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Effect of Hyperbaric oxygen on myelin injury and repair after hypoxic-ischemic brain damage in adult rat

Ruijun Xue [a], Shuyi Pan [a,b,*], Dazhi Guo [b,*]

[a] School of Medicine, South China University of Technology, Guangzhou 510006, China
[b] Department of Hyperbaric Oxygen, 6th Medical Center of PLA General Hospital, Beijing 100048, China

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ABSTRACT

Introduction: Hypoxic-ischemic encephalopathy (HIE) is one of the leading causes of death and neurological disability with limited options for treatment in neonates, children and adults worldwide. The pathogenesis and treatment of white matter (WM) injury in adult patients with HIE remains largely elusive.

Methods: Sixty male Sprague-Dawley rats were randomly divided into control group, sham-operated group (HBO treatment 6 days after sham operation), and Hypoxia-ischemia (HI) induced brain damage group (receiving left carotid arteries ligation + hypoxia treatment), 1.5ATA hyperbaric oxygen group (HI + 1.5ATA HBOT) and 2.5ATA HBOT group (HI + 2.5ATA HBOT). All the rats were evaluated by water maze before operation, and 6 days after operation, and the function of learning and memory was evaluated; Demyelination in the hippocampus and prefrontal cortex was observed by Luxol fast blue staining (LFB) and MBP immunostaining; the number of Myelin Oligodendrocyte Glycoprotein (MOG), glial fibrillary acidic protein (GFAP), ionic calcium-binding adaptor (Iba-1) and NG2 positive cells in the hippocampus and prefrontal cortex were determined by immunofluorescence staining. The expression of interleukin-1β (IL-1β), IL-6 and tumor necrosis factor (TNF-α), Hypoxia Inducible Factor 1 Subunit Alpha (HIF1-α) and Superoxide dismutase (SOD) in brain and serum of rats were measured by Western Blot method and Enzyme linked immunosorbent assay (ELISA).

Results: Compared with those in the normal control group and sham-operated group, in the HI group, the learning and memory abilities of rats were significantly decreased ($P < 0.05$), the intensity of LFB and MBP immunostaining in hippocampus and prefrontal cortex was significantly decreased ($P < 0.05$); the number of MOG positive oligodendrocytes (OLs) significantly decreased ($P < 0.05$), whereas the number of Iba-1, GFAP, NG2 positive microglias, astrocytes and oligodendrocyte precursors (OPCs) was increased ($P < 0.05$); the level of IL-1β, IL-6, TNF-α and HIF-1a in brain and serum were significantly increased ($P < 0.05$), whereas SOD was significantly decreased in brain and increased in serum. Compared with those in the HI group, in both 1.5ATA and 2.5ATA HBOT group, the learning and memory abilities were significantly increased ($P < 0.05$); the intensity of LFB and MBP immunostaining in the hippocampus and prefrontal cortex was significantly increased ($P < 0.05$); the number of MOG positive OLs significantly increased ($P < 0.05$); the number of Iba-1, GFAP, NG2 positive microglias, astrocytes and OPCs was decreased ($P < 0.05$); the level of IL-1β, IL-6, TNF-α and HIF-1a in brain and serum were significantly decreased ($P < 0.05$); the level of SOD was significantly increased in brain and decreased in serum. Morever, compared with those in the 1.5ATA group, 2.5ATA provided better treatment results ($P < 0.05$).

Conclusion: In the present study, we demonstrated the mechanism of different pressure HBOT on HI induced brain injury from three levels: (1) On a tissue level, HBOT protects against HI induced myelin injury; (2) On a cellular level, HBOT attenuates HI-induced OL loss, suppresss the reactive activation of astrocyte and microglia, and may promote OPC to differentiate into OL; (3) On a molecular level, HBOT inhibites neuroinflammation, and balances oxidative damage and antioxidant capacity. Among the above effects, 2.5ATA HBOT is better than 1.5ATA HBOT. Ongoing research will continue to seek out the signalling pathways and molecules mechanisms on different pressure of HBOT-related myelin protection, and possibly expand suitable HBOT use in adult HIE clinically.

* School of Medicine, South China University of Technology, Guangzhou 510006, China and Department of Hyperbaric Oxygen, 6th Medical Center of PLA General Hospital, Beijing 100048, China (S. Pan); Department of Hyperbaric Oxygen, 6th Medical Center of PLA General Hospital, Beijing 100048, China (Dazhi Guo).
E-mail addresses: psy9992011@163.com (S. Pan), guodazhigod1983@163.com (D. Guo).

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1. Introduction

Hypoxia-ischemia (HI)-induced brain damage is common in perinatal hypoxic-ischemic encephalopathy (HIE), cardiac arrest, respiratory failure, shock, poisoning and asphyxia. It is one of the leading causes of death and neurological disability with limited options for treatment in neonates, children and adults worldwide. [1–2] HI-induced brain damage ranges from mild to severe depending on varying the HI duration. [3] Many studies have suggested that white matter (WM) is more susceptible to HI injury. [4–5] Histologic studies have also showed reduced myelination but normal axons in the early onset of HIE. [6] However, the pathogenesis of WM injury in adult patients with HIE remains largely elusive. A better understanding of the pathogenesis is necessary to develop various novel and effective treatments for HI-induced brain injury.

In WM of central nervous system (CNS), there are four main types of glial cells, including oligodendrocyte (OL), oligodendrocyte precursor cell (OPC), microglia and astrocyte, which support axons to transmit messages or signals from one region to different areas of gray matter. OLs are the main myelinating cells in CNS which provide support and insulation to axons. [7–10] They are more vulnerable to hypoxia and ischemia than other neural cells in CNS. [11] OPCs arise in specific regions of the brain, and disperse widely through the adult CNS. Upon pathophysiologica stimuli (e.g., HI, inflammation, demyelination), OPCs are prone to differentiate into mature OLs, astrocytes or neurons in specific region of adult brain. [12–13] Microglias are key mediators of inflammation, characterized by their phenotypic plasticity (namely M1 and M2 phenotype), and exacerbate the inflammatory response, polarizing into a predominant proinflammatory M1 phenotype in the early hours post HI. [14] Astrocytes are the most abundant glial cells in brain and the key determinant of the outcome and prognosis of HIE. [15] These suggest that HI-induced myelin injury may be the result of a series of functional changes in glial cells..

Hyperbaric oxygen therapy (HBOT) is widely used as a treatment for HIE, which can improve cell metabolism and neurological outcome by increasing the amount of oxygen dissolved in blood and tissues and regulating inflammatory factors, certain oxidation-related proteins and enzymes and inhibiting neuronal apoptosis. [16–17] In our previous study, we found that HBOT suppressed the inflammatory response after acute carbon monoxide poisoning by blocking NLRP3 inflammasome activation, and alleviating myelin injury and cognitive deficits in mice. [18] However, the mechanism of HBOT in the treatment HI induced WM injury and the effects to different pressures of HBOT remain unclear. In the present study, we tested the effect of HBOT under different pressure on the cognitive deficits, myelin injury, function of glial cells and neuroinflammation in an adult HI-induced brain damage rat model, thereby providing novel insight into the mechanism and optimum pressure of HBOT for myelin injury induced by HI.

