The Journal of Physiological Sciences (2019) 69:779–790
https://doi.org/10.1007/s12576-019-00694-5

ORIGINAL PAPER

# Intermittent hyperbaric oxygen exposure mobilizing peroxiredoxin 6 to prevent oxygen toxicity

Lichao Zhang[1] · Yanan Zhang[2] · Zhongzhuang Wang[3] · Yuliang Chen[4] · Runping Li[2]

Received: 11 March 2019 / Accepted: 26 June 2019 / Published online: 8 July 2019


## Abstract

Intermittent hyperbaric oxygen exposure (IE-HBO) can protect the body against oxygen toxicity, but the underlying mechanisms are not very clear. Peroxiredoxin 6 (Prdx6) is a special endogenous antioxidative protein. We explored if the protective effects of IE-HBO are related to Prdx6. Mice were exposed to 280 kPa $O_2$ for 60 min, followed by 30-min exposure to 20% $O_2$/$N_2$ mixture with equal pressure, repeated for six cycles. The Prdx6 protein level and non-selenium glutathione peroxidase (NSGPx) activity in the brain and lungs were then measured and the injury degree of lung and the oxidation level of brain and lung were evaluated. On this basis, the relationship between Prdx6 and IE-HBO's protection was explored. Generally, both IE-HBO and continuous exposure to HBO (CE-HBO) could increase the protein and mRNA levels of Prdx6, and such increases were more significant 24 h after cessation of exposure; moreover, the Prdx6 level of IE-HBO was higher than that of CE-HBO in both brain and lung, also more significantly 24 h after cessation of exposure. In addition, IE-HBO exposure could more effectively potentiate the activity of NSGPx and increase GSH content in brain and lung tissues. At the same time, it could reduce oxidation products in these tissues. IE-HBO could also provide protection for the lungs against injuries resulting from prolonged HBO exposure. These data showed that IE-HBO can potentiate the production and the activity of Prdx6 and consequently mitigate oxidative damages in brain and lungs. The influences of IE-HBO on Prdx6 may form an important basis for its protection against oxygen toxicity.

**Keywords** Hyperbaric oxygen · Oxygen toxicity · Intermittent exposure · Peroxiredoxin 6 · Prevention

## Introduction

Prolonged exposure to oxygen at an increased partial pressure may produce damage to body tissues, with symptoms mostly observed in the central nervous system and lung, namely oxygen toxicity [1, 2]. It is a major concern in the application of hyperbaric oxygen in diving operation, clinical hyperbaric oxygen therapy, and submarine emergency [3, 4]. For example, in the US Navy Diving Manual, for decompression illness therapy, the treatment Table 6 and 6A, which are widely used in many countries, formulating the oxygen breathing duration to be 4 h, or even could be lengthened up to more than $6\frac{1}{2}$h to the max. For the decompression procedure with oxygen in a decompression chamber after diving operations at great depth, the oxygen breathing duration can reach 4 h in some cases, according to helium–oxygen decompression table in US Navy Diving Manual. It has long been known that during long-term exposure to hyperbaric oxygen, intermittent exposure to equally pressurized air or other gas mixtures with relatively lower oxygen partial pressure for a short time can significantly delay occurrence of oxygen toxicity. This approach has been widely applied in reality to prevent oxygen toxicity. However, the underlying mechanisms are still unclear [5, 6]. Clarifying the protective mechanisms of IE-HBO would help people find more optimal HBO exposure schedules for different applications and,

Lichao Zhang and Yanan Zhang contributed equally to this work.

✉ Runping Li
smartrpli@163.com

[1] Department of Pharmacy, Shanghai Hospital of Traditional Chinese Medicine, Shanghai, China

[2] Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai 200433, China

[3] Department of Pharmacy, Changhai Hospital, Second Military Medical University, Shanghai, China

[4] Department of Nautical and Aviation Medicine Center, Navy General Hospital, Beijing, China

at the same time, would also be in favor of deeply understanding the pathogenesis of oxygen toxicity.

HBO exposure can stimulate free oxygen radical production, leading to excessive generation of reactive oxygen species (ROS), including $O_2{\cdot}^-$, $H_2O_2$, NO, $ONOO^-$, and ·OH. The oxidative stress induced by these ROS can damage cells and tissues by oxidizing biomolecules [7–9]. In addition, ROS play important roles in cellular metabolism and signal transduction, and are thus of great pathophysiologic importance [10, 11].

Peroxiredoxins (Prdxs) are a ubiquitous family of antioxidant enzymes identified in recent years. There are six peroxiredoxins in mammals, namely Prdx1–6. They are vital cellular detoxification molecules that catalyze the reduction of peroxide substrates using a redox-active cysteine in the active site [12, 13]. We hypothesized that IE-HBO might activate Prdxs so as to eliminate ROS, thus ameliorating oxygen toxicity [14–16]. Herein we investigated the influences of IE-HBO on the expression and reactive activity of Prdx6, a special member of the Prdxs family, in mouse lung and brain.

## Materials and methods

### Animals and groups

All procedures were performed in accordance with Second Military Medical University (SMMU) Guide for the care and use of laboratory animals, and approved by the ethics committee for Animal Experiments of SMMU. Animals were either deeply anesthetized with halothane before killing by decapitation or killed by cervical dislocation.

Male adult C57BL/6 mice with a body weight between 20 and 22 g were purchased from B&K Universal Group Limited, Shanghai. They were divided into five groups: control (not exposed to HBO), continuous HBO exposure (CE), intermittent HBO exposure (IE), 24 h-post-continuous HBO exposure (24 h-PCE), and 24 h-post-intermittent HBO exposure (24 h-PIE) groups.

### Hyperbaric oxygen exposure

Mice were placed in the chamber designed for animal experiment. Before being pressurized, the chamber was flushed with oxygen until the oxygen concentration inside was above 99% as indicated by an oxygen sensor. Then chamber pressure was gradually increased to 280 kPa. The regimen of continuous and intermittent HBO exposure was according to that of Chavko et al. [17]. The CE group was continuously exposed to 280 kPa oxygen for 6 h. The IE group was exposed to 280 kPa $O_2$ ($PO_2$ 280 kPa) for 60 min, followed by a 30-min exposure to 280 kPa $O_2/N_2$ mixture (20% $O_2$, $PO_2$ 56 kPa). This exposure cycle was repeated six times. The HBO exposure regimen was graphically illustrated in Fig. 1.

### Determination of survival time

The CE and IE HBO exposure regimen was graphically presented in Fig. 1. For determination of survival time, after 6-h continuous exposure and six cycles of intermittent exposure, mice were successively and continuously exposed to pure oxygen at the same pressure, until they were dead. Times from the beginning of the subsequent continuous exposure in pure oxygen to the death were recorded as survival time.

