

Hyperbaric Oxygen Combined With Hydrogen-rich Saline Protect Against Acute Lung Injury Induced by Lipopolysaccharide in Rat Model

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Research

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Abstract

Background: To investigate the effects of hydrogen-rich saline (HRS) combined with hyperbaric oxygen (HBO) on acute lung injury (ALI) and its clinical significance.

Methods: 40 adult male Sprague-Dawley rats were randomly divided into 5 groups: the sham, LPS, LPS + HBO, LPS + HRS and LPS + HBO + HRS. LPS at a rate of 3mg/kg was injected into the trachea of the experimental animals to develop ALI model, then the animals were respectively given simple HBO or HRS treatment or combined treatment. 3 days later, we study lung pathological, the levels of inflammatory factors, and cell apoptosis in the pulmonary tissue was detected by Tunel and cell apoptosis rate was calculated accordingly.

Results: In the groups treated with HRS and HBO, pulmonary pathological data, wet-dry weight ratio and inflammatory factors in the pulmonary tissues and alveolar lavage fluid were significantly superior to those of the sham group ($P < 0.05$). Cell apoptosis detection revealed that the simple treatment with HRS or HBO, or combined treatment with both, can all alleviate cell apoptosis, and the combined treatment with HRS and HBO was obviously superior to single treatment ($P < 0.05$).

Conclusions: HRS and HBO could all decrease the release of inflammatory cytokines in lung tissue, reduce accumulation of oxidative products and alleviate apoptosis of pulmonary cells, and could produce good therapeutic effects on ALI induced by LPS. HBO combined with HRS seems to have a synergistic effect on the decrease of cell apoptosis, and in the expression of inflammatory cytokines and the generation of related inflammatory products, the combined use of HBO and HRS showed a decreasing trend as compared with simple application.

Background

Lung injury is one of the most serious injuries that are difficult to handle and is a leading cause of death in patients with thoracic trauma^[1, 2]. Clinical researches indicate that serious thoracic trauma could instantly activate cellular and humoral immunity, trigger effector cells to be involved in the process and activate cascade reaction^[3, 4]. Excess release and over-expression of inflammatory mediators and related inflammatory cytokines could stimulate excess inflammatory reaction, resulting in damage to alveolar-capillary membrane and increased vascular permeability and ultimately acute lung injury (ALI)^[5-7]. Initial pathological changes that could be noted were alveolar internal hemorrhage, atelectasis, edema and parenchymatous degeneration, which could be reversible at early stage and within 12–24 hours after injury they could be progressive. If pathological changes were not timely curbed, acute respiratory distress syndrome (ARDS) could be developed^[8-10]. The treatment of ALI mainly depended on the application of respirator. Up to now, the most remarkable progress in the supportive treatment of patients with ALI still remained at the stage of better management of respirators^[11, 12].

The application of hyperbaric oxygen therapy (HBOT) in China has a history of over 50 years, and so far over 140 diseases could be treated with HBO. HBOT could obviously enhance physical solubility of oxygen in arteries, increase partial pressure of oxygen and improve ischemia in various organs or tissues, decrease acid metabolites and hasten functional recovery of various organs. The indications of HBO for the treatment of lung diseases include pulmonary edema, ARDS, etc. and the earlier the treatment is implemented, the better results will be achieved. Biologists have long held that hydrogen is physiologically an inertial gas, and recent researches have strongly proposed that hydrogen is a novel antioxidative substance, capable of selectively reducing reactive oxygen species (ROS) or oxygen radicals^[13, 14]. Follow-up studies have once again demonstrated its antioxidative effects on the reperfusion of myocardio, liver and small intestine ischemia^[15-18], and the biologic antioxidative effect of hydrogen has its distinctive features.

In our experiment, adult SD rat model of acute lung injury was established by injecting lipopolysaccharide (LPS) into trachea, and life signs and dry-wet ratio of pulmonary tissue were closely observed. The levels of malondialdehyde (MDA) and superoxide dismutase (SOD) were detected in the pulmonary tissue, and the levels of inflammatory factors (TNF- α , IL-1 β , IL-6) in the pulmonary tissue and blood were detected as well. Then, the pathological structure and cell apoptosis of the pulmonary tissue were strictly observed, and the therapeutic effects of HBO combined with HRS on pulmonary injury was closely studied in rats.

