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Research Report

Mechanism of hyperbaric oxygen preconditioning in neonatal hypoxia–ischemia rat model

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ABSTRACT

Hypoxic ischemic (HI) injury in neonates damages brain tissues. We examined the mechanism of hyperbaric oxygen preconditioning (HBO-PC) in neonatal HI rat model. Seven-day-old rat pups were subjected to left common carotid artery ligation and hypoxia (8% oxygen at 37 °C) for 90 min. HBO (100% O₂, 2.5 atmospheres absolute for 2.5 h) were administered by placing pups in a chamber 24 h before HI insult. Brain injury was assessed by the survival rate, 2,3,5-triphenyltetrazolium chloride (TTC), Nissl, TUNEL staining and caspase-3, caspase-9 activities after HI. In HBO preconditioned animals, survival rate was increased, infarct ratio was decreased, and the positive stained TUNEL cells were reduced, accompanied by the suppression of caspase-3 and -9 activities. These results indicate that a single HBO-PC appears to provide brain protection against HI insult via inhibition of neuronal apoptosis pathways.

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1. Introduction

Ischemic preconditioning (IPC) or ischemic tolerance induced by a mild stimulation can produce protective effect to a subsequent lethal insult in a variety of organs including heart, kidney, and brain (Kitagawa et al., 1990). IPC or ischemic tolerance has been reported to be induced by diverse stimuli such as seizures (Plamondon et al., 1999), anoxia (Perez-Pinzon et al., 1996), heat (Kitagawa et al., 1991), oxidative stress (Ohtsuki et al., 1992), polyunsaturated fatty acid (PUFA) pretreatment (Maingret et al., 2000), and by inhibition of oxidative phosphorylation (Riepe

et al., 1997). Those IPC methods cannot be used clinically due to the safety concerns.

Hyperbaric oxygen (HBO) may provide IPC without harmful side effects. HBO therapy has been applied in the treatment of carbon monoxide poisoning, stroke, air embolism and decompression sickness (Sahni et al., 2003). Recently, HBO preconditioning (HBO-PC) has been shown to have neuroprotective effects against ischemic injury in gerbil hippocampus (Wada et al., 2001), rabbit spinal cord (Xiong et al., 2001), transient brain infarct in mouse (Prass et al., 2000) and rat (Xiong et al., 2000) or in primary cultured spinal cord neurons (Li et al., 2007).

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Abbreviations: ANOVA, analysis of variance; ATA, atmosphere absolute; BCIP, 5-bromo-4-chloro-3-indolylphosphate; BDNF, brain derived neurotrophic factor; CAT, catalase; HBO, hyperbaric oxygen; HBO-PC, hyperbaric oxygen preconditioning; HI, hypoxic ischemic; HIBD, HI brain damage group; HIF-1 α , hypoxia-inducible factor-1 α ; IPC, ischemic preconditioning; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SOD, superoxide dismutase; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

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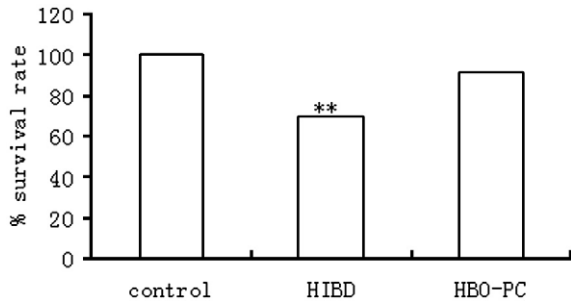


Fig. 1.

Hypoxia-ischemia (HI) is a common cause of brain injury in neonate and causes static encephalopathies in children, by injury of neuronal cells (Aggarwal et al., 2001). The cell death patterns to HI include acute necrosis and delayed apoptosis (Martin et al., 2000; Northington et al., 2001). Apoptosis is a programmed cell death that is characterized by specific ultrastructural changes that include cell shrinkage, nuclear condensation and DNA fragmentation. At the molecular level, apoptosis is activated by the aspartate-specific cysteine protease (caspase) cascade, including the initiative caspase-9 and executive caspase-3 (Elmore, 2007; Rupinder et al., 2007).

