomy was performed after skin shaving and preparation with 10% povidone–iodine solution. For induction of spinal ischemia, a right common iliac artery was isolated, and a 2-French Fogarty catheter (Edwards Life Science, Shanghai, China) was placed into the descending thoracic aorta so that the tip of the catheter reached the level of the left subclavian artery. On the basis of preliminary dissections, this level corresponded to a distance of 9.8–10.2 cm from site of insertion. To induce spinal ischemia, the balloon catheter was inflated with 0.05 mL saline, and blood was allowed to flow into the external reservoir. The efficiency of the occlusion was evidenced by an immediate and sustained loss of any detectable pulse pressure and decrease of MDAP. After 9-min ischemia, the balloon was deflated, and blood was reinfused over a period of 1 min. Protamine sulfate (6 mg) was then injected intraperitoneally to neutralize excessive heparin. Arterial blood was sampled before ischemia, 5 min after the beginning of ischemia, and 10 min after the beginning of reperfusion. The arterial oxygen tension (PaO_2), arterial carbon dioxide tension ($PaCO_2$), pH, plasma glucose, and hematocrit were determined by GEM Premier 3000 analyzer (San Bruno, CA). All arterial lines were then removed, the incisions were closed, and the animals were allowed to recover. Bladder content was compressed manually as required.

The effect of HBO-PC on the spinal cord blood flow (SCBF) was studied in separate three groups of animals ($n = 7$ for each group) at 24 h after the last HBO-PC. After all cannulas were inserted, rats were changed from the supine position to the prone position. SCBF was measured with a laser probe (0.8 mm in diameter; PowerLab data acquisition system; ADInstruments) implanted into the epidural space through a burr hole in the lateral aspect of the L1 vertebral body. SCBF was then continuously monitored before and during spinal cord ischemia and for 30 min of reperfusion using 5-sec averaging cycles.

Assessment of neurological function

Rats were evaluated for neurobehavioral function of hind limb at 1, 6, 12, and 24 h after reperfusion according to a 15-point neurological performance scale (LeMay et al., 1987). A score of 15 indicated normal locomotion, and a score of 0 was given if there was no spontaneous movement. The neurological scoring was performed by two individuals who were unaware of the respective treatment groups.

Nissl stain and in situ detection of DNA fragmentation

After neurological evaluation, rats were transcardially perfused with 100 mL saline followed by 300 mL 4% formaldehyde in 0.01 mmol/L phosphate buffer (pH 7.4). The spinal cords were removed and postfixed in 4% phosphate-buffered formaldehyde for 3 days. L3–6 spinal segments were dissected. Spinal cord segments were embedded in paraffin, and serial transverse sections (4 μm) were obtained.

For Nissl staining, the sections were dewaxed, rehydrated, and immersed in 0.5% cresyl violet for 2 min. After washing in water, the sections were dehydrated in graded alcohols, cleared in xylene, and cover-slipper with Permount.

TUNEL study was conducted in accordance with the detection kit (In Situ Cell Death Detection kit; Roche Diagnostics GmbH, Mannheim, Germany). Briefly, 4-μm-thick paraffin-embedded tissue sections were dewaxed, rehydrated, and digested with proteinase K, and then incubated with reaction mixture of terminal deoxynucleotidyl transferase (TdT) and fluorescein-dUTP at 37°C for 60 min. The slides were washed with PBS. The sections were incubated with antifluorescein–alkaline phosphatase conjugate and colored using substrate solution of NBT/BCIP. For quantitative measurement of the number of cells that underwent apoptosis, an observer without knowledge of group assignment counted apoptotic motor neurons. In four sections from each spinal cord level (L3–6), the number of TUNEL staining–positive neurons in the anterior horn, intermediate gray matter, and dorsal horn was calculated in both sides of the spinal cord.

Tissue sampling for neurochemical assays and mitochondria preparation

The rats were sacrificed with lethal pentobarbital (100 mg/kg, intraperitoneally) before ischemia and at 1, 6, 12, and 24 h after reperfusion. For the biochemical studies, the descending thoracic aorta was cannulated with intravenous tubing, and 300 mL saline at 4°C was infused. Outflow was achieved by opening the right atrium. Rapid laminectomy was performed from the cervical to the sacral vertebrae. Samples of the lumbar spinal cord were harvested at least 2 cm distal to the 12th rib to avoid contamination by non-ischemic tissue.

Spinal cord mitochondria were isolated from the cortex using a Percoll gradient method described by Sims (1991) with minor modifications. The isolation buffer contained 225 mmol/L mannitol, 75 mmol/L sucrose, 0.5 mmol/L ethylenediamine tetraacetic acid (EDTA), 5 mmol/L HEPES, and 1 mg/mL fatty acid–free bovine serum albumin (BSA), pH adjusted to 7.3 with KOH. Spinal cord tissue was homogenized using a glass/glass homogenizer in isolation buffer containing 12% Percoll and carefully layered on the top of a 12%/24%/42% discontinuous gradient of Percoll. After 11 min of centrifugation at 31,000 g, the mitochondrial fraction was collected from the top of the 42% Percoll layer of the gradient and then washed twice. For the final wash, we used isolation buffer with BSA omitted and the EDTA concentration reduced to 0.1 mmol/L. All isolation procedures were performed at 0–2°C. During experimentation, mitochondria were stored on ice at a final concentration of 15–20 mg protein/mL in isolation medium until use. The protein concentration in each preparation was determined by the BCA method using a plate reader (BCA Protein Assay Kit; Byotime Institute of Biotechnology).

Measurements of antioxidant enzymes in mitochondria

The isolated mitochondrial protein extracts were washed three times with cold PBS for next assay.

SOD activity was measured according to the method as previously described (Beyer et al., 1986). SOD activity was measured following the reduction of nitrite by xanthine–xanthine oxidase system, which is a superoxide generator. One unit of SOD is defined as the amount that shows 50% inhibition. Mn-SOD was determined in the same manner ex-

cept that all cuvettes contained 2 mmol/L KCN to inhibit Cu/Zn-SOD activity.

Catalase activity was measured by the method of Beers et al. (1996), and based on decomposition rate of hydrogen peroxide in the samples, the residue of which was neutralized by ammonium molybdate to complex. The activity was calculated by the absorbance reduction of a complex at visible light of 405 nm compared with the control, and was expressed as U/mg protein.