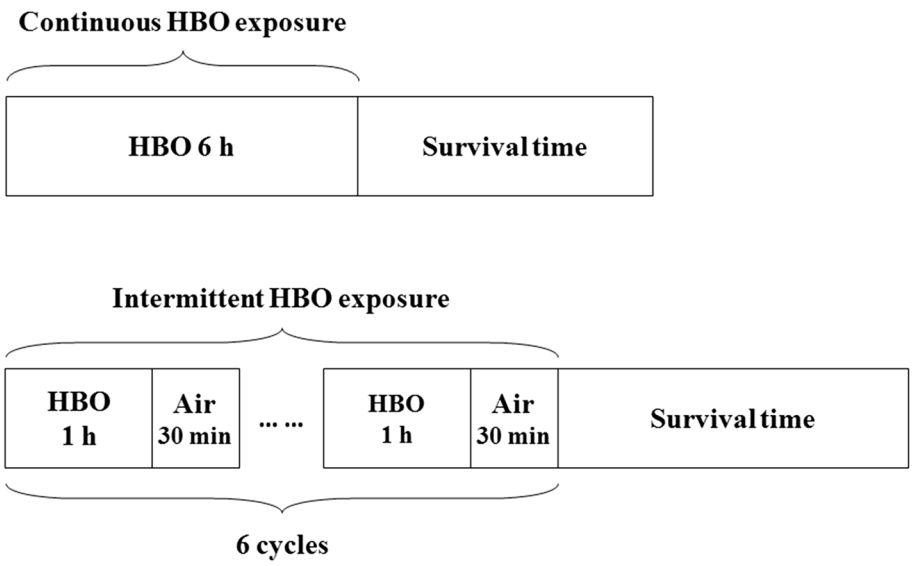


**Fig. 1** HBO exposure regimen

### Histological examination of lung tissues

At the scheduled detecting time point after exposure, mice were removed from the chambers, then immediately anesthetized with pentobarbital and sacrificed by cervical dislocation. Lungs were removed from the thorax and inflated via a cannula by gentle infusion of fixative (10% phosphate-buffered formalin, pH 7.0) over 5 min to reach a constant fluid pressure of 25 cm$H_2O$. The trachea was tied with a ligature, and the lungs were placed in a glass vial containing 10% formalin and kept on ice for 24 h. After fixation, individual lung lobes were embedded in paraffin and blocks were sectioned. Then they were stained with hematoxylin and eosin (H&E). The sections were observed and evaluated by a histopathology expert who was "blinded" to the experimental grouping.

### MDA and $H_2O_2$ measurement

Malondialdehyde (MDA) and hydrogen peroxide ($H_2O_2$) were assayed according to the instructions of assay kits from the manufacturer (Beyotime Institute of Biotechnology, China). $H_2O_2$ was assayed with colorimetric method, which was based upon formation of a complex between Xylenol Orange and ferric iron. Ferric iron was produced by the peroxide dependent oxidation of ferrous iron. This reaction is quantified colorimetrically. MDA was also assayed with colorimetric method. In the assay protocol, the MDA in the sample reacts with thiobarbituric acid (TBA) to generate a MDA-TBA adduct, which can be easily quantified colorimetrically. Protein concentration was determined using BCA Protein Assay kit (Beyotime Institute of Biotechnology, China). MDA and $H_2O_2$ levels were normalized against protein (μmol/g protein).

### Measurement of water content in lung tissue

At the scheduled detecting time point after exposure, immediately after abdominal aorta exsanguination, lungs were excised en bloc and dissected away from the heart and thymus. The right lung tissues were immediately weighed and then placed in a drying oven at 60 °C for 96 h to stabilize dry weight. The ratio of (wet weight − dry weight)/wet weight was used to quantify lung water content.

### BALF collection and total protein concentration measurement in BALF

At the scheduled detecting time point after exposure, bronchoalveolar lavage fluid (BALF) was collected. After cannulation of the main bronchus, mice were irrigated with 1000 μl 5% saline solution to the total lung, repeated three times, at last 600 μl BALF could be got. The collected BALF was centrifuged at 1000 × *g* for 10 min. Protein concentration was determined using the BCA Protein Assay kit (Beyotime Institute of Biotechnology, China).

### Immunohistochemical detection of Prdx6 expression in lung and brain tissues

At the scheduled detecting time point after exposure, mice were exsanguinated by abdominal aorta, the lung and brain tissues were removed and then transferred to 4% formaldehyde for at least 24 h. Then the tissues were paraffin embedded, and sections of 5-mm thickness were cut. Samples were incubated with rabbit anti-Prdx6 antibodies (diluted 1:500, Abcam, UK) at 4 °C overnight, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz, USA) at 37 °C for 1 h. Immunostaining was visualized by DAB incubation. Negative controls were samples incubated with PBS instead of the primary antibody. Image-Pro Plus 6.0 software was used to analysis immunohistochemistry image.

### Western blotting

Mice were sacrificed and the lung and brain tissues were collected immediately. After being washed with PBS and removing residual water, samples were homogenized in precooled lysis buffer. Lysates were cleared by a centrifugation at 12,000 × *g* at 4 °C and supernatants were transferred to a new tube where protein concentration was determined. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (AMERSCO, USA). The PVDF membrane was blocked with 5% fat-free milk at room temperature for 1 h and then incubated with anti-Prdx6 antibodies (diluted 1:1000, Abcam, UK) at 4 °C overnight. Membranes were washed with TBST and incubated with secondary antibody (diluted 1:1000, Santa Cruz, USA) at room temperature for 2 h. After extensive washing, membranes were incubated with ECL (Pierce, USA) to detect Prdx6 proteins. The internal control was β-actin, and mouse monoclonal anti-actin antibody (1:2000) was purchased from Sigma and goat anti-mouse IgG (1:2000) from Santa Cruz. Intensity of Prdx6 protein bands was normalized to that of β-actin and the results were calculated as relative protein levels. The relative protein level of the control group was considered as one and those levels of the other groups were made relative to the control group.

### mRNA quantitation by qRT-PCR

Total RNA was extracted from treated brain and lung tissues using Trizol Reagent (Invitrogen Life Technologies, USA) according to standard procedure and quantified by spectrophotometry. Reverse transcription was

conducted using RevertAid First Strand cDNA Synthesis Kit (Thermo, USA). 2 μg total RNA of each group was used for reverse transcription into cDNA with 1 μl oligo dT15 primer. Then q-PCR was performed with FastStart Universal SYBR Green Master (Rox) (Roche, Switzerland) and a 7300 Real Time PCR System (ABI, USA) according to the manufacturer's instructions. All transcripts were normalized to β-actin as internal control. Data were analyzed using MxPro-Mx3005P 4.1 software. The following primers were used: β-actin, forward 5′-GTGACGTTG ACATCCGTAAAGA-3′, reverse 5′-GTAACAGTCCGC CTAGAAGCAC-3′; Prdx6, forward 5′-TCTATCCTCTAC CCTGCCACCAC-3′, reverse 5′-TACCATCACGCTCTC TCCCTTCT-3′.

### Measurement the antioxidant activity of non-selenium glutathione peroxidase (NSGPx) and GSH level in brain and lung tissues

The antioxidant activity of NSGPx was obtained by subtracting the activity of glutathione peroxidase with selenium (SeGPx) from the total GPx activity. The activities of total GPx and SeGPx were assayed according to the instructions of assay kit (Beyotime Institute of Biotechnology, China). In the assay protocol, for total GPx (SeGPx and NSGPx) determination, GPx oxidizes GSH to produce GSSG as part of the reaction in which GPx reduces cumene hydroperoxide (Cum-OOH), a common substrate of SeGPx and NSGPx. Glutathione reductase (GR) then reduces the GSSG to produce GSH, and in the same reaction consumes NADPH. The decrease of NADPH is proportional to GPx activity. So through measuring the change of NADPH content, the total GPx could be determined. For SeGPx determination, the peroxide substrate provided was *t*-butyl hydroperoxide (*t*-Bu-OOH), which could not be catalyzed by NSGPx, so the final result merely represented SeGPx activity.