2. Experimental procedure

2.1. Animals and experimental design

Six-week-old male Sprague-Dawley rats were purchased from Vital River Laboratory Animal Technology Co., ltd (Beijing, China). Animals were maintained under standard conditions at 22 °C to 25 °C with a 12 h light–dark cycle and were fed a normal diet. All experimental protocols were approved by the Institutional Animal Experiment Committee of the Sixth Medical Center, PLA General Hospital and were conducted in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China). Sixty rats were randomly divided into five groups: control group, sham group (exposed and isolated the left carotid artery without ligation), HI group (receiving left carotid artery ligation + hypoxia treatment), 1.5ATA hyperbaric oxygen group (HI + 1.5ATA HBOT) and 2.5ATA HBOT group (HI + 2.5ATA HBOT).

2.2. HI-induced brain damage adult rat model

Refering to the model reported as Rice JE et al. [19] Briefly, rats were anesthetized with 3 % isoflurane. Gentaly exposed the left carotid artery, isolated from nerve and vein, ligated the proximal and distal ends of the left carotid artery, disconnected in the middle, and then sutured the skin layer by layer. After surgery, rats were placed in an airtight box and exposed to nitrogen–oxygen mixture (8 % oxygen and 92 % nitrogen) delivered at 1.5 L per minute for 3 h. Note: the airtight box was cleaned with nitrogen–oxygen mixture at 2.3 L per minute for half an hour before each experiment. We found that the mortality rate of rats exceeded 70 % for more than 3 h, while there was no neurological abnormality in rats<3 h.

2.3. Hyperbaric oxygen treatment (HBOT)

Rats in the sham, HI + 1.5ATA HBOT and HI + 2.5ATA HBOT groups were exposed to HBOT in a Type NG90-IIC pure oxygen chamber (Hyperbaric Oxygen Chamber Factory, Ningbo, China) immediately after sham surgery or HI. The treatment consisted of administration of 100 % oxygen at 1.5ATA or 2.5ATA for 60 min (including 10 min each for compression and decompression), once daily for six days. The chamber was flushed with 100 % oxygen at a rate of 5 l/m to prevent carbon dioxide accumulation. Decompression was carried out at 0.2 kg/cm2 /minute.

2.4. Morris water maze (MWM) test

MWM test was conducted in a round dark pool with 150 cm in diameter and 60 cm deep. The pool was filled with the 60 cm depth of water maintaining at 25 ± 0.5℃. The escape platform was placed in the center of the second quadrant of the pool and submerged 2 cm beneath the water surface. Training was carried out every day for three days before start of the experiment. Rat were put in the third quadrant of the pool to find the platform, and count the escape latency before surgery and after surgery for 6 consecutive days. The whole escape latency was set at 90 s. After the trial, rats were manually dried with a towel and placed in a warming cage for at least five minutes before it was returned to the home cage. Rats were tested in two trials per day, with an interval of approximately 30 min. All testing was carried out at roughly the same time each day in order to minimize variability in performance. All tracks from all trials were analyzed for a number of behavioral parameters using SMART software (Beijing Zhong Di Chuang Ke Co.).

2.5. Immunofluorescent staining

The paraffin sections were deparaffinized in xylene and rehydrated with various concentrations of ethanol. To deactivate the endogenous oxidase, the sections were treated with 3 % H2O2 for 10 min. The sections underwent antigen retrieval by means of pressure-cooking in citrate buffer (10 mM, pH 6.0) and blocking with 10 % goat serum (Solarbio, China) diluted in PBS 3 times (5 mM, pH 7.4), followed by incubation overnight at 4℃ with primary antibody: rabbit monoclonal anti-GFAP (ab7260, 1:2000), rabbit monoclonal anti-Iba1 (ab178846, 1:2000), anti-NG2(abcam, ab12905, 1:200), anti-MOG(abcam, ab243034, 1:200) and anti-MBP(Thermo Fisher, bs-0308R, 1:100). The sections were then washed with PBS and incubated with HRP-conjugated secondary goat anti-mouse antibody (Jackson, 115-545-003, 1:200) or goat anti-rabbit antibody (Jackson, 111-165-003, 1:500) for 50 min, followed by washed with PBS and incubated with DAPI stain solution (Solarbio, C0060, 1:100) for 10 min. After staining, sections were cleared by PBS, in graded ethanol, and then coverslipped by anti-fluorescence quenching agent sealed tablet. Analysis of mean IOD (integral optical density) and the number of cells was carried out, using Image-Pro Plus 6.0 (IPP) software, based on images acquired on the confocal microscope. At lease three different slides were selected per

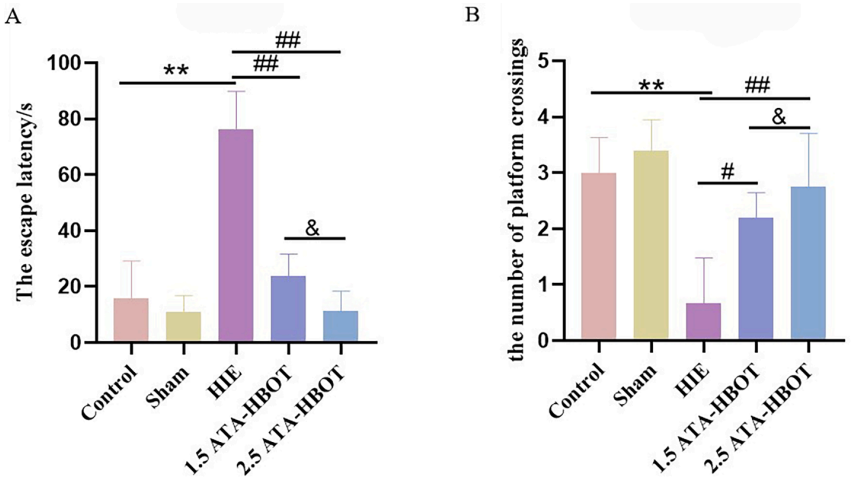

Fig. 1. HBOT improved the learning and memory of HI rats. (A) The escape latency of different groups on the 6th day. (B) The number of platform crossings of different groups on 6th day. Date are represented as mean ± SD, n = 5 in each group. Date were analysed by one-way ANOVA with Turkey's post hoc test. *p < 0.05, ** p < 0.05 vs sham group; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs HI group; $^{\&}$p < 0.05, $^{\&\&}$p < 0.01 vs 1.5ATA group.