Measurements of superoxide and hydrogen peroxide in mitochondria

Bis (N-methylacridinium) (lucigenin) and 5-amino-2,3-dihydro-1,4- phthalazinedione (luminol) were purchased from Sigma (St. Louis, MO). Lucigenin- or luminol-derived chemiluminescence was monitored with a Berthold Biolumat LB 9505 (Bad Wildbad, Germany) at 37°C. For measurement of lucigenin-derived chemiluminescence, the reaction mixture contained 0.1 mg of mitochondrial protein in 1 mL of air-saturated respiration buffer in the presence of exogenous substrate (6 mmol/L succinate) with or without the pharmacological uncoupling agent carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (Sigma). The respiration buffer contained 70 mmol/L sucrose, 220 mmol/L mannitol, 2 mmol/L HEPES, 2.5 mmol/L KH_2PO_4, 2.5 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, and 0.1% BSA, pH 7.4. The lucigenin-derived chemiluminescence was initiated by adding 5 μmmol/L lucigenin and was continuously monitored for 40–60 min. This is a non-redox-cycling concentration of lucigenin. For measurement of luminol-derived chemiluminescence, the reaction mixture contained 0.1 mg of mitochondrial protein in 1 mL of air-saturated respiration buffer plus succinate (6 mmol/L) and horseradish peroxidase (10 μg/mL). The luminol-derived chemiluminescence was initiated by adding 10 μmmol/L luminol and was continuously monitored for 40–60 min.

Western blot analysis

The isolated mitochondrial and cytoplasmic proteins were used for detection of Bcl-2 and cytochrome c, respectively. Protein samples were boiled at 100°C after adding 1/6 (v/v) loading buffer (130 mmol/L Tris pH 6.8, 10% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.06% bromphenol blue), and 300 μg of total protein was electrophoresed on polyacrylamide gels with a continuous gradient from 5% to 12% (Byotime Institute of Biotechnology). The proteins were transferred to nitrocellulose membrane at 110 mA with a transfer buffer consisting of 48 mmol/L Tris-HCl (pH 7.5), 39 mmol/L glycine, 0.037% SDS, and 20% methanol. After the transfer the membrane was placed in 5% nonfat milk powder in TBS/T (10 mmol/L Tris pH 8.0, 150 mmol/L NaCl, and 0.1% Tween 20) to block nonspecific binding for 1 h at room temperature. Then, it was incubated with primary antibodies at 4°C for 20 h. The primary antibodies used were as follows: rabbit polyclonal antibody against Bcl-2 (1:1000; Stressgen Bioreagents, Victoria, Canada) and rabbit polyclonal antibody against cytochrome c (1:500; NeoMarkers, Fremont, CA). Membranes were then washed with TBS/T three times and then exposed to horseradish peroxidase–conjugated goat anti-rabbit IgG (1:1000; Cell Signaling Technology, Danvers, MA) at 25°C for 90 min. Proteins of interest were tested using the enhanced chemiluminescence Western blotting detection

system kit (Pierce Biotechnology, Rockford, IL). The blots were exposed on Kodak Hyper-film. In each blot, β-actin (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) was used as an internal standard. Densitometry was performed after scanning of the immunoblots. The relative density of bands was analyzed by a SmartScape imaging system (Furi Science & Technology, Shanghai, China).

Caspase activity assays

Activation of the caspase-3 and −9 was detected during the first 24 h of reperfusion. The spinal cord tissue was washed with ice-cold PBS and homogenized in caspase buffer containing 50 mmol/L HEPES-KOH (pH 7.4), 0.1%3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid, 100 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L DTT, and 10% glycerol. The lysates were sonicated (Bandelin Electronic, Berlin, Germany) for 30 sec and placed on ice for 30 min. The homogenated solutions were centrifuged at 14,000 g for 15 min, and the supernatant fractions were stored for analysis. Caspase activity was determined using a fluorometric assay (Patel et al., 1996). Briefly, 300 μg of total protein, determined by the BCA protein assay (Byotime Institute of Biotechnology), was incubated with 5 μL of the 1 mmol/L fluorogenic peptide substrate LEHD-AFC (BioVision, Mountain View, CA) and caspase buffer to total volume of 100 μL. After 1 h incubation at 37°C the release of 7-amino-4-trifluoromethyl coumarin was determined spectrofluorometrically using an excitation wavelength of 400 nm and an emission wavelength of 505 nm, as suggested by BioVision.

NO assay

Because NO rapidly converts into nitrate and nitrite, total NO production in sample was determined based on modified cadmium reduction method (Cortas et al., 1990) by measuring *in vivo* nitrite and those reduced from nitrate by spectrophotometry with NO assay kit (Jiancheng Biological Institute, Nanjing, China). Prior to HBO preconditioning (baseline), at 30 min after each session HBO pretreatment and 24 h after the last pretreatment (preischemia), spinal cord tissue was triturated in glycine-NaOH buffer (15 g/L glycine-NaOH pH 9.7), and centrifuged at 1500 g at 4°C for 30 min. The supernatants were deproteinized with Somogyi reagent and centrifuged again. Reduction of nitrate to nitrite was accomplished by catalytic reaction using cadmium, and the nitrite produced was determined by diazotization of sulfonilamide and coupling to naphthylethylene diamine. After a reaction of 60 min, absorbance of this complex was measured at 550 nm using EL×800 Bioelisa Reader (Bio-Tek Instruments, Winooski, VT), and the results were presented as nmol/mg protein.

Statistical analysis

All quantitative data are expressed as mean ± SEM. Mean differences were verified by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test for multiple comparisons. The scores of hind-limb neurological function were analyzed using a nonparametric method (Kruskal–Wallis test) followed by the Mann–Whitney U-test with the Bonferroni correction. The Western blot data were analyzed by one-way ANOVA on ranks followed by the Dunn method. Differences of $p < 0.05$ were considered statistically significant.

Results

Physiologic variables and SCBF

Physiologic values are presented in Table 1. There were no physiologically relevant differences among the groups for all monitored variables with the exception of MDAP and pH. The MDAP was significantly reduced by 32–37 mmHg during aortic artery occlusion, but returned to the preocclusion baseline level after reperfusion. The pH decreased to 7.40 ± 0.06 during the reperfusion.