The reduced GSH level was assayed according to the instructions of assay kit (Beyotime Institute of Biotechnology, China). The assay is based on the GSH recycling system by DTNB and glutathione reductase. DTNB and GSH react to generate TNB, which has a yellow color. Therefore, total GSH (GSH + GSSG) concentration can be determined by measuring absorbance at 412 nm. For reduced GSH assay, the GSH in sample was removed by adding a GSH quencher, the GSSG content was then detected with above method. The content of reduced GSH was calculated by subtracting the GSSG from the total GSH.

Protein concentration was determined using BCA Protein Assay kit (Beyotime Institute of Biotechnology, China). The GPx activity and GSH level were normalized against protein (μ/mg protein and mg/g protein).

### Statistical analysis

Results were shown as mean ± standard deviation (X ± SEM). SPSS17 statistical software was used for statistical analysis. Statistical significance between two groups was judged by *t* test and for significant difference between multiple groups by ANOVA. $P \leq 0.05$ was considered to be significant.

## Results

### Influences of different types of exposure on survival times of mice

Results showed that compared with pre-continuous exposure in pure oxygen, pre-intermittent exposure could markedly extend the survival time of mice in subsequent continuous exposure to pure oxygen (Fig. 2).

### Effect of different exposures on histology of lung tissue

Compared with control group, histological examination in CE and IE groups both revealed increased alveolar wall thickness, severe or moderate interstitial and alveolar edema, alveolar hemorrhage, inflammatory cell infiltration. However, there were no obvious differences between these two groups. 24 h after cessation of exposure, all above pathological changes in both groups were significantly reduced. Besides, compared with 24 h-PCE, the alveolar expansion, alveolar hemorrhage, perivascular edema of capillary vessels and the infiltration of karyocyte in 24 h-PIE were markedly alleviated (Fig. 3).

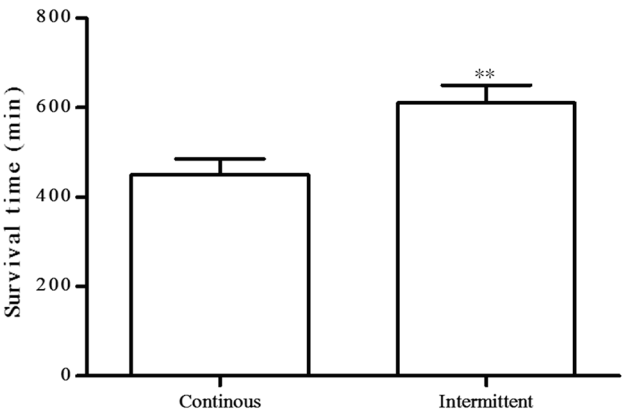


**Fig. 2** Effects of continuous and intermittent HBO exposure on the survival time of mice successively and continuously exposed to pure oxygen after different pre-exposure. $^{**}P < 0.01$, compared with continuous exposure. $n = 12$ for each group

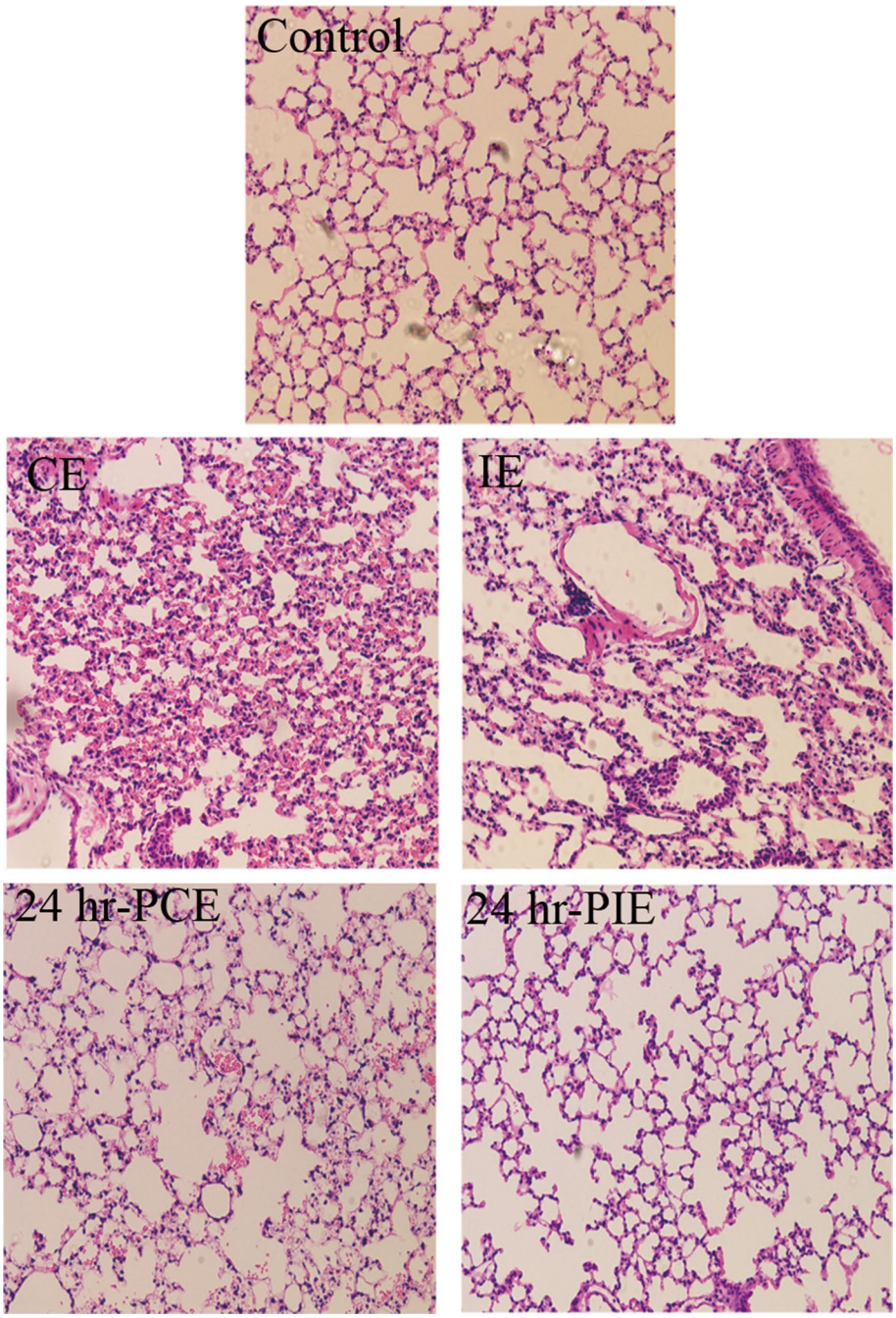


**Fig. 3** Histological examination of lung after different types of HBO exposure. ×200. $n=4$ for each group

## The MDA and $H_2O_2$ levels in brain and lung tissues

Compared with the control, MDA levels of brain tissue in both CE and IE groups were significantly increased, but the increase in CE group was more notable; 24 h after cessation of exposure, MDA levels in both 24 h-PCE and 24 h-PIE groups declined, but the level in the 24 h-PIE group declined more markedly and there was significant difference when compared with 24 h-PCE group. Besides, there was no significant difference between the control and 24 h-PIE group. For lung tissue, the results were almost the same except that immediately after exposure, there was no significant difference between CE and IE groups (Fig. 4a).

