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Hyperbaric oxygen preconditioning protects lung against hyperoxic acute lung injury in rats via heme oxygenase-1 induction

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ABSTRACT

Hyperoxic acute lung injury (HALI) is a clinical syndrome as a result of prolonged supplement of high concentrations of oxygen. Previous studies have shown hyperbaric oxygen preconditioning (HBO-PC) had a protective effect on oxidative injury. In the present study, we investigated the effect of HBO-PC on HALI in rats. The results demonstrated that HBO-PC ameliorated the lung biochemical and histological alterations induced by hyperoxia, decreased oxidative products but increased antioxidant enzymes. Furthermore, HBO-PC up-regulated heme oxygenase-1 (HO-1) mRNA and activity in lung tissues. The administration of HO-1 inhibitor, Zinc protoporphyrin IX, abolished its protective effects. The data showed that HBO-PC could protect rats against HALI and the anti-oxidative effect may be related to the up-regulation of HO-1.

1. Introduction

Prolonged supplement of high concentrations of O_2 (fractional concentrations of $O_2 > 50\%$) is usually used in patients with cardiopulmonary disease, during major surgical interventions or in neonates with respiratory failure in neonatal intensive care units [1,2]. However, long exposure to high concentrations of O_2 may cause hyperoxic acute lung injury (HALI) and increase mortality, causing a therapeutic dilemma. HALI, a term to describe the pulmonary-specific toxic effects of O_2, is characterized by lung alveolar endothelial and epithelial injury, capillary leak syndrome, and enhanced inflammatory cell recruitment [3,4]. The severity of HALI is directly proportional to pO_2 ($FIO_2 > 0.6$) and exposure time [5]. It is generally accepted that increased generation of reactive oxygen species (ROS), which hereafter overwhelms anti-oxidant defenses, is crucial in the etiology of HALI [6]. Although this therapeutic dilemma is well known to clinicians, no effective treatment is available as yet.

Hyperbaric oxygen (HBO) treatment is now routinely used to treat various disorders, such as carbon monoxide poisoning, gas embolism, decompression sickness and neurologic diseases [7]. Recently accumulating evidence has proven that hyperbaric oxygen preconditioning (HBO-PC) has a protective effect on various injuries related to oxidative stress, such as heart ischemia [8,9], spinal cord injury [10], focal cerebral ischemia [11], traumatic and surgical brain injury [12]. However, the effects of HBO-PC on HALI was not yet explored.

The molecular mechanism of HBO-PC remains unknown, but it is suggested that HBO-PC could up-regulate antioxidant enzymes [13], decrease lipid peroxidation [14] and induce heme oxygenase-1 (HO-1) [15], which may play an important role. HO-1 was accepted as an important protectant for hyperoxic injury [16]. He et al found that the up-regulation of HO-1 expression played an essential role in the protection of HBO-PC against renal I/R injury [17], but Soejima's results showed that HBO-PC decreased HO-1 expression at 24 h after transient MCAO in rats and attenuated hyperglycemia enhanced hemorrhagic transformation [18]. These controversial results raised our curiosity to explore the expression and role of HO-1 in the effects of HBO-PC on HALI. Thereby in the present study, we pretreated rats with four daily 2.0 ATA HBO, then observed its protective effects on HALI induced by constant 95% O_2 exposure for 72 h. Next, we examined the effects of HBO-PC on oxidative products and antioxidant enzymes. HO-1 mRNA level and activity were measured to explore its role in the protective effects of HBO-PC.

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2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats, weighing 220–250 g, were used in the study. The study was approved by the institutional animal care and use committee in Changhai hospital animal center and carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The animals were housed in individual cages in Changhai hospital animal center with access to food and water *ad libitum*. The facility was maintained under standard conditions (22 °C room temperature, 33% humidity) with a 12 h light/dark cycle.