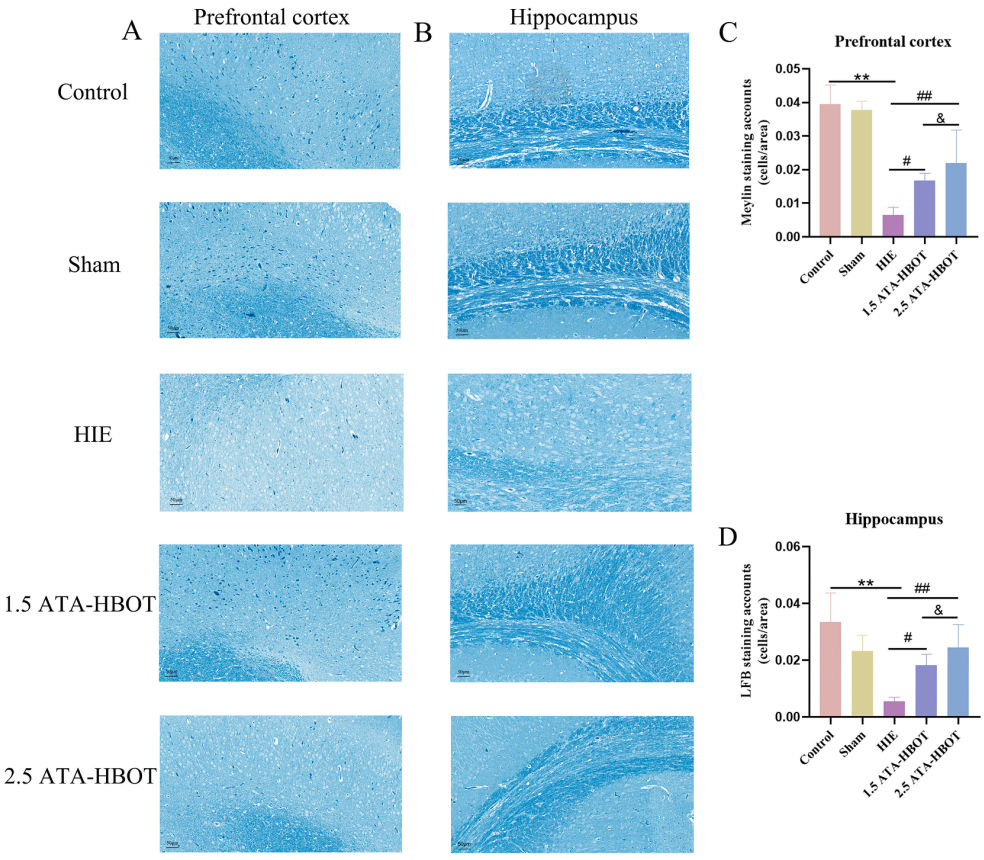

Fig. 2. HBOT protected against HI-induced myelin injury. (A, B) Luxol fast blue staining for myelin injury (blue) on the 6th day in hippocampus and prefrontal cortex. (C, D) Quantitative analysis of mean density for Luxol fast blue staining of different groups on the 6th day after HI. Date are represented as mean ± SD (n = 3 in each group). Date were analysed by one-way ANOVA with Turkey's post hoc test. *p < 0.05, ** p < 0.05 vs sham group; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs HI group; $^{\&}$p < 0.05, $^{\&\&}$p < 0.01 vs 1.5ATA group.

a-

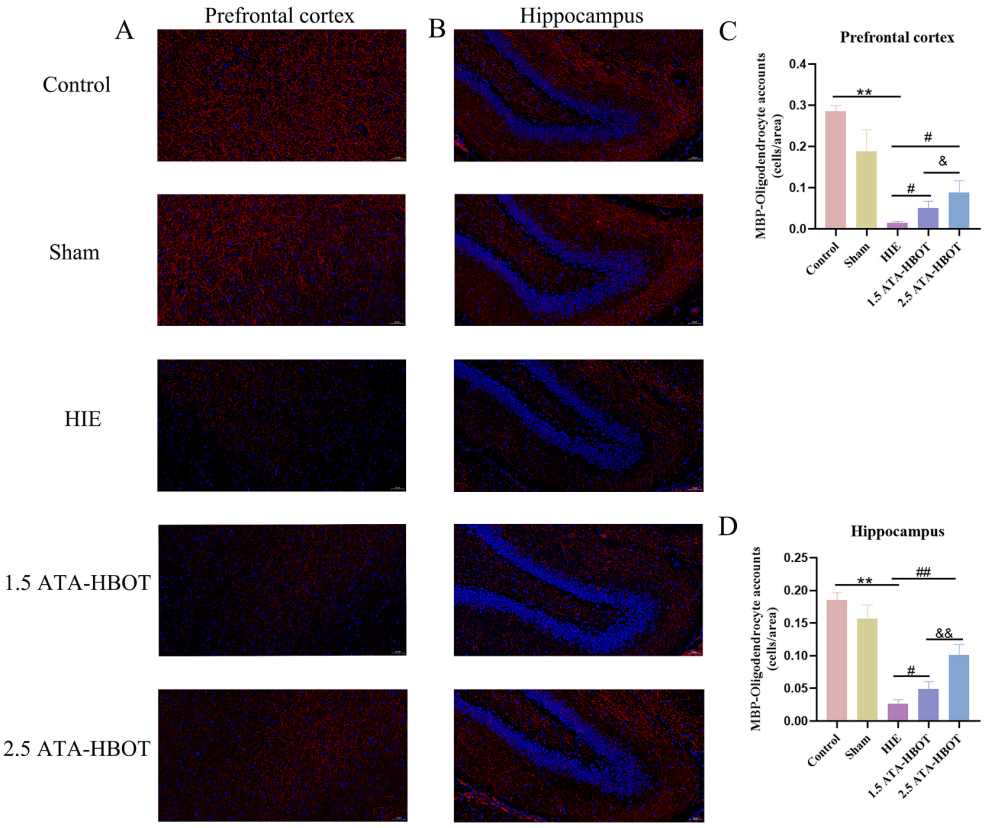

Fig. 3. HBOT promoted the expression of MBP after HI. (A, B) Immunostaining of MBP on the 6th day in hippocampus and prefrontal cortex. (red); Bar: 200 μm, DAPI (blue). (C, D) Quantitative analysis of mean density for MBP staining of different groups on the 6th day after HI. Date are represented as mean ± SD (n = 3 in each group). Date were analysed by one-way ANOVA with Turkey's post hoc test. *p < 0.05, ** p < 0.05 vs sham group; #p < 0.05, ##p < 0.01 vs HI group; &p < 0.05, &&p < 0.01 vs 1.5ATA group.

nimal, and at least three different fields were selected per slide for analysis. A pathology specialist consulted was invited for these analyses.