The changes of $H_2O_2$ level were similar to those of MDA. Continuous and intermittent exposure all could increase $H_2O_2$ level in brain tissue when detected immediately after exposure, but there was no significant difference between the two groups. Twenty-four hours after cessation of exposure, the $H_2O_2$ level in the 24 h-PIE group declined significantly, being close to that of the control group and significantly lower than that of 24 h-PCE group. For lung tissue, the change of $H_2O_2$

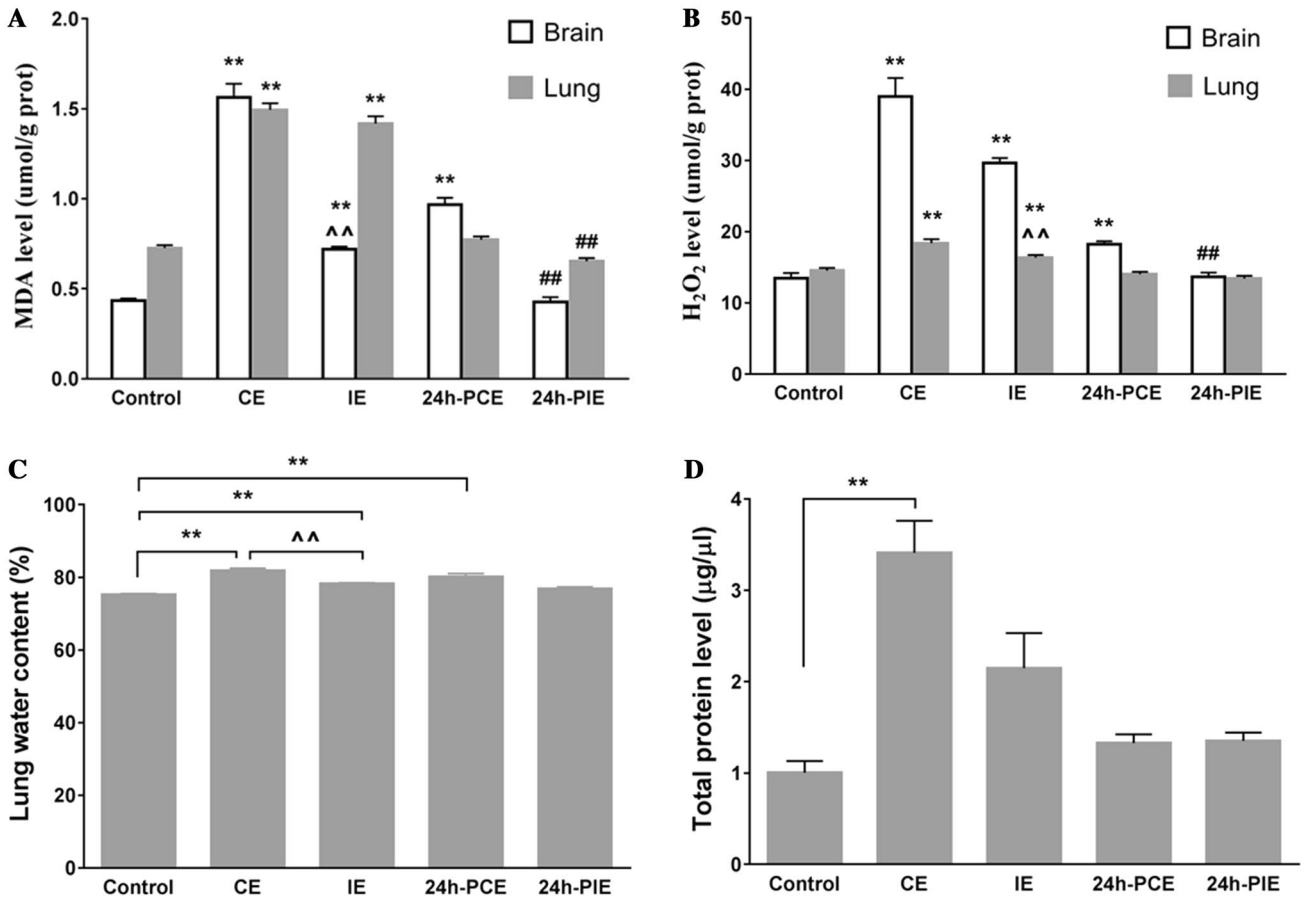


**Fig. 4** Effects of continuous and intermittent exposures on MDA (**a**) and $H_2O_2$ (**b**) contents in brain and lung tissue; water contents (**c**) in lung tissue and total protein concentration in BALF (**d**). Compared with the control, $^{**}P<0.01$; Compared with the group of instant after continuous exposure, $^{\wedge\wedge}P<0.01$; Compared with the group of 24 h after continuous exposure, $^{\#\#}P<0.01$. $n=8$ for each group

level showed a similar trend. However, continuous exposure could increase the $H_2O_2$ level more significantly than intermittent exposure when detected immediately after exposure. Twenty-four hours after cessation of exposure, the $H_2O_2$ level in both the 24 h-PCE and the 24 h-PIE groups declined significantly and there was no significant difference between the two groups (Fig. 4b).

## Effects of different exposures on lung tissue edema

The water contents of lung tissue of CE and IE groups were significantly higher than that of the control. At the same time, the water content of CE group was higher than that of IE group. Twenty-four hours after cessation of exposure, the water content of the 24 h-PIE group declined significantly, being close to that of the control, while the water content of the 24 h-PCE group was still significantly higher than that of the control (Fig. 4c).

## Effects of different exposures on protein concentration in BALF

Compared with the control, the protein content of CE group was significantly higher, but there was no obvious difference between the IE and control groups. Twenty-four hours after cessation of exposure, the protein level in the 24 h-PCE group declined significantly, being close to those of the control and the 24 h-PIE groups (Fig. 4d).

## Effects of different exposures on protein and mRNA levels of Prdx6 in brain tissue

Immunohistochemical results demonstrated that, in brain tissue, Prdx6 was mainly located in astrocytes and oligodendrocytes. Examination performed immediately after exposure revealed that, compared with the control, intermittent exposure could increase Prdx6 level significantly, but continuous exposure had no such effect. Twenty-four hours

after cessation of exposure, the Prdx6 levels of both the 24 h-PCE and the 24 h-PIE groups were significantly higher than that of the control and also significantly higher than those of their correspondent immediate-detecting group, CE, and IE groups (Fig. 5a, b).

The Western blotting results also verified that HBO exposure could increase Prdx6 level in brain. As the time prolonged, the effects were more notable. Compared with continuous exposure, intermittent exposure had a more significant influence when detected 24 h after cessation of exposure (Fig. 5c, d).

The mRNA level of Prdx6 in brain was quantified by qRT-PC. The result showed that the changes of mRNA level in brain tissues were consistent with the results of Western blotting (Fig. 5e).