SCBF in the control group decreased from baseline (100%) to 7.3 ± 3.2% immediately after the balloon inflation and was

TABLE 1. PHYSIOLOGIC VARIABLES ($N = 7$)

	Control	HBO	HBO + NAME
Before ischemia			
pH	7.40 ± 0.06	7.40 ± 0.05	7.39 ± 0.04
PaCO$_2$, mmHg	41 ± 4	37 ± 3	40 ± 4
PaO$_2$, mmHg	187 ± 16	195 ± 17	191 ± 18
Hematocrit, %	37 ± 3	40 ± 2	39 ± 4
Distal MAP, mmHg	60 ± 5	62 ± 6	58 ± 5
Temperature, °C	37.9 ± 0.3	38.2 ± 0.3	37.8 ± 0.2
During ischemia			
Distal MAP, mmHg	4 ± 2[a]	5 ± 2[a]	3 ± 1[a]
Temperature, °C	38.2 ± 0.4	38.0 ± 0.3	38.1 ± 0.5
After ischemia			
pH	7.12 ± 0.03[b]	7.10 ± 0.03[b]	7.10 ± 0.04[b]
PaCO$_2$, mmHg	48 ± 3	47 ± 3	46 ± 3
PaO$_2$, mmHg	193 ± 21	186 ± 22	194 ± 20
Hematocrit, %	36 ± 4	35 ± 4	38 ± 5
Distal MAP, mmHg	58 ± 5	54 ± 6	56 ± 8
Temperature, °C	38.0 ± 0.2	37.8 ± 0.4	38.1 ± 0.3

Data are presented as mean ± SEM.
[a]$p < 0.01$ and [b]$p < 0.01$ compared with before-ischemia value.
MAP, mean arterial blood pressure; PaCO$_2$, arterial carbon dioxide tension; PaO$_2$, arterial oxygen tension.

TABLE 2. CHANGES IN SCBF BEFORE, DURING, AND AFTER AORTIC OCCLUSION ($N = 7$)

	Control	HBO	HBO + NAME
Before ischemia	100	100	100
During ischemia			
1 min after occlusion, %	7.3 ± 3.2^a	6.4 ± 2.1^a	7.8 ± 3.4^a
9 min after occlusion, %	3.1 ± 1.2^a	2.6 ± 1.1^a	2.9 ± 1.5^a
After ischemia			
10 min of reperfusion, %	165.0 ± 26.3^b	155.6 ± 19.2^b	167.0 ± 18.6^b
30 min of reperfusion, %	107.8 ± 12.4	109.6 ± 11.8	115.6 ± 10.4

Data are presented as mean $\pm$ SEM.
[a]$p < 0.01$ and [b]$p < 0.01$ compared with before-ischemia value.

$3.1 \pm 1.2\%$ at the end of 9 min of aortic occlusion. After approximately 10 min of reperfusion, SCBF overshot baseline to $165.0 \pm 26.3\%$ and finally, after 30 min of reperfusion, decreased to the baseline level ($107.8 \pm 12.4\%$). There were no significant differences among the groups throughout the experiment (Table 2).

Neurological outcome

Nine minutes of ischemia caused by occlusion of the descending thoracic aorta in combination with maintaining systemic hypotension (40 mmHg) resulted in immediate hind-limb paralysis in all control animals. There was no improvement in the neurological function over 24 h in control spinal cord ischemia animals. HBO-PC improved neurological function dramatically ($p < 0.01$ vs. control). The effect of HBO-PC was abolished by L-NAME ($p < 0.01$ vs. HBO) (Fig. 1).

Nissl stain

At 24 h after spinal ischemia, moderate shrinkage and darkening of neurons were the most abundant changes in the anterior and intermediate gray matter of the untreated group

FIG. 1. Nine minutes of spinal cord ischemia caused immediate functional deficit of hind limb, which was still present at 24 h after the reperfusion in the control group. HBO-preconditioned rats showed a significant recovery that was noted after reperfusion, but L-NAME abrogated it ($n = 8$ per group; *$p < 0.01$ vs. control; #$p < 0.01$ vs. HBO).

(Fig. 2A, D). Injured neurons with twisted axonal processes and cell loss were noted in the dorsal horn (Fig. 2G). However, similar changes were very few in the HBO group (Fig. 2B, E, H). In the HBO + NAME group, relatively numerous dark neurons were observed as compared to the HBO group in the anterior horn (Fig. 2C), and a large population of cells in the central part and dorsal horn were damaged (Fig. 2F, I).

TUNEL and apoptotic cell count

Cells with double-strand breaks in DNA are detected by TUNEL staining in brown color. At 24 h after ischemia-reperfusion, anterior horn neurons of lumbar spinal cord in the control group were entirely TUNEL positive (Fig. 3A) and so were a large population of cells of the intermediate and dorsal gray matter (Fig. 3D, G). Only a few apoptotic neurons were present in the three regions in HBO-preconditioned rats (Fig. 3B, E, H). In the HBO + NAME group a remarkable increase of TUNEL staining was also observed compared to HBO group all in the anterior horn, central part, and posterior horn (Fig. 3C, F, I). All aforementioned results were quantified by means of percentage of the total cell number, in that HBO-PC significantly reduced the number of TUNEL-positive cells compared with other groups ($p < 0.01$) (Fig. 3J).

Effect of HBO preconditioning on NO

The effect of HBO-PC on NO levels in normal rat lumbar spinal cord was determined by measuring nitrite and nitrate before spinal cord ischemia. After each HBO-PC NO levels in the spinal tissue gradually increased and peaked on the last pretreatment, and remained significantly higher at 24 h after the last HBO-PC compared with the control group ($p < 0.01$). NO content was not increased in animals pretreated with 10 mg/kg L-NAME at 20 min before each HBO-PC ($p < 0.01$ vs. HBO) (Fig. 4A).