Materials And Methods

Drugs and reagents

Lipopolysaccharide solution (2.5mg/ml) (055: B5) was purchased from SIGMA, pentobarbital sodium was bought from Sangon Biotech in Shanghai. 10% formalin was available from the Shanghai Changzheng Hospital. IL-1 β ELISA, IL-6 ELISA and TNF- α ELISA test kits were respectively obtained from Lianke Bioproducts (Biology) Company and Bioscience Company. MDA and SOD test kits were provided by Nanjing Jiancheng Biologic Company. TUNEL test kit was obtained from the American Roche Company. Briefly, the HRS was prepared as follows: hydrogen was dissolved in normal physiological saline at a pressure of 4 ATA by gassing for 6 hours.

Division of the animals and development of animal model

Male Sprague-Dawley rats (6 weeks, 240 \pm 10g) were provided by the Laboratory Animal Center of Second Military Medical University (Shanghai, China) and housed in pathogen-free conditions with a 12h/12h light/dark cycle at 22–24°C and free access to food and water. The experimental protocols were approved by Animal experimental ethics committee of the Second Military Medical University and conducted in compliance to the standards of Experimental Animal Regulation Ordinances defined by the China National Science and Technology Commission. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

According to the report, ALI was induced by trachea injection of LPS. After anesthesia, a small incision at the neck was also performed to expose trachea, in which a proper volume of LPS solution and saline were infused, after puncture of tracheal cavity. Then, the incision was sutured and disinfected. Following development of the endotoxic model. Briefly, rats were injected intratracheally with LPS at a dose of 3mg/kg body weight and was supplemented with 0.3 ml normal saline. Sham controls were injected intratracheally with the same volume of aseptic saline.

40 adult male Sprague-Dawley rats were randomly divided into 5 groups: the sham, LPS, LPS + HBO, LPS + HRS and LPS + HBO + HRS. The LPS group: a small incision at the neck was also performed to expose trachea, in which a proper volume of LPS solution (3mg/kg) was infused and was supplemented with 0.3 ml normal saline, after puncture of tracheal cavity. Then, the incision was sutured and disinfected. Following development of the endotoxic model. The LPS + HBO group: the animals were exposed to HBO at a pressure of 2.5 ATA, one session every 12 hours and the duration of HBO exposure a session was 120 minutes. The LPS + HRS group: the animals received abdominal injection of HRS at a dosage of 5 ml/kg, once every 12 hours. The LPS + HBO + HRS group: the animals also received abdominal injection of HRS at a dosage of 5 ml/kg, and treated with HBO. once every 12 hours.

Seventy-two hours later, Rats were euthanized with an overdose of sodium pentobarbital (100 mg/kg, ip), and serum, pulmonary and lung lavage samples were collected for laboratory detection.

Evaluation of pulmonary pathology

Middle lobe of the right lung was taken for HE staining, and then pathological results were evaluated using the modified lung tissue damage scale by Yamane L et al^[19].

Detection of the rat lung dry and wet weight ratio

The upper lobe of right lung was placed on the paper to weigh wet weight of lung (W) by an electronic scale and the obtained data were recorded. Then, the pulmonary tissue sample was placed in a 70°C thermostatic baking oven for 48 hours, and the dry lung weight of each sample was measured, and the dry-wet weight ratio was calculated. Lung dry-wet (W/D) weight ratio = lung wet weight (W)/lung dry weight (D).

Biochemical testing

The levels of inflammatory cytokines in the pulmonary homogenate were detected with ELISA. MDA and SOD in the pulmonary tissue and serum were determined using the reagent which is from Nanjing Jiancheng Biologic Company.

Detection of cell apoptosis

The nucleus of the apoptotic cell withered and presented a dark-brown color, with a spherical, crescentic or irregular shape. By using the Image-pro Plus 6.0 software, the same dark-brown cell nucleus was selected as the criteria for positive rate assessment of all photos, and the same blue cell nucleus was

chosen as other cells. In accordance with positive cell counts and total cell counts shown in the photos, apoptosis index (AI) could be obtained. Apoptosis index (AI)= $\times 100$.

Statistical analysis

The SPSS17.0 statistical software and GraphPad Prism 6 were used for data analysis. The continuous data were expressed as mean \pm standard deviations (SD). The data between the groups were compared either by one-way ANOVA and LSD-*t* method or Kruskal-Wallis *H* and Dunnett's *C* method for multiple comparisons. Detection level $\alpha = 0.05$, and the difference with $P < 0.05$ was considered statistically significant.