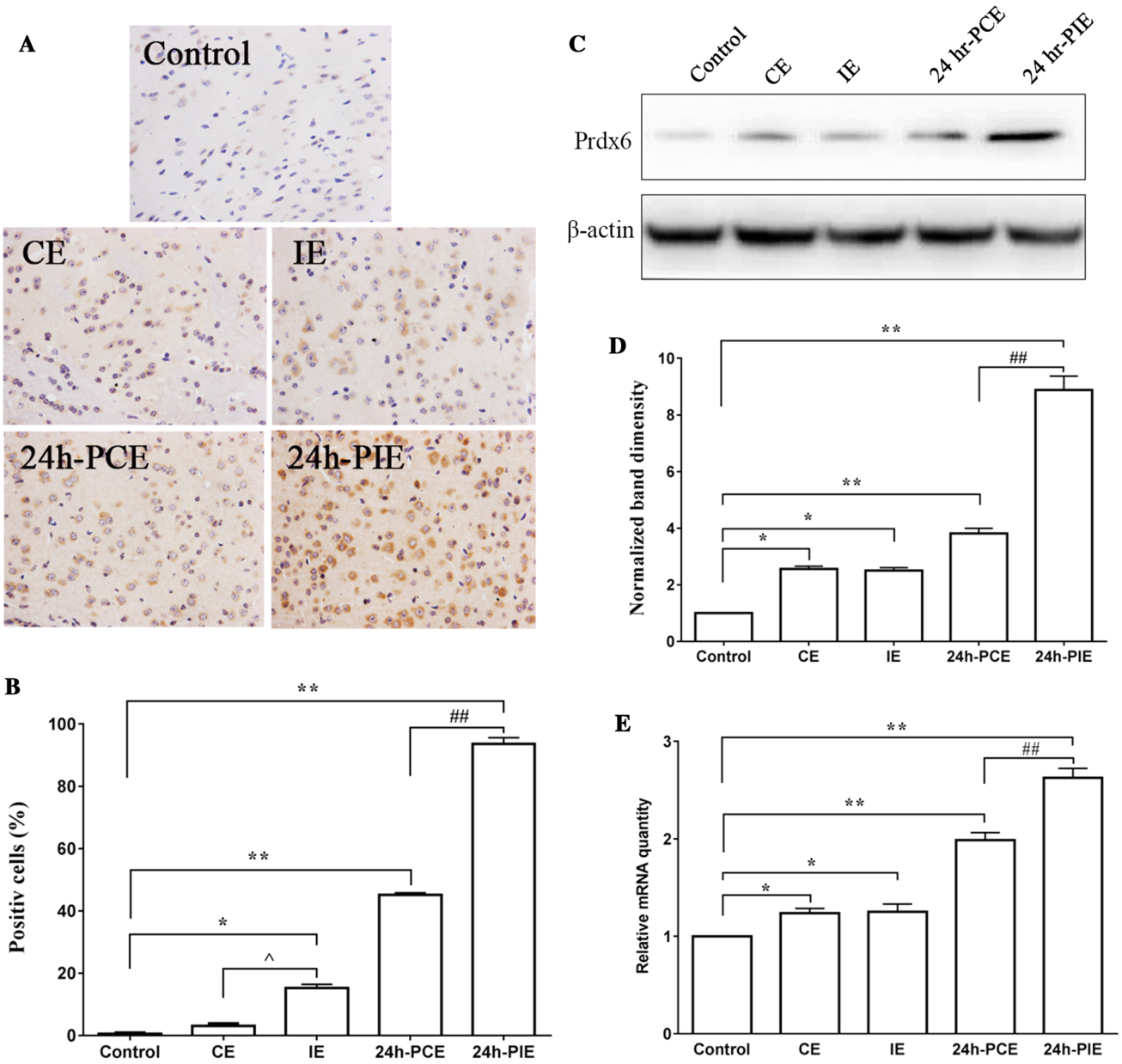


**Fig. 5** Effects of continuous and intermittent exposures on protein and mRNA levels of Prdx6 in mouse brain. **a** Results of immunohistochemical examination, ×400; **b** percentage of positive immunohistochemical staining cells. **c** Results of Western blotting. **d** Quantification of band densities relative to the control after normalization to β-actin. **e** qRT-PCR assay of Prdx6 mRNA levels, the values represent fold changes to the control. Compared with the control, $^{*}P<0.05$, $^{**}P<0.01$; Compared with the group of instant after continuous exposure, $^{\wedge}P<0.05$; Compared with the group of 24 h after continuous exposure, $^{\#\#}P<0.01$. $n=4$ for each group

### Effects of different exposures on protein and mRNA levels of Prdx6 in lung tissue

Immunohistochemical results showed that, in lung tissues, Prdx6 was mainly located in alveolar epithelial cells, bronchiolar epithelial cells, and vascular endothelial cells. HBO exposure could increase Prdx6 levels, but being significant only 24 h after cessation of exposure, and compared with continuous exposure, intermittent exposure had more significant influence (Fig. 6a, b).

The Western blotting results also showed that HBO exposure could increase Prdx6 levels in lung tissues, even when detected immediately after HBO exposure. As the immunohistochemical results, intermittent exposure had more significant influence than continuous exposure 24 h after cessation of exposure (Fig. 6c, d).