Antioxidant enzyme activities

Mn-SOD and catalase enzyme activities in mitochondria were quantified at preischemia and after spinal cord ischemia. At 24 h after the last HBO-PC, activities of Mn-SOD and catalase in lumbar spinal cord tissue in HBO group were higher than that of the control and HBO + NAME groups ($p < 0.01$). A higher activity of Mn-SOD was found in the HBO group
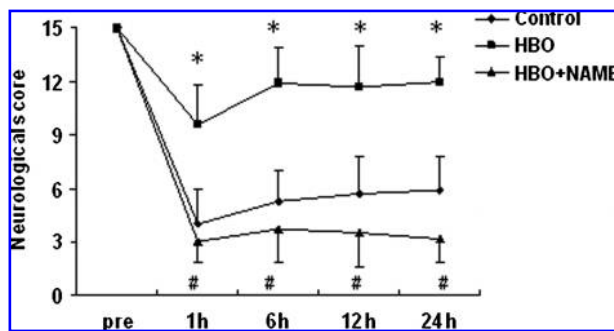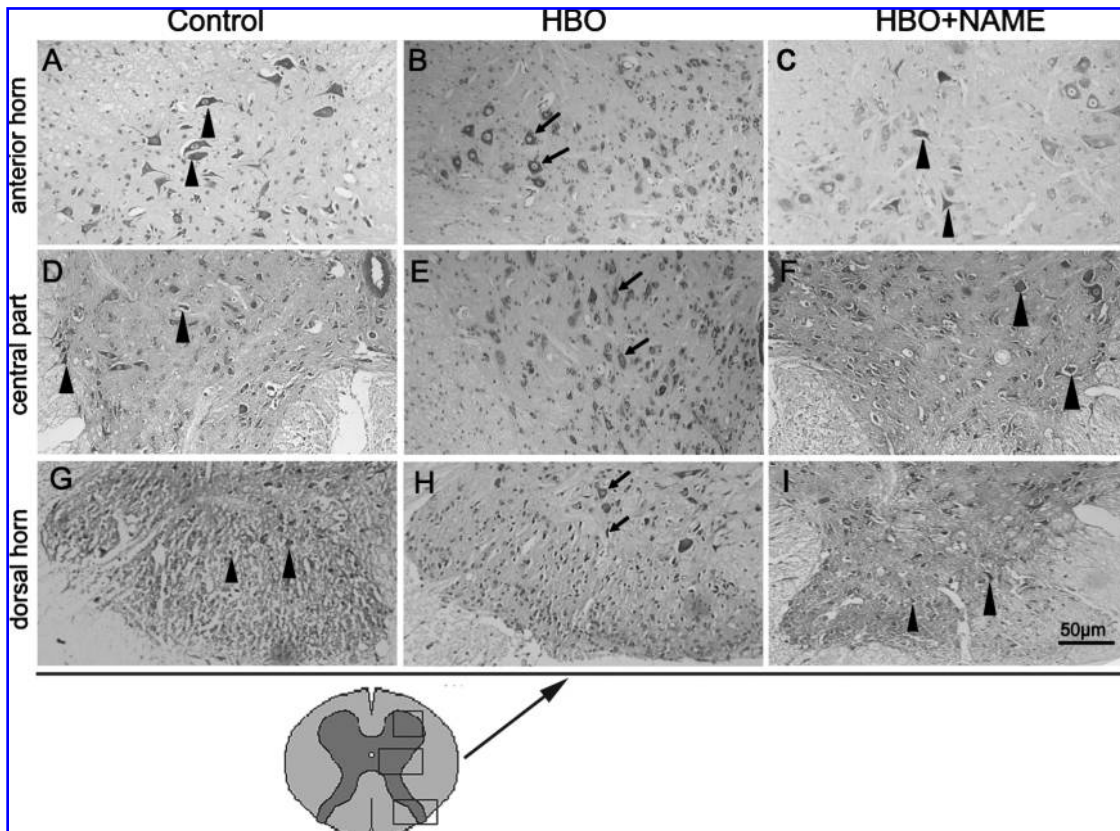
FIG. 2. Representative histological panels show ischemic cell change (arrowheads) in the anterior horn, central part, and dorsal horn of the lumbar spinal cord at 24 h after spinal cord ischemia (**A**, **D**, and **G**). In the HBO-preconditioned group, the majority of neurons presented undisturbed morphology (**B**, **E**, and **H**; arrows). However, transverse sections taken from the HBO + NAME group were associated with the presence of ischemic neurons (arrowheads) in the anterior, intermediate, and dorsal gray matter (**C**, **F**, and **I**).

than the other two groups within 24 h after reperfusion ($p < 0.01$ vs. control and HBO + NAME for all the time points). The comparison of catalase activity among three groups revealed similar results to that of Mn-SOD after reperfusion ($p < 0.01$ vs. control and HBO + NAME for 1 and 6 h after reperfusion; $p < 0.05$ vs. control and HBO + NAME for 12 and 24 h after reperfusion). No significant difference was observed between the control and HBO + NAME groups at corresponding time points. In addition, Mn-SOD and catalase activities in all three groups decreased at 12 and 24 h after reperfusion, compared with preischemic state ($p < 0.05$) (Fig. 4B, C).

ROS generation in mitochondria

The mitochondria isolated from the lumbar spinal cords of control and HBO + NAME rats at 1 h following ischemia-reperfusion displayed high levels of superoxide generation compared with HBO-preconditioned animals (Fig. 5A). Mitochondrial fractions from the HBO-PC lumbar spinal cord contained less hydrogen peroxide (H_2O_2) (Fig. 5B). Total superoxide and H_2O_2 (the areas under the curves) measured from these experiments in comparison with preischemic state are shown in Figure 5C. At 1 h following ischemia-reperfusion, there was an increase of nearly 10 times, re-

spectively, in superoxide and H_2O_2 in mitochondria isolated from control group ischemic spinal cord compared to pre-ischemia normal spinal cord tissues. Both superoxide and H_2O_2 were markedly reduced in HBO group, while treatment with L-NAME resulted in major enhancement of ROS production. There were no differences in findings among three groups before spinal cord ischemia.

Bcl-2 expression and cytochrome c release

We examined the effect of HBO-PC on the expression of mitochondrial Bcl-2 and the release of cytochrome c to cytoplasm in rat lumbar spinal cord at preischemia (24 h after the last HBO-PC) and during early reperfusion (Fig. 6). HBO-PC significantly increased the expression of Bcl-2 in the mitochondria at preischemia, and decreased the release of cytochrome c throughout the early stage of reperfusion compared with the control group. These effects were reversed by L-NAME (*$p < 0.05$ vs. control and HBO + NAME; #$p < 0.01$ vs. control and HBO + NAME).