2.2. Experimental protocol

Rats were randomized into five groups (10 rats per group): Control, NBA-PC, NBO-PC, HBA-PC and HBO-PC. The treatments were as follows:

(1) Normal group: received no pretreatment or hyperoxic exposure;
(2) Control group: received four daily normobaric air pretreatment, rested for 24 h, then exposed to 95% O_2 for 72 h;
(3) NBO-PC group: received four daily normobaric oxygen pretreatment, rested for 24 h, then exposed to 95% O_2 for 72 h;
(4) HBO-PC group: received four daily hyperbaric oxygen (2.0 ATA) pretreatment, rested for 24 h, then exposed to 95% O_2 for 72 h;
(5) HBA-PC group: received four daily hyperbaric air (2.0 ATA) pretreatment, rested for 24 h, then exposed to 95% O_2 for 72 h;

For NBA-PC or NBO-PC treatment, animals were placed in cages in a 7-L Plexi glas chambers and exposed continuously to air or 100% O_2 for 1 h per day. For HBA-PC or HBO-PC treatment, the chamber was first ventilated with 100% air or O_2 at a flow rate of 20 L/min to minimize CO_2 accumulation. Then the pressure in the chamber was increased at a rate of 1.0 ATA/min to 2.0 ATA and maintained for 1 h. After the exposure was done, rats were then decompressed at a rate of 1.0 ATA/min. For Zinc protoporphyrin IX (ZnPP-IX) treatment, ZnPP-IX (Sigma, MO, United States) was given intraperitoneally (3 mg/kg of body weight) daily during HBO-PC exposure.

2.3. Hyperoxic exposure

Animals were placed in cages in a 7-L Plexi glass chambers exposed continuously to 95% O_2 for 72 h. The gas in the chamber was continuously ventilated with O_2 (with a flow rate of 1 L/min) to minimize pCO_2 changes. The CO_2 and O_2 concentrations were recorded with an analyzer equipped with a strip-chart recorder (Servomex, MA, USA). The animals were supplied food and water *ad libitum* during the exposure. The temperature in the chamber was maintained at 22–25 °C and the humidity was routinely 60%–70%. Once a day, chambers were open for 10 min to supply water, food and new bedding.

2.4. Sample collection

After hyperoxic exposure, the rats were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneal injection). After lungs were exposed, bronchoalveolar lavage fluid (BALF) were taken from the left lung with 4 mL phosphate balanced saline (PBS) in 2.5-mL aliquots after cannulation of the left trachea. 1 mL of the BALF was used for total cell count. The remaining BALF was centrifuged at 1,000*g* for 10 min, then the supernatant was collected and stored at −80 °C for later protein and lactate dehydrogenase (LDH) activity assays. Next, the lungs were removed en bloc and drained of blood. The right lung was cut in half. One half for hematoxylin and Eosin (H&E) assay; the other half for oxidative products, antioxidant enzymes and HO-1 expression assays.

2.5. Total cell count, total protein and LDH measurement in BALF

The total cell numbers in BALF was counted on a fresh fluid specimen using a hemocytometer. Total protein content in BALF was determined with BCA protein assay reagents (Pierce, Rockford, IL, USA). The activity of LDH was quantified with the LDH measurement kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. LDH activity was expressed as U/L, using a LDH standard.

2.6. Measurement of malondialdehyde (MDA), protein carbonyl and 8-hydroxy-2-deoxyguanosine (8-OHdG) in lung tissues

100 mg Lung tissue was homogenized in 2 mL PBS on ice. After centrifugation at 12,000*g* for 20 min, the MDA and 8-OHdG content in the supernatant were measured using the corresponding kits.

MDA content was measured with a commercial kit (Nanjing Jiancheng, Nanjing, China). It was determined with thiobarbituric acid (TBA) fluorometric method at 553 nm with excitation at 515 nm using 1,1,3,3-tetramethoxypropane as the standard. The method was to obtain a spectrophotometric measurement of the color produced during the reaction of TBA with MDA at 553 nm.