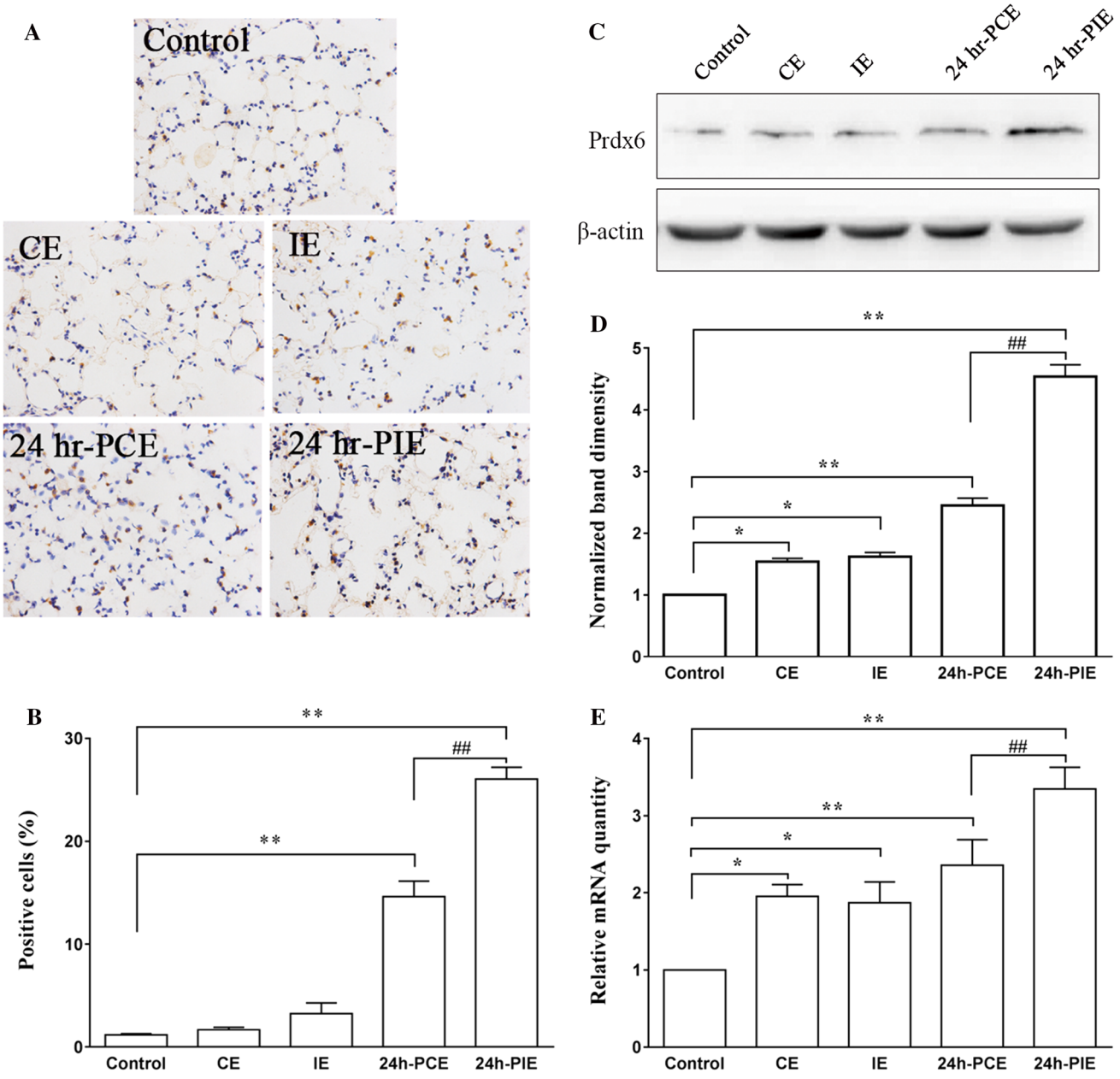


**Fig. 6** Effects of continuous and intermittent exposures on protein and mRNA levels of Prdx6 in mouse lung. **a** Results of immunohistochemical examination, ×400; **b** percentage of positive immunohistochemical staining cells. **c** Results of Western blotting. **d** Quantification of band densities relative to the control after normalization to β-actin. **e** qRT-PCR assay of Prdx6 mRNA levels. The values represent fold changes to the control. Compared with the control, $^{*}P<0.05$, $^{**}P<0.01$; Compared with the group of 24 h after continuous exposure, $^{\#\#}P<0.01$. $n=4$ for each group

The changes of mRNA level of Prdx6 in lung tissues quantified by qRT-PCR were consistent with the results of Western blotting (Fig. 6e).

### The antioxidant activity of NSGPx and levels of reduced GSH in brain and lung tissues

In brain tissue, HBO exposure could increase the antioxidant activity of NSGPx no matter immediately or 24 h later after cessation of exposure. Besides, compared with continuous exposure, intermittent exposure had more significant influence at both of the time points. The antioxidant activity of NSGPx in lung tissue presented consistent changes with the results in the brain tissue (Fig. 7a).

In brain and lung tissues, continuous exposure did not alter the reduced GSH levels when detected immediately after cessation of exposure. Intermittent exposure could increase the reduced GSH level in brain significantly, but not in lung. Twenty-four hours later after cessation of exposure, for no matter immediate or intermittent exposure, the reduced GSH level in both brain and lung tissues increased, and compared with continuous exposure, intermittent exposure could increase reduced GSH level more significantly in both tissues (Fig. 7b).

## Discussion

Breathing of molecular oxygen at increased partial pressures raises body oxygen concentration. A portion of the oxygen excess in body tissues is rapidly reduced to generate large amounts of $O_2^{\cdot -}$ and $H_2O_2$, which can, in turn, further participate in reactions to produce more reactive oxygen species (ROS), including NO, $ONOO^-$, and ·OH. These ROS can damage intracellular structures and plasma membranes to severely impair cellular functions, resulting in oxygen toxicity [18]. In the body, central nervous system (brain), lungs, and retina are the targets being most frequently attacked by oxygen toxicity. For diving and hyperbaric operations, CNS and pulmonary oxygen toxicity have always been among the limiting factors of safety and efficiency, and must be paid attention to.

Hyperbaric oxygen exposure in an intermittent manner can significantly delay the occurrence of oxygen toxicity but the underlying mechanisms remain unclear [17]. Some studies have examined the activity changes of several redox enzymes during intermittent hyperoxic exposure; however, the results are not consistent. It was found that, after exposure to 96–101 kPa (0.95–1.0 ATA) hyperoxia for 48 h, 24 h of subsequent "rest" in room air can render rats able to tolerate a continuing hyperbaric oxygen exposure for 3–7 days with only mild pulmonary edema and no lethality. The increase in oxygen tolerance is due to the upregulation of activities of antioxidant enzymes in lungs, including those of superoxide dismutase, catalase and glutathione peroxidase. Reducing air breathing time to 6 h did not cause lethality. However, reducing exposure to hyperbaric oxygen from 48 to 36 h led to only 83% of experimental rats surviving the subsequent 72 h re-exposure; and these rats showed obvious lung damage. These results indicate that a recovery in room air after exposure to toxic hyperbaric oxygen can induce antioxidant enzyme expression during the subsequent re-exposure [19].

Harabin et al. [20] exposed guinea pigs and rats to 282 kPa (2.8 ATA) oxygen delivered either in a continuous or intermittent manner, and then measured activity of antioxidant enzymes in brain and lung. The intermittent exposure consisted of repeated cycles of 10 min of hyperoxic exposure followed by 2.5 min of room air (57 kPa $O_2$, 0.56 ATA). The authors found that it took longer for intermittently exposed animals to reach convulsion and death. They further compared antioxidant enzyme activity in brain and lung tissues between the two groups of exposed animals. Superoxide dismutase activity showed no obvious difference between

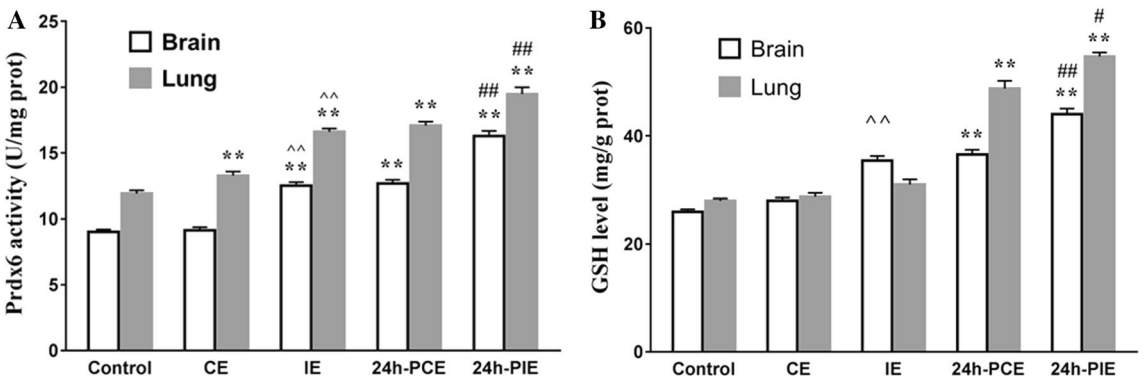


**Fig. 7** The NSGPx activity (**a**) and the GSH levels (**b**) in brain and lung tissues. Compared with the control, $^{**}P<0.01$; Compared with the group of instant after continuous exposure, $^{\wedge\wedge}P<0.01$; Compared with the group of 24 h after continuous exposure, $^{\#}P<0.05$, $^{\#\#}P<0.01$

brains of the two groups. Catalase activity was decreased in brains of both continuously and intermittently exposed groups of guinea pigs. Glutathione peroxidase activity was diminished in brains of both guinea pigs and rats regardless of either continuous or intermittent exposure. Lung superoxide dismutase activity was elevated in intermittently exposed guinea pigs, and also elevated in both intermittently and continuously exposed rats. Both exposure patterns led to a diminution in lung catalase activity in both guinea pigs and rats. Gluthione peroxidase activity was decreased in guinea pig lungs by both kinds of exposures, but not in rat lungs. Intermittent exposures caused a remarkable change in antioxidant enzyme activity in guinea pigs but not in rats. Thus, the authors speculated that the prolonged time to onset of convulsion and lethality was not only due to changes in antioxidant enzyme activity, but to additional oxygen tolerance mechanisms.