The purpose of this study was to investigate whether a single administration of HBO-PC offers neuroprotection by reducing HI-induced caspase-dependent apoptosis.

2. Results

2.1. Mortality

Fig. 1 shows the survival rate that 70% pups (56/80) survived HI injury in the HI alone group (HIBD) which is significantly lower than 91.2% (73/80) pups that survived HI insult in HBO-PC group.

2.2. TTC staining

Fig. 2 shows representative photographs of TTC-stained sections from 14-day-old rats in each group, at seven days after the initial HI insult. The infarct ratio in HIBD group (10.5%) was markedly higher ($P < 0.01$) than that in HBO-PC group (0.868%) which was not significantly different ($P > 0.05$) from normal controls.

2.3. Nissl staining

Fig. 3 shows representative samples of Nissl staining from the cerebral cortex and hippocampus of pups at 24 h after HI insult. Extensive neuronal changes in the cortex and CA1 sector of the hippocampus were noticed with features of considerable dark, pyknotic neurons in HIBD group (B1–4). More Nissl-stained cells (C1–4) were observed in HBO-PC group than in HIBD group ($P < 0.01$).

2.4. TUNEL staining

TUNEL, which is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of fragmented DNA, has become the most widely used in situ test for the study of apoptosis. Fig. 4 shows that TUNEL-positive cells were markedly increased in cortex and hippocampus of HIBD group (B1,B3). HBO-PC reduced the number of TUNEL-positive cells (C1,C3). At higher magnification, the nuclei of cells were clearly stained in both hippocampus and cortex (B2,B4, C2,C4). A few TUNEL-positive cells were identified in samples from normal control pups (A1–4).

2.5. Activities of caspase-3 and -9

The activities of caspase-3 and -9 were measured at 24 h after HI insult as shown in Fig. 5. The activity of caspase-3 was 1.16 ± 0.23 in cortex and 2.18 ± 0.62 in hippocampus in HIBD group. HBO-PC significantly reduced the activity of caspase-3 in the

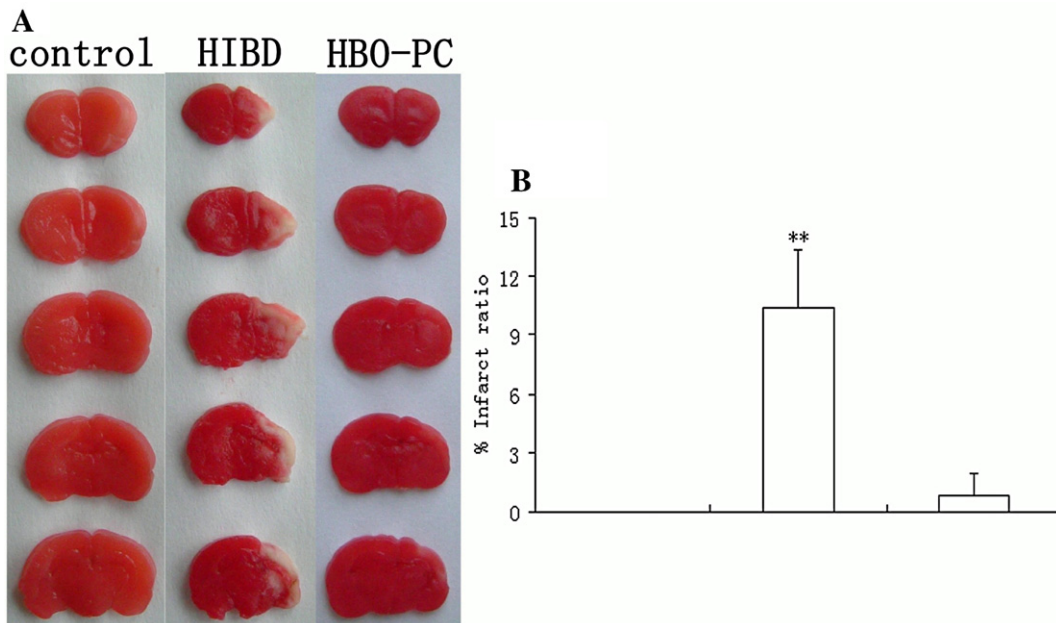


Fig. 2.