For 8-OHdG assay, DNA was extracted from the tissue with a DNA Extraction Kit (DNA Extractor Wb Kit, Wako Chemical; Osaka, Japan). Then the isolated DNA was digested by the method of Valls-Belles et al. [19]. DNA was washed twice with 70% ethanol, dried, and dissolved in 200 μL of 10 mM Tris–HCl (pH 7.0) for its enzymatic digestion. Next, DNA samples were added to plate wells pre-coated with anti-8-OHdG antibody (Fukuroi, Japan), then incubated for 45 min at 37 °C. After washed for 3 times, the wells were sequentially treated with IgG for 30 min and Streptavidin-Horseradish Peroxidase for 30 min. A substrate containing 3,3′,5,5′-tetramethylbenzidine was added and the wells were incubated for 15 min. Then reaction was terminated by the addition of a sulphuric acid. Finally the absorbance was read at a wavelength of 450 nm.

Protein carbonyls were measured using a commercial quantitative assay kit (Cayman Chemical, CA, USA). Samples were homogenized in PBS containing 1 mM EDTA, pH 6.7, and then centrifuged at 10,000*g* for 5 min under 4 °C. The supernatant was collected for assay. The protein concentration was determined using a standard BCA protein assay kit. All the oxidative products levels were expressed as nmol/mg protein.

2.7. Measurement of SOD, catalase (CAT) and GSH-px activities in lung tissues

Lung tissues were collected and washed in saline, and then homogenized immediately on ice in 1 mL saline. The homogenates were centrifuged at 3,000*g* at 4 °C for 15 min. Then supernatant was collected for SOD, CAT and GSH-px assay using chemical colorimetry assay kits from Beyotime Institute of Biotechnology (Shanghai, China). SOD activity was measured using nitroblue tetrazolium (NBT) reduction assay. One unit of SOD is defined as the amount that shows 50% inhibition. CAT activity was quantified according to the kit manufacturer's instructions, in which the initial rate of hydrogen peroxide decomposition was determined. GSH-px activity was determined by the method of Rickett [20].

All of these enzymes were expressed as units per milligram tissue (U/mg).

2.8. Real-time (RT)-PCR analysis

Lung tissues were collected and washed in saline, then homogenized immediately on ice in 1 mL saline. The total RNA was isolated with Isogen (Nippon Gene, Toyama, Japan), then reverse transcribed into cDNA and used for RT-PCR analysis (Invitrogen, CA, USA). The PCR was first pre-amplified under 50 °C for 2 min and 95 °C for 10 min, then amplified under 95 °C for 15 s and 60 °C for 1 min for 40 cycles. The products were resolved by 1.5% agarose gel electrophoresis and then stained with ethidium bromide. It was finally photographed under ultraviolet light for quantification. GAPDH mRNA expression was used as a reference gene.

2.9. HO-1 activity assay

The HO-1 assay was performed by the method of Velislava et al. [21]. Briefly, the lung tissue was homogenized in 4 °C homogenization buffer (1 M Tris–HCl, 250 mM sucrose). The homogenized tissue was then centrifuged at 12,000*g* for 30 min at 4 °C. The supernatant was collected and incubated with heme (18 μM), biliverdin reductase (30 U mL^{-1}) and NADPH (2.75 mM). The reaction was conducted in the dark for 40 min at 37 °C and terminated by placing the samples on ice for 10 min. The amount of extracted bilirubin was calculated by the difference of absorbance between 470 and 530 nm using an extinction coefficient of 40 mM^{-1} cm^{-1}. HO-1 activity was expressed as pmol bilirubin min^{-1} mg $protein^{-1}$.

2.10. H&E staining

The lower lobe of the left lung were harvested and flushed with saline, transferred to 4% formaldehyde for 48 h, and embedded in paraffin. Next, butterfly shaped sections of 4-mm thickness were cut and stained with H&E staining for histopathological analysis.