Caspase activities

Figure 7 showed time course of the caspase activities in three groups. Activities of caspase-3 and −9 were significantly increased at 6 h, peaked at 12 h, and appeared to lessen at 24 h
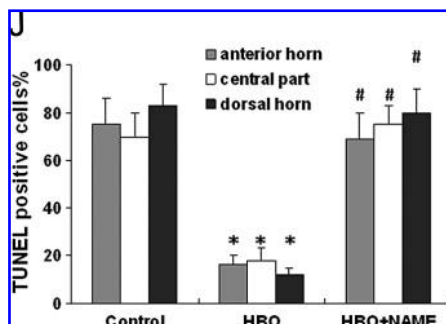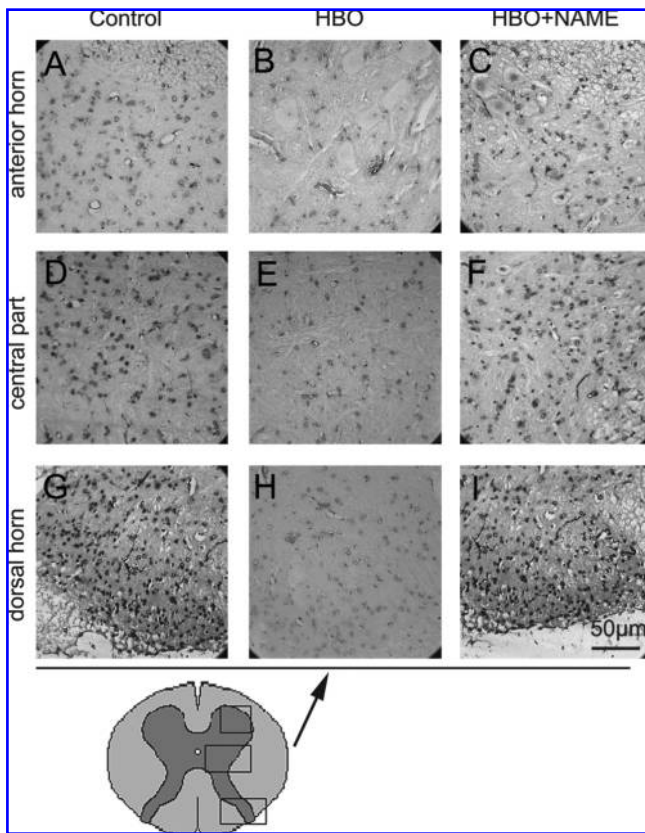
FIG. 3. Effect of HBO preconditioning on neuronal apoptosis (**A–I**) and apoptotic cell counts at 1 day after spinal cord ischemia (**J**). A significant increase in the number of TUNEL-positive cells occurred in the anterior, intermediate, and dorsal gray matter of lumbar spinal segments in the control group (**A**, **D**, and **G**). Scarce TUNEL-positive cells were also seen in the HBO group (**B**, **E**, and **H**). More abundant TUNEL-positive cells (**C**, **F**, and **I**) were all seen in the anterior horn, central part, and dorsal horn of the HBO + NAME group. Apoptotic cells containing positively labeled nuclei were scored and expressed with a percentage of the total cell number, which was presented as mean ± SEM. Note an apparent decrease from the HBO group ($n = 8$ per group; $^*p < 0.01$ vs. control; $^\#p < 0.01$ vs. HBO).

after spinal cord ischemia compared to preischemic state ($p < 0.001$ vs. preischemia). HBO-PC markedly reduced the caspase activities, and treatment with L-NAME before each HBO-PC abrogated the effects of HBO-PC ($p < 0.01$ vs. control and HBO + NAME).

Discussion

In this rat model of transient spinal cord ischemia, we have demonstrated both early and delayed molecular changes. The early changes include ROS formation and cytochrome c release within the first hour after reperfusion. The delayed changes are the activation of caspase-3 and −9 and apoptosis at the 24 h after reperfusion. HBO-PC potentiated the levels of Mn-SOD, catalase, and Bcl-2 in the mitochondria in normal spinal cord at 24 h after the last HBO-PC procedures, and the elevation of these factors seems responsible for the reduction of apoptotic cell death and the improvement of neurological functions at 24 h after ischemic spinal cord injury. NO production occurred during each session of HBO pretreatment. The mechanisms of elevation of Mn-SOD, catalase, and Bcl-2 and the reduction of apoptosis seem mediated by NO, because the neuroprotective effect of HBO-PC is abolished by a nonspecific NOS inhibitor, L-NAME.

Our observation of Mn-SOD and catalase is consistent with a previous report of HBO-PC in a rabbit spinal cord ischemia model, which, however, used a 5-day HBO-PC protocol (Nie et al., 2006). Because 5 days' HBO-PC is difficult for clinical application, we modified HBO-PC into a 2-day, four-treatment protocol, and we have observed similar neuroprotection. In our study, we also expanded mechanistic approach and identified that NO may play a crucial role in HBO-PC–induced tolerance in spinal cord ischemia. Other HBO-PC protocols have been tested, including one of our previous studies that one HBO-PC application produced strong ischemic tolerance in a neonatal hypoxia-ischemia rat model (Li et al., 2008). Recently, a 1-day, three HBO-PC protocol was applied in a clinical cardiovascular surgery setting (Alex et al., 2005). The present study and recent reports indicate that shortened HBO-PC protocol, which is more feasible for clinical application, seems effective at least for some brain and spinal cord ischemic injury.

The mechanisms of antioxidant activity of Mn-SOD and catalase and the antiapoptotic effect of Bcl-2 are established. Our results showed that HBO-PC induces an increase in activities of Mn-SOD and catalase in mitochondria. Mn-SOD is a nuclear-encoded mitochondrial enzyme that scavenges superoxide radicals generated by the electron-transport chain to form H_2O_2 and oxygen (Hirai et al., 2004). Catalase exerts protection against oxidative injury by converting H_2O_2 to water and oxygen. This is consistent with the degree of the reduction of regional ROS production after HBO-PC in this spinal cord ischemic model. We found that HBO-PC induced overexpression of Bcl-2 in the mitochondrial fractions. Bcl-2 stabilizes the mitochondrial membrane barrier function and inhibits the permeability transition pore complex, prevents channel formation by atractyloside-treated ANT, and neutralizes the cooperation between Bax and ANT (Brenner et al., 2000; Belzacq et al., 2003). Moreover, inhibition of the mitochondrial permeability transition itself attenuated ROS production at complex I of the respiratory chain (Batandier et al., 2004).

The major observation of this study is that NO seems playing a key role in HBO-PC–induced tolerance and L-NAME abolished the neuroprotective effect of HBO-PC. It is established that NO has complicated opposing actions, detrimental or protective effect depends on the amount of NO generated. High micromolar concentrations of NO are often