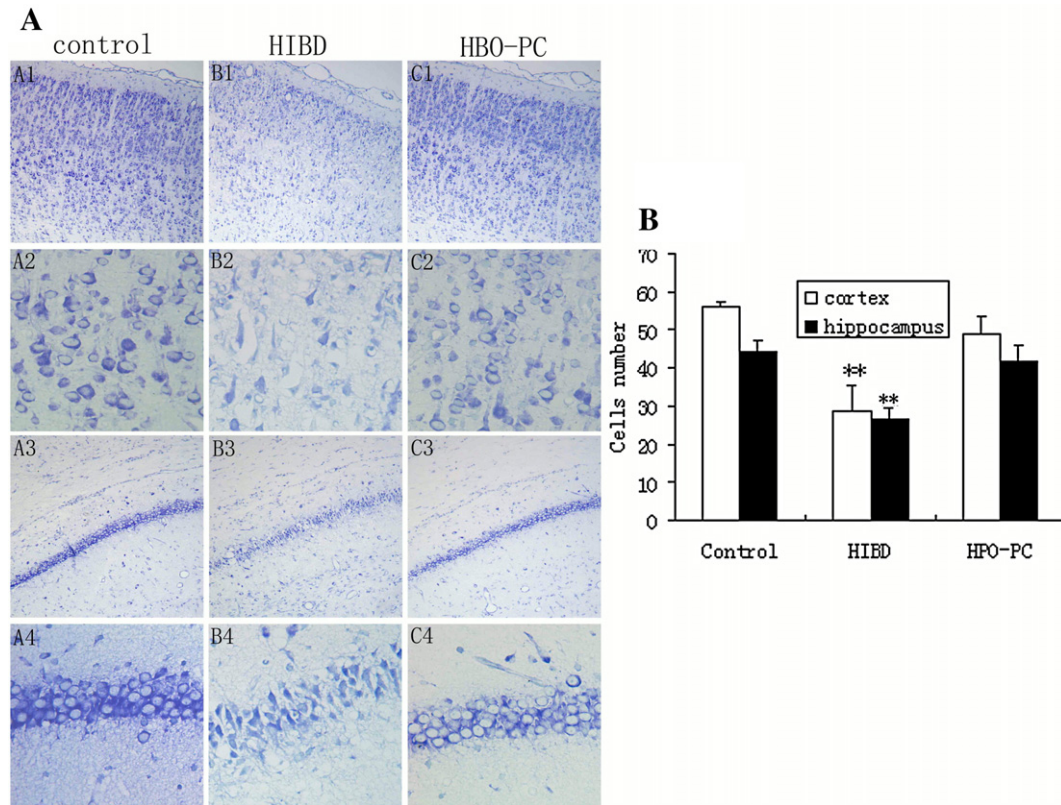


Fig. 3.