2.11. Statistical analysis

Values were presented as mean ± SD. Statistical analysis was done using the SPSS 17.0 with one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Changes in LDH, total cells and protein concentration in BALF

Rats exposed to hyperoxia exhibited significant increase in the LDH level in BALF compared to Normal. A significant decrease of LDH was observed in HBO-PC group compared to that in Control group. Total cells and protein concentration in the BALF showed similar trend, which were significantly increased in all groups compared to Normal group ($P < 0.05$). The increase in the HBO-PC group, however, was significantly less than Control group. There was no significant difference in these biomarkers between NBO-PC, HBA-PC and Control (Table 1).

Table 1
Analysis of LDH, total cells and protein concentration in the BALF from rats exposed to 95% oxygen for 72 h.

Groups	LDH (U/mL)	Total cells (1×10^4/mL)	Protein concentration (μg/mL)
Normal	10.53 ± 0.75	19.33 ± 2.08	37.53 ± 4.46
Control	66.23 ± 3.75※	69.86 ± 6.78※	95.63 ± 6.44※
NBO-PC	75.89 ± 3.57※	67.23 ± 7.03※	90.47 ± 6.35※
HBA-PC	63.86 ± 4.12※	59.63 ± 4.29※	76.98 ± 5.07※
HBO-PC	23.86 ± 2.13※,△	28.44 ± 2.51※,△	37.63 ± 2.42※,△

Normal group received no pretreatment or hyperoxic exposure; Control group received normobaric air pretreatment before exposed to hyperoxia; NBO-PC group received four daily normobaric oxygen pretreatment before exposed to hyperoxia; HBO-PC group received four daily hyperbaric oxygen pretreatment before exposed to hyperoxia; HBA-PC group received four daily hyperbaric air pretreatment before exposed to hyperoxia; values are the mean ± SD. $N = 10$ per group.
※ $P < 0.05$ compared to Normal group.
△ $P < 0.05$ compared to Control group.

3.2. Protein carbonyl, 8-OHdG and MDA Measurements in Lung Tissues

As shown in Fig. 1, lung-tissue protein carbonyl, 8-OHdG and MDA assays revealed significant lung oxidative stress changes in the Control group ($P < 0.05$ compared to Normal). HBO-PC significantly decreased the protein carbonyl, 8-OHdG and MDA levels compared with those in Control rats ($^*P < 0.05$ versus Control). NBO-PC group and HBA-PC group showed no significant reduction of protein carbonyl, 8-OHdG or MDA levels ($P > 0.05$ versus Control).

3.3. Effect of HBO-PC on antioxidant enzymes GSH-px, CAT and SOD activities

Colorimetry assay showed that GSH-px, CAT and SOD activities were markedly decreased in the Control group compared with Normal group (Fig. 2, $P < 0.05$). HBO-PC significantly inhibited the reduction of GSH-px, CAT and SOD activities ($P < 0.05$). NBO-PC and HBA-PC group showed no significant change of GSH-px, CAT or SOD activities compared to Control group ($P > 0.05$). Treatment with HBO-PC alone (no hyperoxia exposure) significantly increased SOD activity ($P < 0.05$ compared to Normal), while GSH-px or CAT activity was not altered.

3.4. HO-1 mRNA level and activity

RT-PCR showed that hyperoxia treatment slightly increased HO-1 mRNA level in lung tissues (Fig. 3A). After HBO-PC treatment, the HO-1 mRNA level was greatly enhanced compared to that in hyperoxia-treated rats ($P < 0.05$ compared to hyperoxia group). After rats were given ZnPP-IX, HO-1 mRNA was significantly decreased to a level close to hyperoxia group ($P < 0.05$ compared to HBO-PC group; $P > 0.05$ compared to hyperoxia group). Similar effects were observed in the HO-1 activity assay (Fig. 3B).

3.5. Lung histopathology changes

As shown in Fig. 4, the lung tissues were severely injured in the Control group, NBO-PC group and HBA-PC group, with notable lung hemorrhage, edema, alveolar septal thickening, influx of inflammatory cells, and fibrin deposition. In HBO-PC group, moderate hemorrhage, edema and inflammatory cell infiltration were evident, indicating reduced HALI severity. The administration of ZnPP-IX successfully counteracted the protective effects of HBO-PC, as demonstrated by severe lung histopathological changes.