2.6. Luxol fast blue

Luxol fast blue was used to determined the variation of myelin in the injury area. The experimental rats were anesthetized by intraperitoneal injection of 10 % chloral hydrate. After fixation by perfusion, the brain tissues of the rats' prefrontal cortex and hippocampus were made into paraffin sections. The paraffin sections were dewaxed to water, and the sections were immersed in solid blue staining solution and incubated overnight at 60℃. After staining, sections were dehydrated in graded ethanol, and cleared in xylene before being coverslipped by neutral resin. Three 200x fields were randomly selected for each section in each group, and the positive cumulative optical density of each photo was obtained by analyzing each photo.

2.7. Western blot

Rat brain tissues were cut out on ice and placed in EP tubes. Added 200-300ul lysis buffer for 45 min, and then centrifuged with 12,000 rpm at 4℃ for 15 min. The concentrations of protein samples were measured by BCA kit (Beyotime-Bio, Shanghai, China), then the 5X protein loading buffer was added into the supernates and boiled for 5 min. Protein samples were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-interleukin (IL) 1β (abcam, ab254360, 1:500), anti-IL-6 (abcam, ab9324, 1:500), anti-tumor Necrosis Factor Alpha (TNF-α) (abcam, ab205587, 1:500), anti-hypoxia Inducible Factor 1 Subunit Alpha (HIF1-α) (abcam, ab179483, 1:500), and anti-superoxide dismutase (SOD) (proteintech, 10269-1-AP, 1:500) at 4℃ overnight. The PVDF membranes were washed with TBST and then incubated with HRP-conjugated secondary goat anti-rabbit antibody (Beyotime-Bio, A0208, 1:1000) or goat anti-mouse antibody (Beyotime-Bio, A0216, 1:1000) at 37℃ for 2 h. Finally, colored and exposed the PVDF membranes. Quantification was performed by scanning Tianneng automatic chemiluminescence analyzer analysis which were normalized to proper internal control proteins and expressed as relative folds of sham treated control. Results were from three independent experiments.

2.8. Enzyme linked immunosorbent assay (ELISA)

The protein levels of IL-1α,IL-6, IL-8, HIF-1α, SOD and TNF-α in serum were quantitated using ELISA kits (IL-1α ELISA kit, YM-Bio, Wuhan, China; IL-6 ELISA kit, JYM-Bio, Wuhan, China; IL-8 ELISA kit, JYM-Bio, Wuhan, China; HIF-1α ELISA kit, JYM-Bio, Wuhan, China; SOD ELISA kit, JYM-Bio, Wuhan, China and TNF-α ELISA kit, JYM-Bio, Wuhan, China) according to the manufacturers' instructions.

2.9. Statistical analysis

Data are presented as mean ± SEM. The statistical significance of differences was evaluated using one-way ANOVA. Turkey's post hoc test was conducted for multiple group comparison, using GraphPad Prism 8.0.1 (Graphpad Inc., San Diego, U.S.) soft ware; p < 0.05 indicated significant difference.

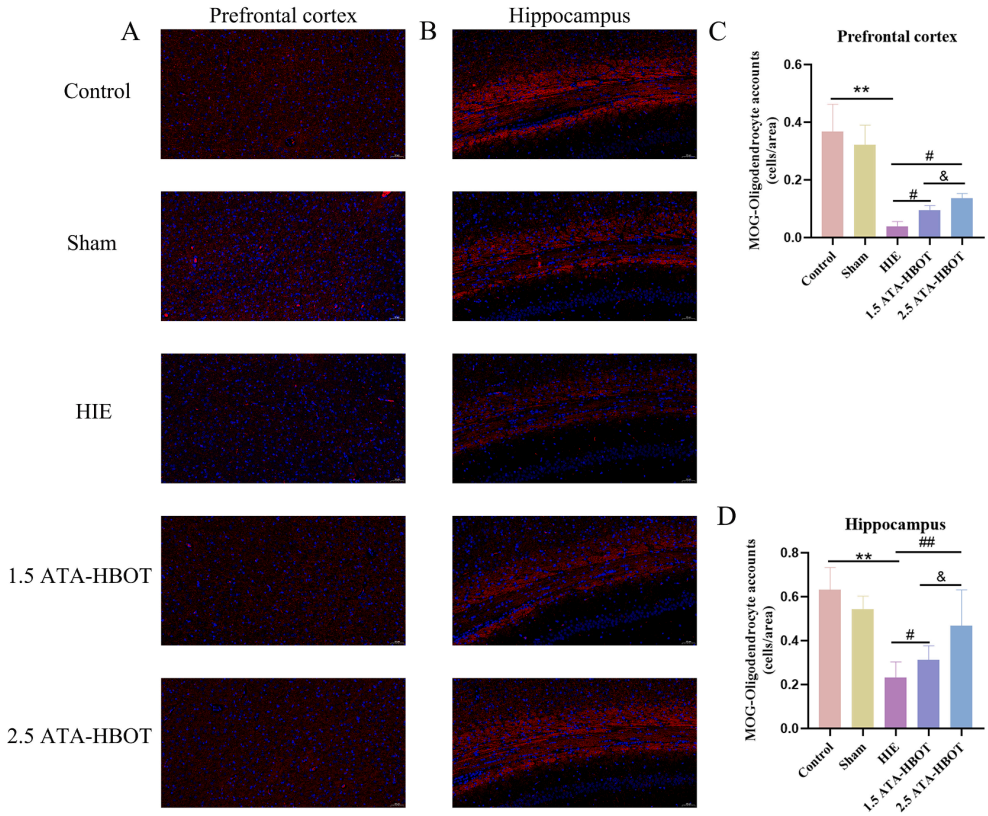

Fig. 4. HBOT attenuated HI-induced OLs loss. (A, B) Immunostaining of MOG on the 6th day in hippocampus and prefrontal cortex. (red); Bar: 20 μm, DAPI (blue). (C, D) Quantitative analysis of counts for MOG positive OLs of different groups on the 6th day after HI. Date are represented as mean ± SD (n = 3 in each group). Date were analysed by one-way ANOVA with Turkey's post hoc test. *$p < 0.05$, ** $p < 0.05$ vs sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs HI group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs 1.5ATA group.

3. Results

3.1. HBOT improved the learning and memory after HI in adult rats

Cognitive deficits was assessed with the Morris water maze test by measuring escape latency and number of platform crossings before and 6 days after HI. There was no significant difference in either parameter of different groups. Compared with control and sham surgery group, the escape latency was significantly longer on day 6 in HI group ($P < 0.05$). Compared with HI group, the escape latency was significantly shorter in the HBOT group ($P < 0.05$), and the 2.5ATA HBOT group had a shorter escape latency than that in the 1.5ATA HBO group ($P < 0.05$) (Fig. 1A). Compared with control and sham surgery group, the number of platform crossings was significantly reduced on day 6 in the HI group ($P < 0.01$). Compared with HI group, the number of platform crossings was significantly increased in HBOT group ($P < 0.05$), and the increase was more obvious in 2.5ATA HBOT group ($P < 0.05$) (Fig. 1B).