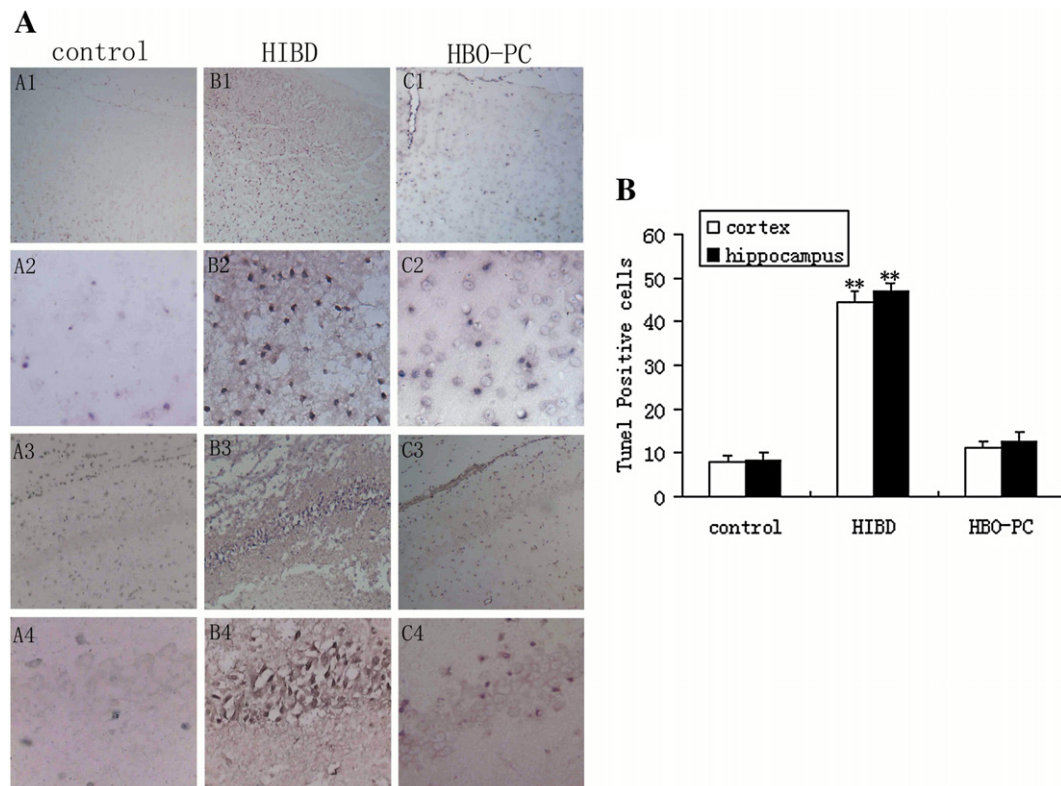


Fig. 4.

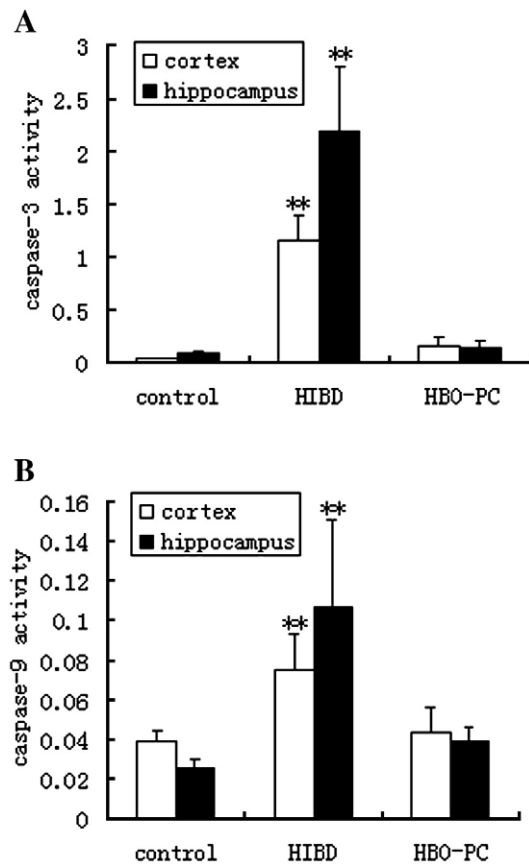


Fig. 5.

cortex (0.16 ± 0.09) and hippocampus (0.15 ± 0.06) ($P < 0.01$ vs. HIBD). Similarly, higher caspase-9 activity was obtained in cortex and hippocampus in HIBD group which was reduced by HBO-PC ($P < 0.01$ vs. HBO-PC).