4. Discussion

The present work was to explore the effects of HBO-PC on HALI. The HALI was assessed by the level of LDH, total cell count and protein concentration in the BALF, as well as histological analysis by H&E staining. Both biochemical and histopathological assays showed that HBO-PC effectively reduced the severity of HALI in rats. Rats in the HBO-PC group exhibited dramatic decreases in

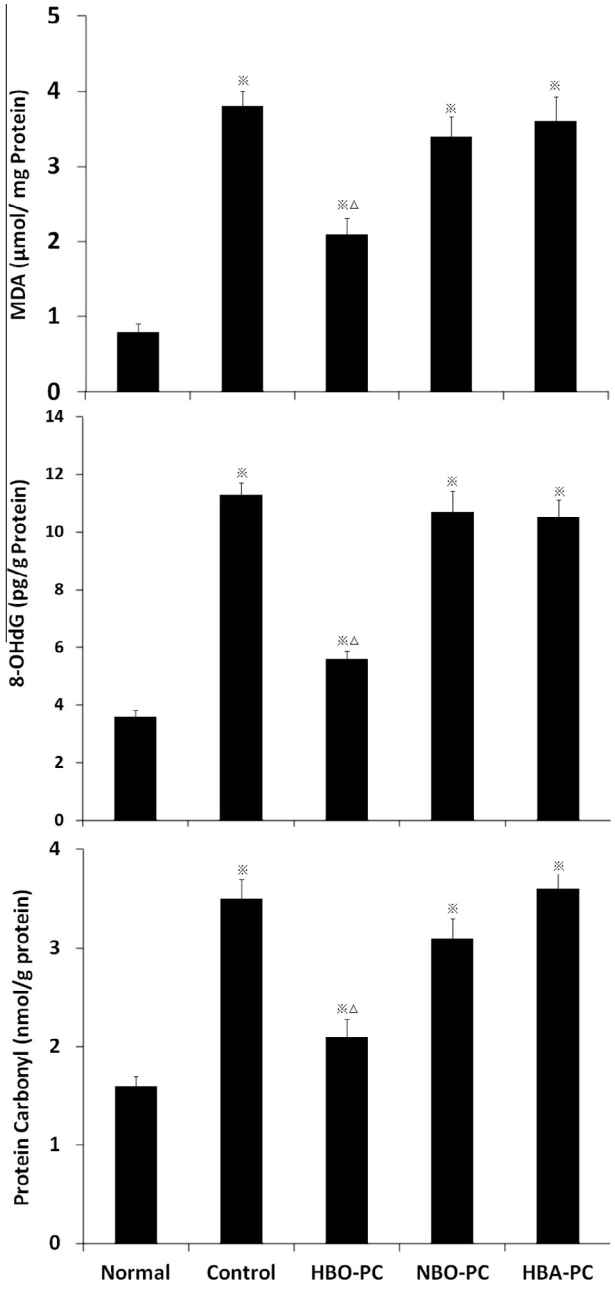

Fig. 1. Protein carbonyl, 8-OHdG and MDA in lung tissue. Lung oxidative injury was measured using Protein carbonyl, 8-OHdG and MDA assays. Normal group, Control group, NBO-PC group, HBO-PC group and HBA-PC group received treatment as described earlier in Table 1 legend. Values are the mean ± SD. ※$P < 0.05$ compared to Normal group; △$P < 0.05$ compared to Control group. $N = 10$ per group.