Chavko et al. [17] revealed that intermittent HBO exposure could protect against pulmonary oxygen toxicity by inhibiting inflammation, and the mechanism may involve the antiinflammatory and antioxidative effect of HO-1. Meanwhile, the authors also considered some other mechanisms may be involved in protection, like inhibition the activation of inducible nitric oxide synthase (iNOS) and the consequent protein nitration.

Prdxs is a peroxidase superfamily identified in recent years. There have been six Prdxs discovered in mammals and they can be divided into two classes according to the number of conserved cysteine (Cys) in the active site, namely 1-Cys (1 conserved Cys) and 2-Cys (2 conserved Cys). Among them, Prdx1–5 belong to the 2-Cys class and Prdx6 belongs to the 1-Cys class [13]. Prdx6 contains 224 amino acids with a molecular weight of 25,100 and is a homodimer [21]. It is highly enriched in lung tissues, especially in type II alveolar epithelial cells and bronchiolar Clara cells [22]. This protein is a bifunctional enzyme with non-selenium glutathione peroxidase (NSGPx) and non-calcium phospholipase A2 (PLA2) activities [23, 24]. It can reduce inorganic and organic oxides such as $H_2O_2$ and phospholipid hydroperoxides using glutathione. Over-expression of Prdx6 can increase the cell's capability to reduce $H_2O_2$ and phospholipid hydroperoxides. Prdx6 knock-out mice demonstrate substantial susceptibility to lung damage by higher oxygen concentrations or chemicals [25, 26]. Thus, Prdx6 may play an important role in the antioxidant capabilities and phospholipid metabolism of lung [27].

To elucidate the mechanisms of protection against pulmonary hyperbaric oxygen toxicity by intermittent exposure, Chavko et al. compared the efficacy of several intermittent exposure schedules in extending survival time in hyperbaric oxygen [17]. They found that 60 min of oxygen – 30 min air for six cycles was a protective intermittent schedule for hyperbaric oxygen toxicity. Moreover, this schedule was also convenient for operating. So in our study, we adopted this intermittent exposure schedule. It was verified that such a schedule could indeed extend the survival time when oxygen toxicity occur. Meanwhile, our data also showed that such a schedule was able to protect lung from hyperoxia injuries, including keeping the structure of lung tissue relative intactness, alleviating pulmonary edema and reducing protein level in BALF.

Oxidant stress is a potent inducer of Prdx6 expression. Previous studies have shown that exposure to hyperoxia resulted in an approximate doubling of Prdx6 expression in rat and mouse lungs and alveolar type II cells [28]. On the other hand, mice over-expressing Prdx6 were more resistant to the oxidant stress generated by exposure to oxygen at elevated partial pressures. By contrast, Prdx6 null mice showed a significant increase in sensitivity to the toxic effects of hyperoxia [28]. These results indicate that the relationship between Prdx6 and hyperoxia is very close. In our studies, we also found HBO exposure could increase the protein and mRNA expression of Prdx6. In lung, prolonged exposure led to a remarkable increase in Prdx6 level; at 24 h after ceasing exposure, the increase was more notable. Particularly, this increasing effect was more significant in the intermittent exposure group. For either protein level or mRNA level, they all presented coincident ascending trend in this group. The results indicated that Prdx6 in lung was very responsive to HBO exposure. Since Prdx6 possess antioxidant property and is protective, its increase must be favorable to lung to antagonize the toxic effect of HBO and repair oxidative injury. In addition, our results showed intermittent exposure mode was more effective for elevating Prdx6 level, this was also consistent with the fact that intermittent exposure can provide protection against oxygen toxicity. So intermittent HBO exposure promotes Prdx6 increment may explain its protective effects to a certain extent. For the Prdx6 level in brain tissue, the situation was similar to that in lung. Especially for the Prdx6 protein level, the alterations along with the time and mode of HBO exposure were almost the same as those in lung, reflecting in brain tissue, Prdx6 was also very responsive to HBO exposure and more Prdx6 was needed to resist the oxidative injury. Collectively, these results indicate that Prdx6 may be a very reactive molecular response to HBO exposure in most organs. With its antioxidative property, largely increased Prdx6 can potentiate the antioxidant ability of tissue and resist HBO toxicity. Most importantly, our investigation also demonstrated that intermittent exposure could promote Prdx6 generation more effectively. Such a result provided solid experimental evidence for its protective effects in delaying and antagonizing oxygen toxicity.

Prdx6 is the only mammalian 1-Cys member of the peroxiredoxin superfamily and is expressed in all major organs, with high levels of expression in lung, brain, testis, kidney,

and liver. The expression level of Prdx6 mRNA and protein is greatest in the lung [14, 28, 29]. With its catalytic Cys at position 47 and uses GSH as the physiological reductant, Prdx6 is known to be functionally a glutathione peroxidase (GPx) and to play a critical role in antioxidant defense. In addition to reducing a wide range of hydroperoxide substrates, Prdx6 is the only enzyme in the peroxiredoxin family that can reduce PLOOH through its peroxidase activity. Different from the ubiquitous cytosolic GSH peroxidase (GPx1), which is selenium-dependent, Prdx6 possesses an activity of non-selenium GSH peroxidases (NSGPx) [14, 28, 29]. Since in mammals, most GPx are selenium-dependent, only a very small part of it are non-selenium dependent, and since there is an abundant distribution of NSGPx-type Prdx6 in lung and brain, it could be inferred that the majority of NSGPx activity come from Prdx6; and indeed, Prdx6 was called NSGPx at one time in previous reports [29]. In our studies, through measuring the total GPx and GPx with selenium, respectively, we assayed the activity of NSGPx, in which Prdx6 activity may take up a large proportion. Our results showed that in both lung and brain tissues, HBO exposure could potentiate the antioxidant activity of NSGPx (Prdx6), and coincident with the activity potentiation, the GSH content was also increased. Such potentiation and increment became greater over time extended. Of particular importance, intermittent exposure had a more significant effect for the antioxidant activity of NSGPx (Prdx6). This undoubtedly was closely related with its protection against the toxic effect of elevated oxygen partial pressure. The higher level of Prdx6 activity in intermittent HBO exposure may be correlated with two factors: (1) At a sufficiently high oxygen pressure for a sufficiently prolonged duration of exposure, excessive production of ROS can overwhelm the antioxidant defenses, including all kinds of antioxidases. Periodic interruption of less toxic degrees of hyperoxic exposure by restoration of inspiratory normoxia can alleviate the damage effect of continuing attack of ROS, thus allowing antioxidase to be maintained at a relatively high level. As in our study, immediately after exposure, the antioxidant activity of Prdx6 in IE group was higher than that in the CE group, while there was no significant difference for the protein content of Prdx6 between these two groups. (2) Periodic air break during HBO exposure could increase the production of antioxidase [19], thus increasing the enzyme activity. This factor might be more prominent with the lapse of time after exposure. As shown in our study, at the time of 24 h post exposure, the activity and the protein content of Prdx6 in IE group were all markedly higher than those in the CE group.

Corresponding with the potentiation of the expression and activity of Prdx6, meaning more powerful antioxidant defense ability, the lipid peroxidation and tissue injuries resulted from intermittent exposure would be less when compared with continuous exposure. This was verified in our studies, manifesting as less MDA and $H_2O_2$ production, less lung edema, and less total protein concentration in BALF.