FIG. 4. Effect of HBO pre-conditioning on NO production and activities of mitochondrial antioxidant enzymes in lumbar spinal cord. (A) NO metabolites were measured for NO levels before HBO preconditioning (baseline), at 30 min after each pretreatment of four times, and 24 h after the last pretreatment (preischemia). NO content gradually increased after each HBO pretreatment and maintained significantly higher at 24 h after the last HBO pretreatment compared with the control group. No increase was observed in NO content with 10 mg/kg L-NAME given before each HBO pretreatment. (B and C) In the HBO group, activities of Mn-SOD and catalase in mitochondria were increased 24 h after the last pretreatment (preischemia) and, despite a gradual decrease, maintaining a higher level during the first 24 h after reperfusion in comparison with those of control and HBO + NAME groups ($n = 6$ for every time point per group; $*p < 0.01$ vs. control and HBO + NAME; $+p < 0.05$ vs. control and HBO + NAME; $\#p < 0.05$ vs. preischemia).
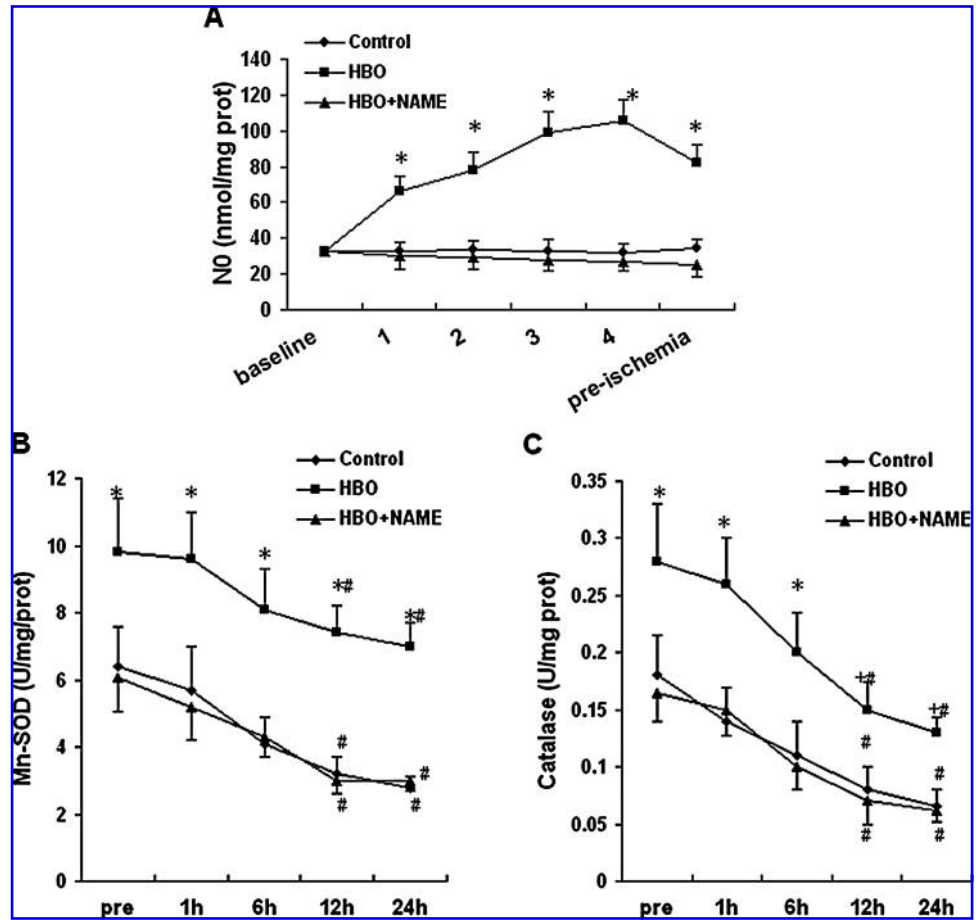
associated with inflammation and may be harmful in most cases (Anggard, 1994); however, a relatively small increase of NOS expression played a critical role in neuroprotection induced by many preconditioning stimuli (Huang, 2004). Our measurements of NO quantities during the period of HBO-PC were at extent of nanomolar. NO has been shown to participate in the mechanisms of preconditioning in the brain. Gidday et al. (1999) demonstrated that the nonselective inhibitor of NOS, nitro-L-arginine, blocked hypoxic preconditioning in a neonatal mode of ischemic brain injury. Using an *in vitro* model, Gonzalez-Zulueta et al. (2000) demonstrated that ischemic tolerance induced by oxygen–glucose deprivation depends on nNOS-derived NO. More recently, iNOS-derived NO has also been implicated in the preconditioning induced by volatile anesthetics (Kapinya et al., 2002), bilateral common carotid artery occlusion, and lipopolysaccharide (Cho et al., 2005). The elevation of NO is accompanied by elevated activities of Mn-SOD, catalase, and Bcl-2 in normal spinal cord tissues after HBO-PC. The upregulation of Mn-SOD, catalase, and Bcl-2 may contribute to the reduction of neuronal death and neurological functional improvement after spinal cord ischemia. However, the sources of NO production, iNOS, nNOS, or eNOS (Bernareggi et al., 1999; Elayan et al., 2000; Atochin et al., 2003), in the HBO-PC and the role of NO in the potentiation of Mn-SOD, catalase, Bcl-2, and reduction of

caspase-3 and −9, and cytochrome c release remain unclear and require further studies. Existing literatures offered some speculations. NO in low concentration of less than one micromolar exerted its biologic actions by reacting with transitional metal centers (Gross and Wolin, 1995). NO reacted with heme groups of enzymes, such as catalase, modulating their activity (Gross and Wolin, 1995), and the transcription factors with thiol groups to promote expression of antiapoptosis protein Bcl-2 (Genaro et al., 1995). NO via Ras activation stimulated downstream ERK1/2 cascade (Gonzalez-Zulueta et al., 2000; Huang, 2004), which has been shown to activate posttranscriptionally Mn-SOD expression and activity (Santillo et al., 2001; Scorziello et al., 2007). Recently, it has been reported that peroxynitrite could exert a neuroprotective action in cortical neurons through PI3/Akt pathway or glucose-6 phosphate dehydrogenase activity (Garcia-Nogales et al., 2003; Delgado-Esteban et al., 2007).