3.2. HBOT protected against HI-induced myelin injury

We utilized LFB staining and MBP immunochemistry to determin whether the cognitive deficits is relate to myelin injury after HI in adult rats. It was showed that myelin injury on day 6 after HI was more extensive than in control and sham groups; this effect was mitigated by HBOT treatment ($P < 0.01$), and the extent of 2.5ATA HBOT was better than 1.5ATA HBOT (Fig. 2A, B, C, D). Similarly, compared with the control and sham groups, the expression of myelin basic protein (MBP) was markedly reduced on day 6 after HI, and compared with HI group, it was increased in the HBOT group on day 6. Morever, the extent of 2.5ATA HBOT was also better than 1.5ATA HBOT ($p < 0.01$) (Fig. 3A, B, C, D).

3.3. HBOT attenuated HI-induced OLs loss and suppressed the reactive activation of astrocyte and microglia

To investigate the cytological mechanisms of HBOT on myelin injury after HI, we examined the number of OLs, astrocytes and microglias in brain, we observed the number of MOG-positive OLs, GFAP-positive astorcytes and Iba-1-positive microglias in the hippocampus and prefrontal cortex of rats. Compared with the control and sham groups, the number of MOG-positive OLs was markedly reduced on day 6 after HI. However, compared with HI group, the number of MOG-positive OLs declined to a lesser extent on day 6 in the HBOT group. Morever, the number of MOG positive OLs was higher in 2.5ATA HBOT group than that in 1.5ATA HBOT group ($p < 0.01$) (Fig. 4A, B, C, D). On day 6 after HI, the number of GFAP-positive astorcytes and Iba-1-positive microglias was significantly higher in hippocampus and prefrontal cortex than that incontrol and sham groups ($p < 0.01$), wheras it was significantly suppressed by HBOT ($p < 0.01$), the extent of inhibition was more pronounced in 2.5ATA HBOT group than 1.5ATA HBOT group ($p < 0.01$) (Fig. 5A, B, C, D and Fig. 6A, B, C, D).

3.4. HBOT reduced HI-induced increase in the number of NG2-positive OPCs

During demyelination in the adult brain parenchyma of rats, OPCs

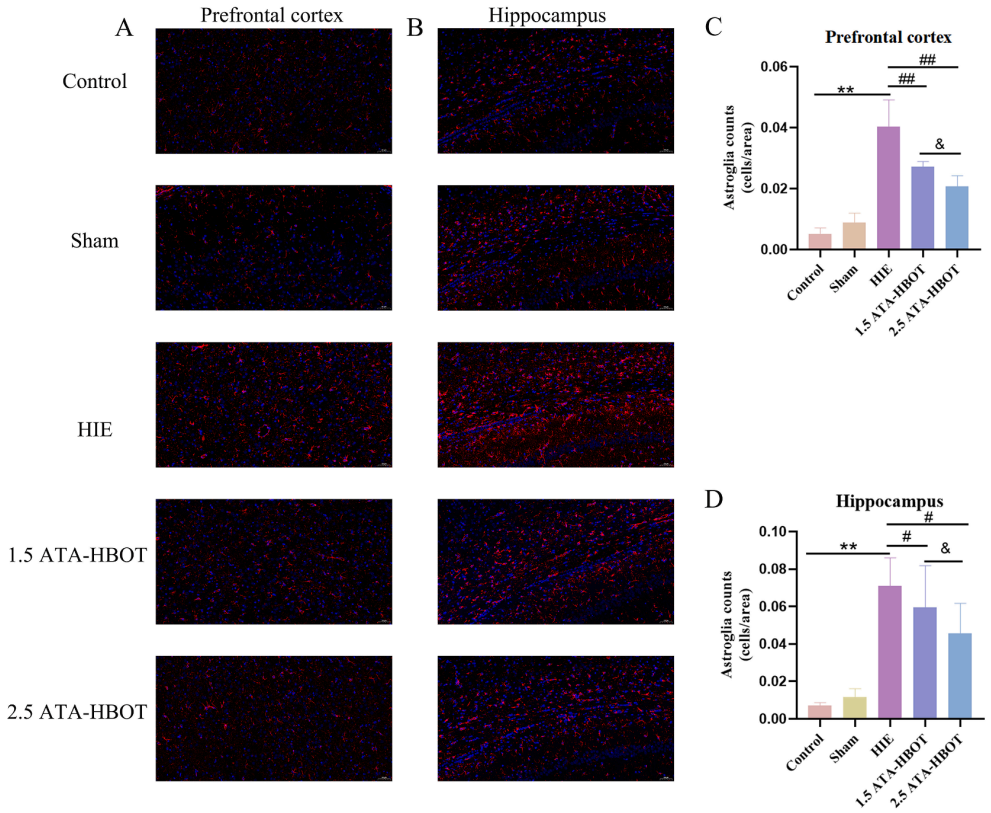

Fig. 5. HBOT suppressed the reactive activation of GFAP-positive astrocyte. (A, B) Immunostaining of GFAP on the 6th day in hippocampus and prefrontal cortex. (red); Bar: 20 μm, DAPI (blue). (C, D) Quantitative analysis of counts for GFAP positive OLs of different groups on the 6th day after HI. Date are represented as mean ± SD (n = 3 in each group). Date were analysed by one-way ANOVA with Turkey's post hoc test. *p < 0.05, ** p < 0.05 vs sham group; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs HI group; $^{\&}$p < 0.05, $^{\&\&}$p < 0.01 vs 1.5ATA group.

possess the potential to give rise to OLs, and improves myelin injury. [20] We also detected the variation in the number of OPCs in the hippocampus and prefrontal cortex of rats after HI. Compared with the control and sham groups, the number of NG2-positive OPCs significantly increased on day 6 in HI group ($p < 0.01$). However, compared with the HI group, it decreased in hippocampus ($p < 0.05$) and prefrontal cortex ($p < 0.01$) in both 1.5ATA and 2.5ATA HBOT group, and it was more significant in the 2.5ATA HBOT group ($p < 0.05$) (Fig. 7A, B, C, D).

3.5. HBOT inhibited neuroinflammation and balanced oxidative damage and antioxidant capacity after HI

To further investigate potential cytokines of HBOT on the myelin injury after HI, we performed a multiplex assay on brain tissues from lesion areas by Western Blot analysis, including interleukin (IL) 1β, IL-6 and tumor necrosis factor alpha (TNF-α), hypoxia inducible factor 1 subunit alpha (HIF1-α) and superoxide dismutase (SOD). It was showed that compare with the control and sham groups, HI induced significantly increased level of the pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), HIF1-α and decreased levels of SOD ($p < 0.0001$). HBOT remarkly inhibited the expression level of IL-1β, IL-6, TNF-α, HIF1-α and promoted the expression of SOD ($p < 0.05$), and the effects was more obvious in 2.5ATA group ($p < 0.05$) (Fig. 8 A, B). We also used enzyme linked immunosorbent assay (ELISA) to test the level of above cytokines in serum. By one-way ANOVA, compared with control and sham groups, the levels of IL-1β, IL-6, TNF-α, HIF1-α and SOD in serum had meaningfully boosted after HI ($p < 0.01$). Compared with HI group, there was a significant reduction in the level of IL-1β, IL-6, TNF-α, HIF1-α, SOD ($p < 0.01$) in 2.5ATA HBOT group. However, in 1.5ATA HBOT group, only the level of IL-6, TNF-α and SOD was significantly inbibited after HI (Fig. 9).