Apart from its peroxidase activity, Prdx6 has another very important enzyme activity, that is phospholipase A2 (PLA2) activity [14, 28]. Previous study indicated that the PLA2 activity of Prdx6 was also very important in the repair of peroxidized phospholipids in cell membranes and the recovery of lung cells from peroxidative stress resulted from hyperoxia exposure, which involved remodeling pathways of phospholipid component of surfactant [12, 30]. On the contrary, there was also a study that indicated blocking Prdx6 PLA2 activity significantly protected lungs against damage from hyperoxia, presumably by preventing the activation of NOX2 and the amplification of lung injury associated with inflammation [23]. If and how intermittent exposure can influence the PLA2 activity of Prdx6 need further investigation.

In summary, our studies indicated that intermittent HBO exposure can stimulate Prdx6 levels and consequently mitigate oxidative damage in lungs and brain. These results may give some help for more comprehensively interpreting how intermittent oxygen exposure can resist oxygen toxicity. At the same time, more mechanisms about intermittent HBO exposure mobilizing Prdx6 to resist against CNS and pulmonary oxygen toxicity is worth probing further.

**Acknowledgements** This study was supported National Natural Science Foundation of China (No. 81471813).

## Compliance with ethical standards

**Conflict of interest** No conflicts of interest, financial or otherwise, are declared by the author(s).

## References

1. Eynan M, Krinsky N, Biram A, Arieli Y, Arieli R (2014) A comparison of factors involved in the development of central nervous system and pulmonary oxygen toxicity in the rat. Brain Res 1574:77–83
2. van Ooij PJ, Hollmann MW, van Hulst RA, Sterk PJ (2013) Assessment of pulmonary oxygen toxicity: relevance to professional diving; a review. Respir Physiol Neurobiol 189:117–128
3. Fock A, Harris R, Slade M (2013) Oxygen exposure and toxicity in recreational technical divers. Diving Hyperb Med 43:67–71
4. Gore A, Muralidhar M, Espey MG, Degenhardt K, Mantell LL (2010) Hyperoxia sensing: from molecular mechanisms to significance in disease. J Immunotoxicol 7:239–254
5. Chavko M, McCarron RM (2006) Extension of brain tolerance to hyperbaric $O_2$ by intermittent air breaks is related to the time of CBF increase. Brain Res 1084:196–201
6. Clark JM, Lambertsen CJ, Gelfand R, Troxel AB (2006) Optimization of oxygen tolerance extension in rats by intermittent exposure. J Appl Physiol 100:869–879

7. Maezawa T, Tanaka M, Kanazashi M, Maeshige N, Kondo H, Ishihara A et al (2017) Astaxanthin supplementation attenuates immobilization-induced skeletal muscle fibrosis via suppression of oxidative stress. J Physiol Sci 67:603–611
8. Ozeki M, Nagasu H, Satoh M, Namikoshi T, Haruna Y, Tomita N et al (2009) Reactive oxygen species mediate compensatory glomerular hypertrophy in rat uninephrectomized kidney. J Physiol Sci 59:397–404
9. Nuoc TN, Kim S, Ahn SH, Lee JS, Park BJ, Lee TH (2017) The analysis of antioxidant expression during muscle atrophy induced by hindlimb suspension in mice. J Physiol Sci 67:121–129
10. Szabo C, Ischiropoulos H, Radi R (2007) Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat Rev Drug Discov 6:662–680
11. Kikusato M, Toyomizu M (2015) Moderate dependence of reactive oxygen species production on membrane potential in avian muscle mitochondria oxidizing glycerol 3-phosphate. J Physiol Sci 65:555–559
12. Li H, Benipal B, Zhou S, Dodia C, Chatterjee S, Tao JQ et al (2015) Critical role of peroxiredoxin 6 in the repair of peroxidized cell membranes following oxidative stress. Free Radical Biol Med 87:356–365
13. Brinkmann C, Brixius K (2013) Peroxiredoxins and sports: new insights on the antioxidative defense. J Physiol Sci 63:1–5
14. Manevich Y, Fisher AB (2005) Peroxiredoxin 6, a 1-cys peroxiredoxin, functions in antioxidant defense and lung phospholipid metabolism. Free Radical Biol Med 38:1422–1432
15. Rhee SG, Kang SW, Netto LE, Seo MS, Stadtman ER (1999) A family of novel peroxidases, peroxiredoxins. BioFactors 10:207–209
16. Trujillo M, Ferrer-Sueta G, Radi R (2008) Peroxynitrite detoxification and its biologic implications. Antioxid Redox Signal 10:1607–1620
17. Chavko M, Mahon RT, McCarron RM (2008) Mechanisms of protection against pulmonary hyperbaric O(2) toxicity by intermittent air breaks. Eur J Appl Physiol 102:525–532
18. Zhu H, Traore K, Santo A, Trush MA, Li YR (2016) Oxygen and oxygen toxicity: the birth of concepts. React Oxyg Species (Apex) 1:1–8
19. Frank L, Iqbal J, Hass M, Massaro D (1989) New "rest period" protocol for inducing tolerance to high $O_2$ exposure in adult rats. Am J Physiol 257:L226–L231
20. Harabin AL, Braisted JC, Flynn ET (1990) Response of antioxidant enzymes to intermittent and continuous hyperbaric oxygen. J Appl Physiol 69:328–335
21. Rivera-Santiago RF, Harper SL, Zhou S, Sriswasdi S, Feinstein SI, Fisher AB et al (2015) Solution structure of the reduced form of human peroxiredoxin-6 elucidated using zero-length chemical cross-linking and homology modelling. Biochem J 468:87–98
22. Nevalainen TJ (2010) 1-Cysteine peroxiredoxin: a dual-function enzyme with peroxidase and acidic $Ca^{2+}$-independent phospholipase A2 activities. Biochimie 92:638–644
23. Benipal B, Feinstein SI, Chatterjee S, Dodia C, Fisher AB (2015) Inhibition of the phospholipase A2 activity of peroxiredoxin 6 prevents lung damage with exposure to hyperoxia. Redox Biol 4:321–327
24. Lien YC, Feinstein SI, Dodia C, Fisher AB (2012) The roles of peroxidase and phospholipase A2 activities of peroxiredoxin 6 in protecting pulmonary microvascular endothelial cells against peroxidative stress. Antioxid Redox Signal 16:440–451
25. Wang Y, Feinstein SI, Fisher AB (2008) Peroxiredoxin 6 as an antioxidant enzyme: protection of lung alveolar epithelial type II cells from $H_2O_2$-induced oxidative stress. J Cell Biochem 104:1274–1285
26. Wang Y, Phelan SA, Manevich Y, Feinstein SI, Fisher AB (2006) Transgenic mice overexpressing peroxiredoxin 6 show increased resistance to lung injury in hyperoxia. Am J Respir Cell Mol Biol 34:481–486
27. Ambruso DR (2013) Peroxiredoxin-6 and NADPH oxidase activity. Method Enzymol 527:145–167
28. Fisher AB (2011) Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospholipase A(2) activities. Antioxid Redox Signal 15:831–844
29. Fisher AB (2017) Peroxiredoxin 6 in the repair of peroxidized cell membranes and cell signaling. Arch Biochem Biophys 617:68–83
30. Fisher AB, Vasquez-Medina JP, Dodia C, Sorokina EM, Tao JQ, Feinstein SI (2018) Peroxiredoxin 6 phospholipid hydroperoxidase activity in the repair of peroxidized cell membranes. Redox Biol 14:41–46