The HBO-PC–produced ischemic tolerance seems not artifacts related to the experimental conditions because all groups are temperature controlled and monitored carefully. It is also unlikely that HBO-PC–induced ischemic tolerance is due to eNOS-derived NO that augments cerebral blood flow (CBF) through vasodilatation, because CBF changes last only a few minutes to hours during the HBO exposure (Demchenko et al., 2003). Further, the SCBF reductions produced by the aortic
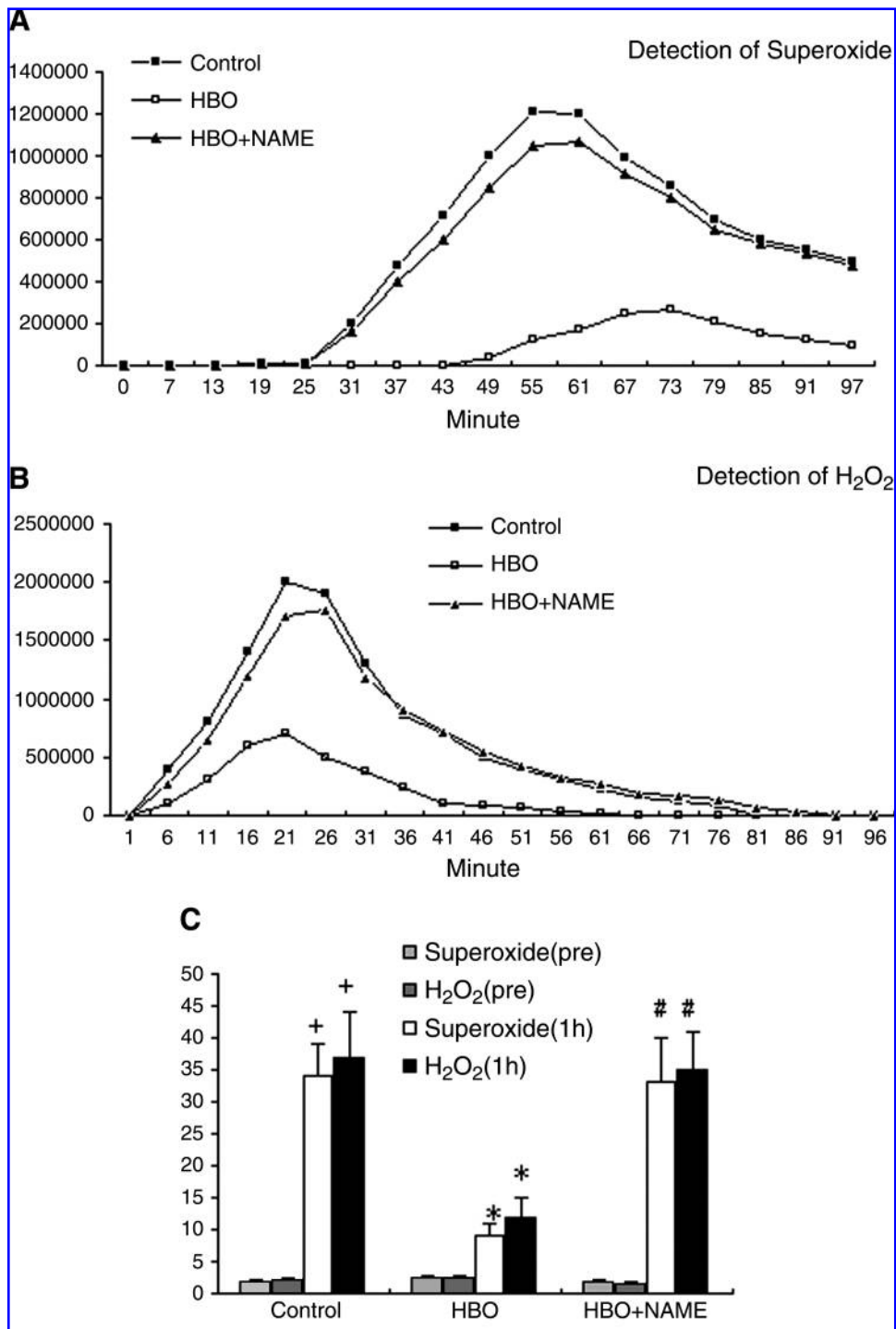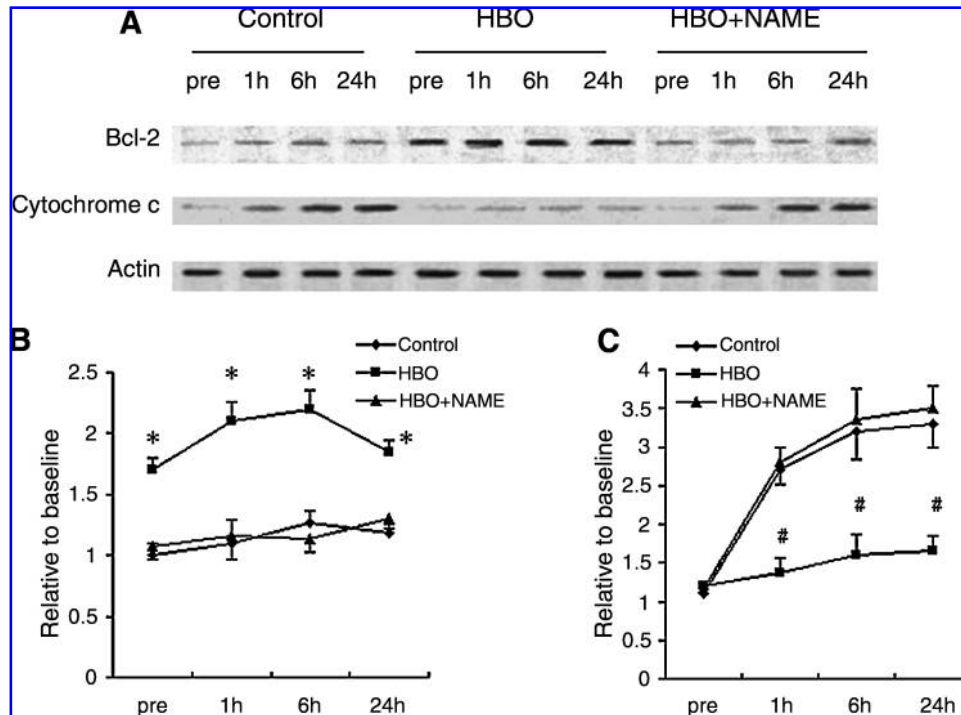
FIG. 5. Effect of HBO preconditioning on ischemia-induced mitochondrial superoxide (**A**) and H_2O_2 generation (**B**) in rat lumbar spinal cord at 1 h of reperfusion. (**C**) ROS formation (area under the curve) in rat spinal cord of three groups compared with preischemic state. At 1 h following ischemia-reperfusion, there was an increase of nearly 10 times, respectively, in superoxide and H_2O_2 in mitochondria isolated from control group ischemic spinal cord compared to preischemia normal spinal cord tissues. Both two ROS formation was markedly reduced in HBO group, while treatment with L-NAME prior to each HBO-PC resulted in major enhancement of ROS production again. There are no differences in findings among three groups before spinal cord ischemia ($n = 6$ per group from preischemia and $n = 7$ per group from reperfusion; $^+p < 0.001$ vs. preischemia; $^*p < 0.01$ vs. control; $^\#p < 0.01$ vs. HBO).

occlusion were comparable in the different groups of rats throughout the experiment. Therefore, the findings of the present study seem not resulted from confounding factors or artifacts of the used methods.