4. Discussion

The main findings of this study were that (1) both 1.5ATA and 2.5ATA HBOT protected against myelin injury, attenuated OLs loss, suppressed the reactive activation of astrocyte and microglia and promoted the differentiation of NG2-positive OPC, thereby improving cognitive deficits following HI-induced brain damage. (2) both 1.5ATA and 2.5ATA HBOT possibly exerted above effects by affecting the expression level of pro-inflammatory cytokines, HIF1-α and SOD in lesion areas and serum. (3) the effect of 2.5ATA HBOT was more obvious than 1.5ATA HBOT.

Survivors after HIE suffer from mild motor and cognitive deficits to cerebral palsy and severe cognitive deficits. [21] WM is mainly composed of myelinated nerve fibers and essential for the development and maintenance of human cognitive function. [22] Myelin of CNS is very sensitive to hypoxia and ischemia, with relatively little blood supply and disproportionate and poor collateral circulation in brain. [23] Therefore, Myelin is the first attact after HI, causing substantial injury to neural cells in white and gray matter. In this study, we first induced hypoxia and ischemic brain damage in adult rats based on the model of neonatal HIE. Water Maze analysis, LFB and MBP immunostaining showed that the rats in HI group exhibited obvious myelin injury and cognitive deficits 6 days after HI. This indicates that our model is able to successfully to simulate the early process of HI-induced brain injury in adult rats.

It is well accepted that exposure to HBOT, namely breathing 100 %

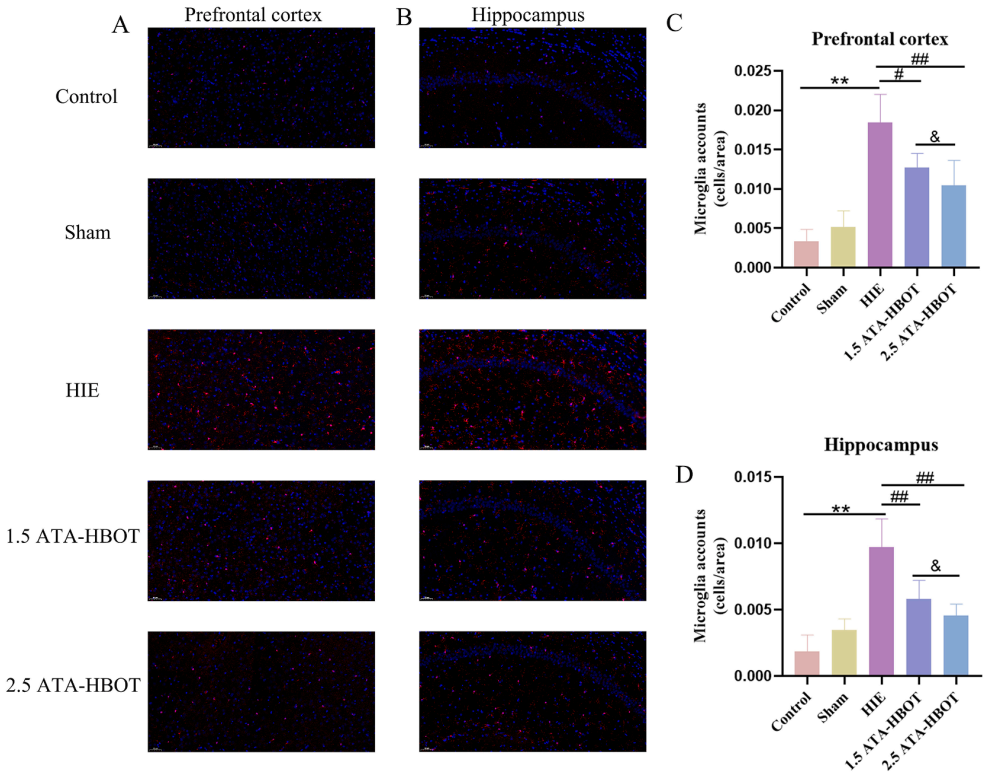

Fig. 6. HBOT suppressed the reactive activation of Iba-1-positive microglia. (A, B) Immunostaining of Iba1 on the 6th day in hippocampus and prefrontal cortex. (red); Bar: 20 μm, DAPI (blue). (C, D) Quantitative analysis of counts for Iba1 positive OLs of different groups on the 6th day after HI. Date are represented as mean ± SD (n = 3 in each group). Date were analysed by one-way ANOVA with Turkey's post hoc test. *p < 0.05, ** p < 0.05 vs sham group; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs HI group; &p < 0.05, $^{\&\&}$p < 0.01 vs 1.5ATA group.

oxygen at greater than atmospheric pressure (1 ATA) has positive effect in the treatment of carbon monoxide intoxication, central retinal artery occlusion, crush injury, gas gangrene etc. [24] Growing animal and clinical research has also determined that early HBOT can decrease the mortality and disability in neonatal and adult HIE. [16,25] It is suggested that HBOT could result in the proliferation of BrdU-positive neural stem cells and alleviate the myelin damage following HIE in neonatal rats. [26] In our study, We used water maze analysis, LFB and MBP immunostaining and found that HBOT protected against myelin injury and improved cognitive deficits of HI adult rats. Oxygen, like any other drug available, can be both high-dosage and low-dosage, depending on how it is used. The primary concern of oxygen inhalation is neurotoxicity especially when pressure was greater than 3 ATA. [27]In China, 1.5 and 2.5 ATA HBOT were the usually preferred pressure values for monoplace and multiplace chamber in clinical practice, depending on the disease, for example, 1.5ATA is more commonly used for traumatic brain injury (TBI), sudden deafness etc, wheras 2.5ATA is often more commonly used for acute carbon monoxide poisoning, wounds etc, suggesting that different pressure of HBOT may has different effect and mechanism. [28] Thus, we chose 1.5ATA and 2.5ATA HBOT for consecutive 7 times treatments to the HI adult rats, and compared their difference on myelin injury in brain and cognitive deficits, we found that 2.5ATA HBOT provided better treatment results.