3. Discussion

HBO-PC has been reported to render brain tissues more resistant to the subsequent lethal hypoxic or ischemic insult (Wada et al., 2001; Prass et al., 2000; Xiong et al., 2000, 2001; Li et al., 2007). However, the optimal timing and duration of HBO-PC have not been established and the five days HBO-PC protocol (Wada et al., 2001) is difficult to apply clinically. An alternative approach was used by prolong normobaric hyperoxia exposure (Dong et al., 2002; Zhang et al., 2004) for 24 h which offers protective effect but may associated with toxicity (Al-Motabagani, 2005). In this study, we tested the neuroprotective effect of a single HBO-PC exposure in a well established HI brain injury pup model. We have found that a single HBO-PC markedly reduced brain injury induced by HI insult and decreased the mortality. The neuroprotective effect of HBO-PC in our study in neonatal hypoxia–ischemia brain injury is consistent with the observation by Freiburger et al. (2006).

The mechanisms of HBO-PC remain unclear. One of the plausible mechanisms is prevention of apoptosis (Dosenko et al., 2006; Falcao et al., 2007; Cserepes et al., 2007). Apoptosis is one of the key factors in brain injury after HI insult and

caspase inhibitors have been reported to provide some neuroprotection in pup models (Pulera et al., 1998; Cheng et al., 1998; Han et al., 2002). Therefore, we examined apoptotic changes after neonatal hypoxia–ischemia insult and the effect of HBO-PC. Morphological and biochemical evidences obtained from this study indicate that HBO-PC reduces apoptosis in rat pup hippocampus and cortex after HI insult, demonstrated by a reduction of TUNEL-positive cells and reduced activities of caspase-3 and -9.

The mechanisms of HBO-PC induced ischemic tolerance remain to be explored. One of the likely candidates is oxidative stress which has been reported to occur after HBO in the forms of superoxide anion, hydrogen peroxide, hydroxyl radicals, and nitric oxide (Benedetti et al., 2004; Conconi et al., 2003). Oxidative stress leads to accumulation of hypoxia-inducible factor-1 α (HIF-1 α) (unpublished observations). Recently, Ostrowski et al. (2008) presented another possible pathway that mediated the protective effects of HBO-PC. Their experiments suggested that multiple HBO preconditioning increased brain derived neurotrophic factor (BDNF) in the cerebral cortex and CA1 area immediately after global ischemia, and suppressed the levels of phosphorylated p38/MAPK, caspase-3, and early apoptosis. Even though different HBO-PC applications were used, our observation indicates that a single HBO-PC reduces the activities of caspase-3 and -9 after HI insult.

In summary, we have observed that a single administration of HBO preconditioning increased the survival rate, decreased the infarct size, reduced the number of apoptotic cells via suppressing the activities of caspase-3 and -9, thereby offered neuroprotective effects. This one time HBO-PC has clinical potentials because it is safe, easy to apply, and is neuroprotective.

4. Experimental procedures

4.1. Experimental groups

Unsexed 7-day-old (day 0, day of birth) Sprague-Dawley rats were randomly assigned to the following three groups: 1) control group (no carotid ligation, hypoxia) ($n=80$), 2) HIBD group ($n=80$), and 3) HBO-PC group ($n=80$). Each group was composed of pups from each litter to obtain parity within the groups. 17 in control, 11 in HIBD and 14 in HBO-PC group were included in the Nissl and TUNEL staining analysis. Same numbers of animals were used for the activity analysis.