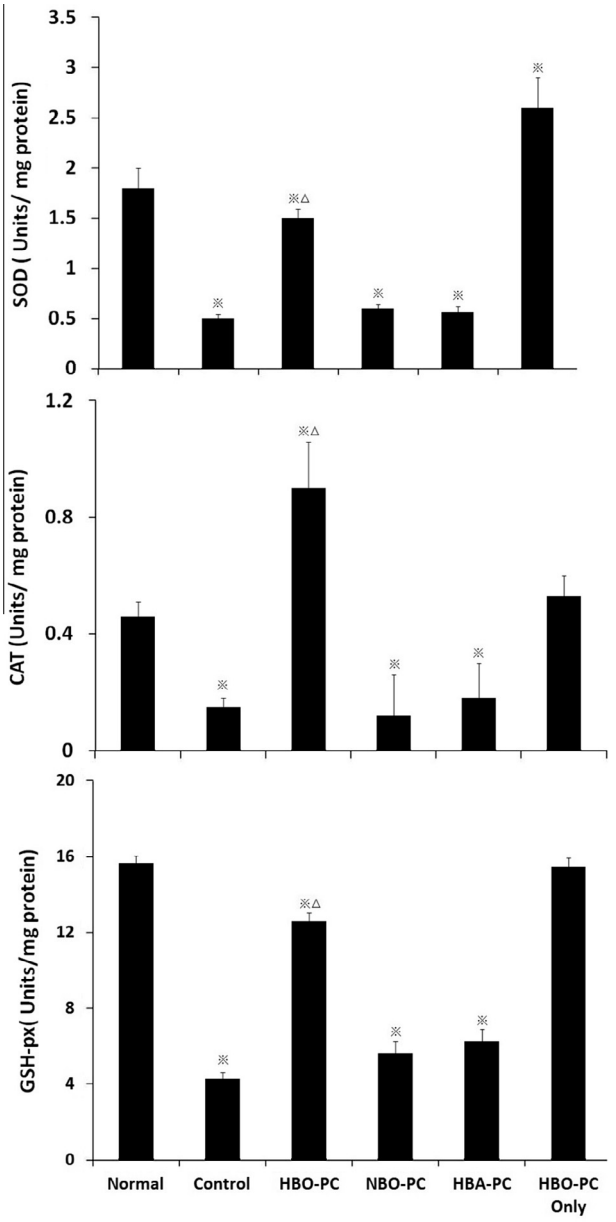

Fig. 2. GSH-px, CAT and SOD activities in lung tissues. Lung antioxidant enzymes were measured using protein carbonyl, 8-OHdG and MDA assays. Normal group, Control group, NBO-PC group, HBO-PC group and HBA-PC group received treatment as described earlier in Table 1 legend. HBO-PC only group received four daily hyperbaric oxygen pretreatment but no hyperoxia exposure. GSH-px, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase. Values are mean ± SD. ※$P < 0.05$ compared to Normal group; △$P < 0.05$ compared to Control group. $N = 10$ per group.

lung injury, as manifested by decreased cell injury biomarkers and less morphologic abnormality.

HALI has been a major concern for critically ill patients who need long time treatment of high concentrations of O_2 to get sufficient O_2 support [22]. The pathogenesis of HALI is believed to be mediated by the production and accumulation of toxic levels of ROS, which overwhelm cellular antioxidant defenses [6]. Excess ROS can cause injury to lung cells directly [23] and indirectly by activating inflammatory cells [24]. As oxidative stress plays an essential role in the development of HALI, we assessed three parameters of oxidative injury (MDA, protein carbonyl and 8-OHdG) and three antioxidant enzymes (SOD, CAT and GSH-px). The results showed that hyperoxic exposure caused oxidative injury to protein, lipid and DNA, which may at least partly contribute to HALI. HBO-PC effectively inhibits these oxidative parameters and restored antioxidant enzymes. It was also found that HBO-PC treatment alone could enhance the level of SOD. The mechanism of ROS production and elimination in HALI remains unclear, but it is believed that excess O_2 could produce superoxide anion (O_2^-) by obtaining a electron in mitochondrial respiration (most notably by Complex I and Complex III). O_2^- could be further converted into more deleterious ROS, including hydroxyl radical. SOD can first catalyze the dismutation of O_2^- into O_2 and H_2O_2, which was in turn decomposed to H_2O and O_2 by catalase [25] or glutathione