The maintenance of myelin depends on the "microenvironment" of axons and their surrounding glial cells, including myelin-producing OL, microglia, astrocyte. [29] Hypoxia and ischemia can activate neuroinflammatory responses and induce the death of OLs and the activation of microglias and astrocytes, further attacking and destroying myelin sheath. [30] Therefore, inhibition of microglia and astrocyte activation is an important mechanism to protect OL apoptosis, myelination, and even neuronal damage. Previous studies have shown that HBOT can reduce HI-induced activation of microglia and astrocyte, and promote OL generation and remyelination. [31] In our study, we used immunostaining MOG, GFAP and Iba-1 to examin the number of OLs, astrocytes and microglias in the hippocampus and prefrontal cortex of rats, and found that HBOT could attenuated HI-induced MOG-positive OL loss and suppressed the reactive activation of GFAP-positive astrocyte and Iba-1-positive microglia. Similar to our myelin pathology and behavioral findings of rats, the effect of 2.5ATA HBOT is also better than 1.5ATA HBOT. Taken together, these results suggest that HBOT may protect against HI-induced myelin damage by preventing OL apotosis and the activation of microglia and astrocyte. Higher pressure of HBOT is better for the restoration of cellular energetics, reduction of oxidative stress/inflammation, and repair of cellular damage and recovery. [32] Morever, we also detect the number of OPCs by NG2 immnostaining, and found that HI induced significant increasiong of NG2-positive OPCs in the hippocampus and prefrontal cortex of rats, and 7 sessions of HBOT significantly reduced HI-induced increase in the number of NG2-positive, it was more obvious in 2.5ATA HBOT group. We speculate that this may relate to the possibility that HI induces the compensatory proliferation of OPCs to replenish the lost OLs or inhibits the differentiation of OPCs, and HBOT may promote OPCs to differentiate into OLs for myelin repair.

Strong evidence indicates that inflammation plays an important role in pathogenesis and progression of HIE. [33] IL-1β, IL-6 and TNF-α in cerebrospinal fluid (CSF) correlate with the severity of brain injury and can predict neurological deficits in infants who suffered from HIE. [34] HBOT can reduce inflammatory and mediators like IL-1β, HIF1-α, matrix metallopeptidase 9 (MMP-9), cyclooxygenase-2, nogo-A, and myeloperoxidase, promoting angiogenesis and the migration and

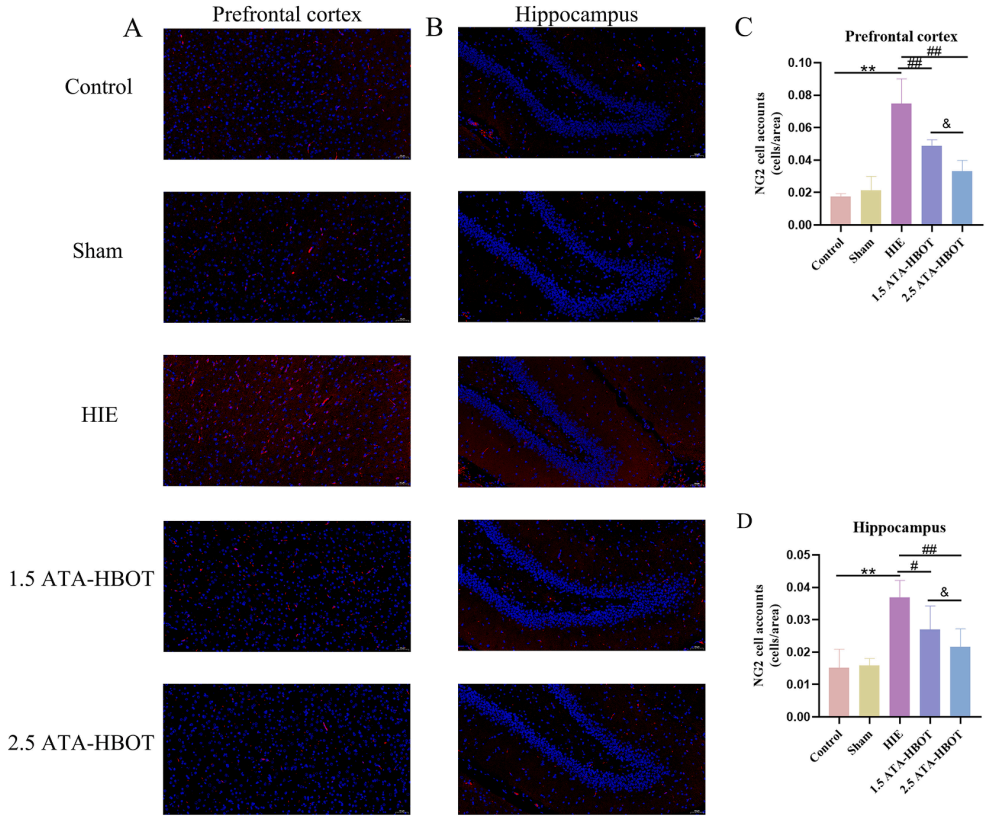

Fig. 7. HBOT reduced HI-induced increase in the number of NG2-positive OPCs. (A, B) Immunostaining of NG2 on the 6th day in hippocampus and prefrontal cortex. (red); Bar: 20 μm, DAPI (blue). (C, D) Quantitative analysis of counts for NG2 positive OLs of different groups on the 6th day after HI. Date are represented as mean ± SD (n = 3 in each group). Date were analysed by one-way ANOVA with Turkey's post hoc test. *p < 0.05, ** p < 0.05 vs sham group; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs HI group; $^{\&}$p < 0.05, $^{\&\&}$p < 0.01 vs 1.5ATA group.

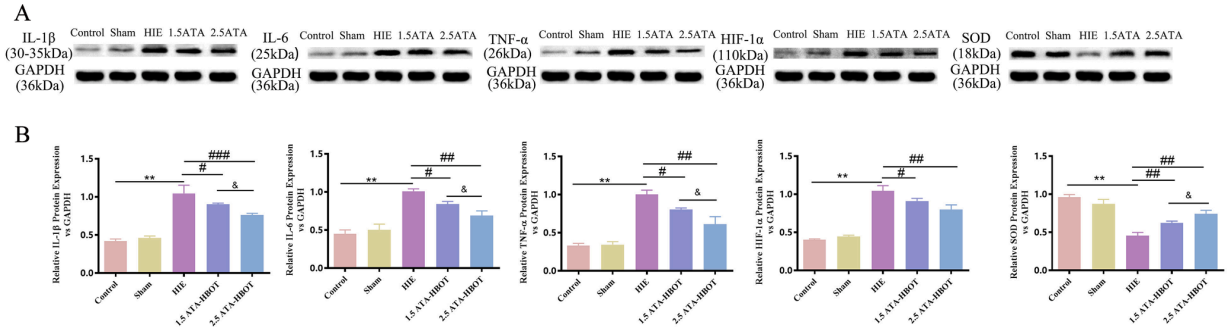

Fig. 8. Effect of HBOT on neuroinflammation and oxidative damage in brain tissue following HI. (A) Detection of IL-1β, IL-6 and TNF-α, HIF1-α and SOD proteins on the 6th day by Western blot. **(B)** Quantitative analysis of IL-1β, IL-6 and TNF-α, HIF1-α and SOD proteins expression levels. Compare with the control and sham groups, HI induced significantly increased level of IL-1β, IL-6, TNF-α and HIF1-α and decreased the level of SOD. HBOT remarkly inhibited the expression level of IL-1β, IL-6, TNF-α and HIF1-α and promoted the expression of SOD, and the effects was more obvious in 2.5ATA group. (n = 3 per group at each time point). Date were analysed by one-way ANOVA with Turkey's post hoc test. *p < 0.05, ** p < 0.05 vs sham group; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 vs HI group; $^{\&}$p < 0.05, $^{\&}$&p < 0.01 vs 1.5ATA group.