4.2. Hypoxia–ischemia model

The model used in this study was based on the Rice–Vannucci model (Vannucci et al., 1999). Pups were housed with the dam under a 12:12 h light–dark cycle, with food and water available ad libitum throughout the studies. These neonatal rats were anesthetized by inhalation with Diethyl Ether. The rats were kept at a temperature of 37 °C as the left common carotid artery of each pup was exposed and ligated with 5-0 surgical sutures. After operation, the pups were returned to the holding container. Anesthesia and surgery time averaged 5 min per pup. Surgery was completed for an entire litter, and the pups were allowed to recover with their dams for 1 h (for rehydration via nursing). Then they were placed in a jar

perfused with a humidified gas mixture (8% oxygen balanced nitrogen) for 90 min. Both the jar and mixture were kept at 37 °C to maintain a constant thermal environment. All surviving pups were returned to their dams after hypoxia exposure.

4.3. HBO preconditioning paradigms

The pups were placed into an animal HBO chamber (100% oxygen, 2.5 atmospheres absolute, ATA) for 2.5 h at 24 h before HI insult. Chamber compression and decompression was performed gradually (0.25 atmospheres/min) using continuous temperature monitoring to avoid compression-related temperature changes of more than 2. Fresh gas ventilation was maintained throughout treatments.

4.4. Measurement of infarct ratio

On postnatal day 14 (at seven days after HI insult), the pups were decapitated and the left brain hemispheric volumes were measured. After decapitation, the brains were quickly removed and placed in cold saline for 5 min, cut at 2-mm intervals from the frontal pole into 5 coronal sections. After incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 8 min at 37 °C, the brain slices were fixed in 4% formalin for 24 h. The volumes of each of the sections were summed by an image analysis system (ImageJ, a public domain image analysis program, developed at the National Institutes of Health). The percentage of infarction (infarct ratio) was calculated by dividing the infarct volume by the total volume of the slices.

4.5. Nissl staining

For Nissl staining, the 4- μ m sections were hydrated in 1% toluidine blue at 50 °C for 20 min. After rinsing with double distilled water, they were dehydrated and mounted with permount. The cortex and the CA1 area of hippocampus from each animal were captured and Imaging-Pro-Plus (LEIKA DMLB) was used to perform quantitative analysis of cell numbers.

4.6. TUNEL staining

TUNEL staining was performed on paraffin-embedded sections by using the in situ cell death detection kit (Roche). According to standard protocols, the sections were de-waxed and rehydrated by heating the slides at 60 °C. Then these sections were incubated in a 20 μ g/ml proteinase K working solution 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. Dried area around sample and added Converter-AP on samples for 1 h at 37 °C. After rinsing with PBS (5 min, 3 times), sections were coloured in dark with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

4.7. Cell counting

Six visual fields (0.6 mm²) of the cerebral cortex and CA1 were photographed in each section. The number of staining cells in each field was counted at higher magnification ($\times 40$). The data were represented as the number of cells per high-power field.

4.8. Caspase activity assay

Brain samples from the cortex and hippocampus were taken from the impaired hemispheres of neonatal rats at 24 h after HI insult. The activities of caspase-3 and -9 were measured with caspase-3/CPP32 Fluorometric Assay Kit and caspase-9/CPP32 Fluorometric Assay Kit (BIOVISION Research Products 980 Linda Vista Avenue, Mountain View, CA 94043 USA). Briefly, brain samples were homogenized in ice-cold cell lysis buffer and kept at 4 °C for 1 h. Brain homogenate was centrifuged (Eppendorf, 5810R) at 12,000 g for 15 min at 4 °C. The supernatant was removed and stored at -80 °C until use. Protein content was measured by using the Enhanced BCA Protein Assay Kit. 20–200 μ g cell lysates were incubated in a 96-well plate with 50 μ l of 2 \times Reaction Buffer. The reaction was started by adding 5 μ l of the 1 mM DEVD-APC substrate. After incubation in the dark at 37 °C, the plate was read in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter.

4.9. Data analysis

All quantitative data are expressed as mean \pm SD. The significance of differences between means was verified by ANOVA followed by Tukey test. For the analysis of cell count results, a non-parametric Kruskal–Wallis ANOVA was used followed by Dunn's test. Mortality rates were analyzed using the chi square test. $P < 0.05$ was considered significant.

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