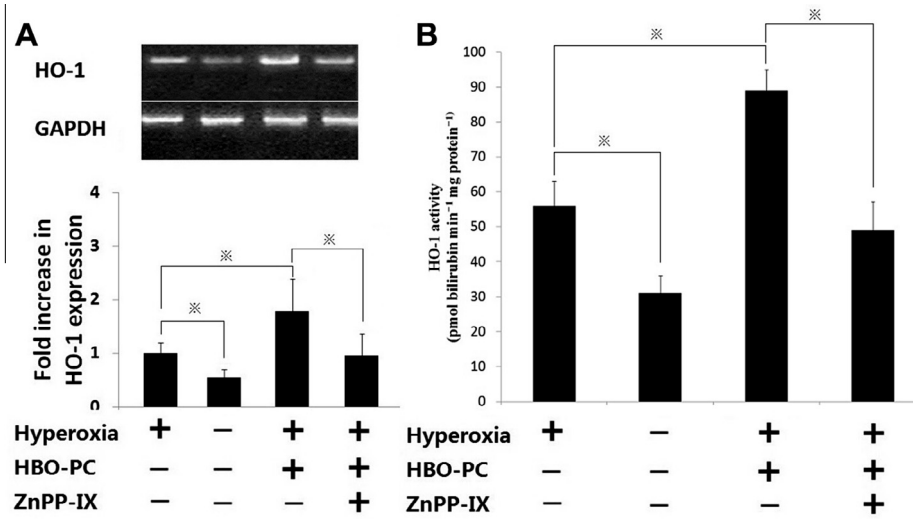

Fig. 3. Effects of HBO-PC and ZnPP-IX on HO-1 mRNA level and activity. HO-1 mRNA level was analyzed with RT-PCR assay (A) and HO-1 activity was measured with biliverdin reductase reaction. Hyperoxia, exposed to 95% O_2 for 72 h; HBO-PC, pretreated with four daily hyperbaric oxygen exposures. ZnPP-IX, received ZnPP-IX intraperitoneally daily during HBO-PC exposure. Values are expressed as mean ± SD. ※$P < 0.05$ between compared groups. $N = 10$ per group.