differentiation of endogenous neural stem cells, improving the brain damage and prognosis in rats and patients with HI-induced brain damage. [35] In our study, we tested the expression of IL-1β, IL-6, TNF-α and HIF1-α in brain and serum, and found that HBOT remarkly supressed the high expression level of IL-1β, IL-6, TNF-α, HIF1-α after HI, and it was also more obvious in 2.5ATA group, indicating that HBOT may alleviate HI-induced myelin damage by inhibit the release of pro-inflammatory factors from astrocyte and microglia etc. It is also suggested that the higher the pressure, the more obvious the anti-inflammatory effect of HBOT. However, following hypoxia and ischemia, reactive oxygen species (ROS) production rapidly increases and overwhelms antioxidant defenses, leading to a cascading inflammatory response, and protease secretion. [36] Moreover, HBOT is also a double-edged sword, and early HBOT may exert both antioxidant effects and oxidative damage,

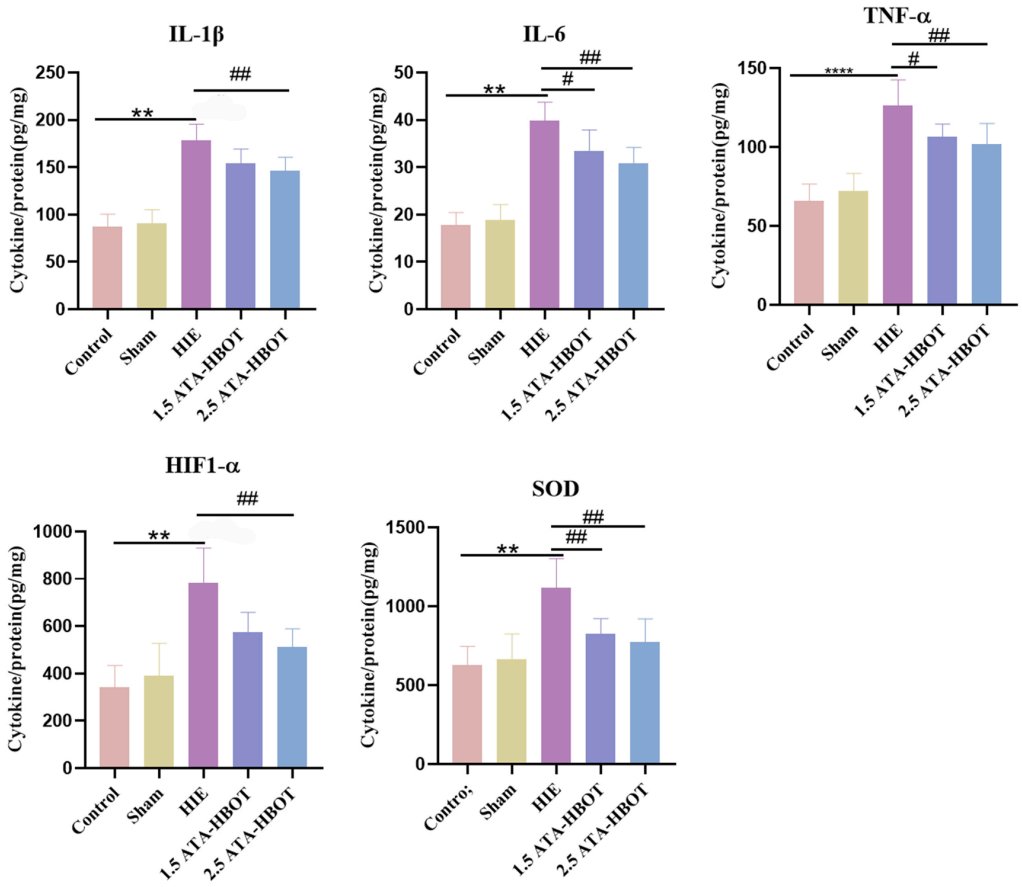

Fig. 9. Effect of HBOT on neuroinflammation and oxidative damage in serum following HI. Quantitative analysis of IL-1β, IL-6, TNF-α, HIF1-α and SOD proteins in serum on the 6th day by ELISA. Compared with control and sham groups, the levels of IL-1β, IL-6, TNF-α, HIF1-α and SOD in serum had meaningfully boosted after HI. Compared with HI group, there was a significant reduction in the level of IL-1β, IL-6, TNF-α, HIF1-α, SOD in 2.5ATA HBOT group. However, in 1.5ATA HBOT group, only the level of IL-6, TNF-α and SOD was significantly inbibited after HI (n = 3 per group at each time point). Date were analysed by one-way ANOVA with Turkey's post hoc test. *$p < 0.05$, ** $p < 0.01$ vs sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs HI group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs 1.5ATA group.

depending on its pressure. [37] Thus, we also detect the level of SOD, an important antioxidant enzyme and protecting from oxidant stress, and found that SOD was significantly decreased in the lysates of brain and increased in serum in HI group, and HBOT reversed this change. This might be explained that antioxidant scavenger activity is elevated in site of brain damage after 6 days (7 sessions) of HBOT, helping the mitochondria function without disturbing the redox balance and even enhancing their activity, while HBOT also causes mitochondria to reduce their activity, which partially decreases ROS production, thereby balancing between free radical levels and antioxidant levels following hypoxia and ischemia. In addition to this, 2.5ATA HBOT did not exhibit higher oxygen toxicity.

5. Conclusion

In the present study, we demonstrated the mechanism of different pressure HBOT on HI induced brain injury from three levels: (1) On a tissue level, HBOT protects against HI induced myelin injury; (2) On a cellular level, HBOT attenuates HI-induced OL loss, suppresss the reactive activation of astrocyte and microglia, and may promote OPC to differentiate into OL; (3) On a molecular level, HBOT inhibites neuroinflammation, and balances oxidative damage and antioxidant capacity. Among the above effects, 2.5ATA HBOT is better than 1.5ATA HBOT. Ongoing research will continue to seek out the signalling pathways and molecules mechanisms on different pressure of HBOT-related myelin protection, and possibly expand suitable HBOT use in adult HIE clinically.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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