Because there are no differences in spinal blood flow between anterior, intermediate gray matter, and posterior horn in an experimental model of spinal cord ischemia (Jacobs et al., 1992), we identified the injury pattern within motor neurons, sensory neurons, and other overlapping populations. In the present study, TUNEL signals, a delayed phase of apo-

ptosis, were detected at 24 h postischemia along with earlier cytochrome c release and caspase-3 and −9 processing. After 15 min of rabbit spinal cord ischemia, TUNEL-positive cells appeared 1 day after reperfusion (Hayashi et al., 1998), whereas nuclei of some motor neurons were positively labeled within the first hour of reperfusion after 30 min of ischemia (Roseborough et al., 2006). This suggests that only quite early intervention (e.g., HBO-PC) may be effective as it can reduce the amount of the initial damage. Interestingly, we have also found no evidence of the second wave of caspase

FIG. 6. Effect of HBO preconditioning on the expression of mitochondrial Bcl-2 and the release of cytochrome c to cytoplasm in rat lumbar spinal cord at preischemia (24 h after last HBO pretreatment) and during early reperfusion. The isolated mitochondrial protein extracts and cytosolic proteins were, respectively, used for detection of Bcl-2 and cytochrome c by immunoblot analysis. (**A**) The film images of the bands are representative of three independent experiments. (**B** and **C**) The graphic presentation of Bcl-2 and cytochrome c protein abundance quantified by integrating the volume of bands from 5 to 7 rats for each experimental condition and normalizing the data by those of actin. Values in graphs are mean ± SEM of the fold changes over the preischemic state of the control group that is set as 1. HBO preconditioning significantly increased the expression of Bcl-2 in the mitochondria at preischemia, and decreased the release of cytochrome c throughout the early stage of reperfusion compared with the control group. These effects were reversed by L-NAME (*$p < 0.05$ vs. control and HBO + NAME; #$p < 0.01$ vs. control and HBO + NAME).

activation occurring after 24 h of reperfusion that would be assumed a new apoptosis resulting from the late onset of spinal inflammation (Jacobs et al., 1987).

Our behavioral studies tested performance of animals associated with a function of spinal cord (sensory-motor tests) and found a significant improvement in both instances in the HBO-preconditioned group within the first 24 h of reperfusion. As a first step to evaluate the neuroprotective effect of HBO-PC in spinal cord ischemia, we focus on the early mechanisms of HBO-PC protection, and we have identified that HBO-PC reduced oxidative stress damage within hours after spinal cord injury. Our future studies will target the long-term improvement of HBO-PC on spinal cord functional recovery. The long-term neuroprotection by HBO-PC is likely because in another study with animals preconditioned and stroked in an identical fashion, we found long-term improvement, with no deterioration, in neurological scores at day 7 as compared to untreated rats, which suggests a durable effect of HBO-PC.

We have demonstrated that spinal cord ischemia caused severe gray matter damage, and that HBO-PC reduced neuronal cell death and improved locomotor function. A limitation of this study is that we did not examine the white matter injury of spinal cord, which also contributed to motor dysfunction after spinal cord ischemia. Recent evidence showed that white matter injury was of equal importance to gray matter damage (Follis et al., 1993; Kanellopoulos et al., 2000), and white matter

was even more vulnerable to ischemia than gray matter (Kudo et al., 2006). Horiuchi et al. (2008) indicated that vacuolation and pallor of the white matter were very marked 24 h after ischemia that reflected the segmental swelling of myelinated axons, the formation of spaces between myelin sheaths and axolemma, and astrocyte swelling. A previous study also showed that spinal cord ischemia induced amyloid precursor protein accumulated at the regions of swollen axons of the ventral and ventrolateral white matter in rats, which is a marker of axonal damage (Kurita et al., 2005). Further study will be required to clarify the neuroprotective efficacy of HBO-PC on white matter injury, which may lead to a fuller understanding of its mechanism-mediated white matter protection.

Based on our overall data, short-term HBO-PC appears as a very effective modality that may be used to achieve durable spinal cord protection through a reduction of the early apoptosis before major surgeries that may cause spinal cord ischemia.
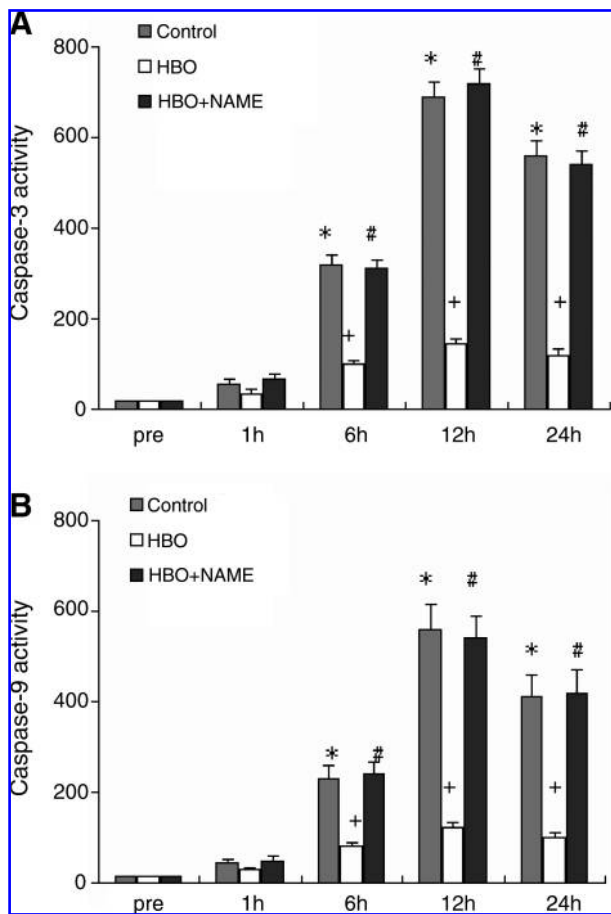
FIG. 7. Activities of caspase-3 (**A**) and −9 (**B**) were both significantly increased at 6 h and peaked at 12 h, and remained higher levels 24 h after spinal cord ischemia compared to preischemic state. HBO preconditioning markedly weakened the activities of the two caspases. Treatment with L-NAME before each HBO pretreatment abrogated the effects of HBO preconditioning ($n = 6$ for every time point per group; *$p < 0.001$ vs. preischemia; +$p < 0.01$ vs. control; #$p < 0.01$ vs. HBO).

Author Disclosure Statement

No competing financial interests exist.

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