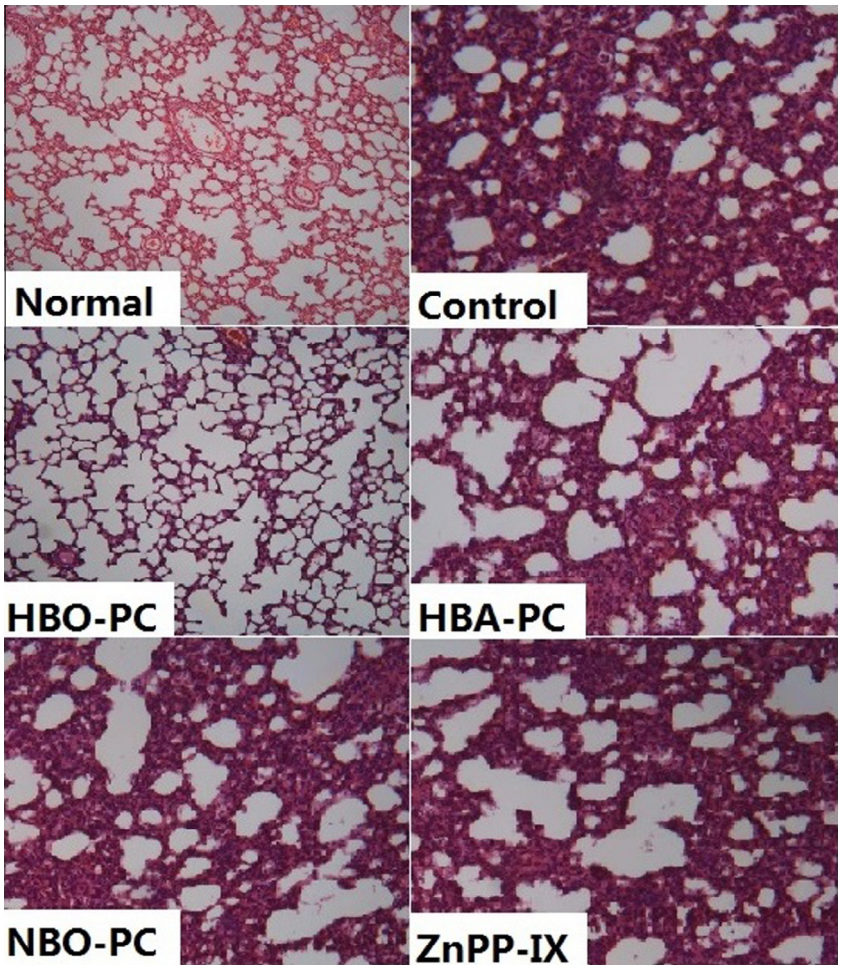

Fig. 4. Effects of HBO-PC and ZnPP-IX on histopathological examination. Lung histopathological injury was measured using H&E staining. Normal group, Control group, NBO-PC group, HBO-PC group, HBA-PC group and ZnPP-IX group received treatment as described above. Notable lung hemorrhage, edema, alveolar septal thickening, influx of inflammatory cells, and fibrin deposition were observed in rats in Control group, NBO-PC group, HBA-PC group and ZnPP-IX group. Significant differences were observed in lung histology between HBO-PC group and other groups.

peroxidase (GSH-px) [26]. HBO-PC is believed to increase antioxidant enzymes, such as catalase, GSH and SOD to scavenge ROS [14] and prevent ROS-induced lipid peroxidation in membranes [13]. In the present study, it was showed that HBO-PC treatment alone did not increase the activities of CAT and GSH-px directly, but significantly enhanced the activity of SOD. HBO-PC was reported to up-regulate the activity of SOD in several studies [27–29], in which it is not clear whether HBO-PC could directly increase SOD activity or indirectly through eliminating ROS and decreasing oxidative stress. The present study clarified that HBO-PC directly increased SOD activity, not CAT or GSH-px activities. The different stimulating effects of HBO-PC on these enzymes needs further investigation.

HO-1 plays a critical protective role in HALI [30,31]. Inhibition of HO-1 function by silencing of HO-1 increased animal mortality in hyperoxia [31], while over-expressing HO-1 significantly reduced pulmonary edema, parenchymal inflammation, and apoptosis after hyperoxia [32]. The results of RT-PCR and HO-1 activity in the present study showed that: (1) HO-1 mRNA and activity was slightly enhanced after hyperoxic exposure; (2) HBO-PC significantly up-regulated HO-1 mRNA and activity in lung tissues, and the administration of HO-1 inhibitor ZnPP-IX reversed the HO-1 expression and activity. To confirm the effects of HBO-PC and HO-1, we investigated the histopathologic changes in lungs. Striking differences were observed between hyperoxic group and HBO-PC group. HBO-PC group exhibited less lung hemorrhage, edema and influx of inflammatory cells. Administration of ZnPP-IX, however, abolished the protective effects of HBO-PC, indicating that up-regulation of HO-1 was involved in the protective effects of HBO-PC.

Many studies have explored the mechanism of the protective role of HO-1 against oxidative injury, although no agreement was accomplished. One explanation is that HO-1 catalyzes the degradation of free heme, which is toxic when released from injured lung cells induced by hyperoxia and causes cell damage in two ways: (1) directly induces lung cells injury; (2) produces toxic hydroxyl radicals [33]. Another assumption is that carbon monoxide (CO), a catalytic byproduct of HO-1 degradation of heme, plays an important role in the cytoprotection against oxidant-induced lung injury [34]. Zhang et al. found out that STAT3 is essential for the protective effects of HO-1 in oxidant-induced lung injury [35]. STAT3-deficience abolished HO-1's protective effects against lung injury, while STAT3 over-expression increased Akt, Bcl-2, and Bcl-xL expression in lung, which were shown to provide protection against hyperoxia [36]. Which specific mechanism was involved in the present study needs further exploration.

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