Results

As shown in Fig. 1, the structure of alveolar air cavity in the rats of the sham group remained intact, uniform, with smooth walls, and infiltration by red and white cells was not visible, while in the animals of the LPS group, damage of alveolar structure, atelectasis, thickened interstitium and excessive white blood cells in interstitial space were noticeable, and hemorrhage and white cell infiltration in alveolar airspace were also noticed. In the animals of the LPS + HBO and LPS + HRS groups, thickened alveolar wall, white cell infiltration in alveoli and interstitium, as well as sporadic red cell infiltration were clearly visible. However, for the animals of the LPS + HRS + HBO group, the structure of alveoli was intact with mild alveolar interstitial edema and there were less inflammatory cells present, and the morphological image was obviously better than those of the LPS + HBO and LPS + HRS groups.

Lung dry-wet weight ratio and pathological evaluation

As shown in Fig. 2, there were significant differences when comparisons were made between the LPS and the sham groups (** $P < 0.01$), and statistical significance could all be seen, when comparisons were made between the LPS and the LPS + HRS and the LPS + HBO and the LPS + HRS + HBO groups ($*P < 0.05$). There were also statistical differences between the LPS + HRS + HBO and the LPS + HRS groups ($*P < 0.05$). Pathomorphologic results revealed that combined therapy with HRS and HBO alleviated pulmonary interstitial edema induced by LPS, decreased cell and protein leakage and retained integrity of pulmonary tissue, indicating that HRS and HBO displayed a synergetic effect.

lung dry-wet ratio could reflect the seriousness of pulmonary protein leakage. There were statistical differences in the dry-wet ratios, when the LPS group was compared with the other groups ($*P < 0.05$). However, no statistical significance could be noted, when comparisons were made between the LPS + HRS, LPS + HBO and the LPS + HRS + HBO groups, so there was no indication that the combined therapy with HRS and HBO could synergistically enhance pulmonary absorption.

Changes in TNF- α , IL-1 β and IL-6 levels in pulmonary tissue and serum

As shown in Fig. 3, inflammatory cytokine detection in lung and serum revealed that there was no difference in TNF- α level in the pulmonary tissue and serum when comparisons were made between the groups. The IL-1 β level in pulmonary tissue for the LPS group was obviously higher than those of the control group and other treatment groups ($\#P < 0.005$). However, no statistical significance could be found, when comparison were made between the LPS + HRS, LPS + HBO and the LPS + HRS + HBO groups. IL-6 detection results showed that there was statistical significance in IL-6 level in the LPS group, as compared with those of the simple HRS and simple HBO treatment groups ($*P < 0.05$), however, no statistical significance could be found as compared with that of the combined HRS and HBO group. Statistical significance could neither be seen in serum IL-6 level, when comparisons were made between the groups.

TNF- α and IL-1 β levels in pulmonary lavage fluid

As shown in Fig. 4, TNF- α level detection in pulmonary lavage fluid revealed that the level of the LPS group was higher than those of the control and intervention groups ($*P < 0.05$). There was no statistical significance when comparisons were made between the LPS + HRS, LPS + HBO and LPS + HRS + HBO groups. Statistical analysis showed that the level of IL-1 β in pulmonary lavage fluid was similar to that of TNF- α , and statistical significance could be found when comparisons were made between the LPS and other treatment groups. However, there were no significant differences when comparisons were made between the treatment groups.

Levels of MDA and SOD in the pulmonary tissue

From A in Fig. 5, we can see that there was statistical significance in MDA level in the pulmonary tissue, when comparisons were made between the PLS group and the sham and various treatment groups ($*P < 0.05$), while no statistical significance could be seen, when comparisons were made between the LPS + HRS, the LPS + HBO and the LPS + HRS + HBO groups. Figure B showed that SOD activity in the LPS group was lower than those of the other groups ($*P < 0.05$), and statistical significance could neither be seen, when comparisons were made between various treatment groups.

Detection of cell apoptosis

As shown in Fig. 6, cell apoptosis detection by Tunel method demonstrated that the color of apoptosis cells was dark brown. Pathomorphological detection indicated that the No. of apoptosis cells in the LPS group was the highest, with an average of 13.7/1000, and pulmonary lesion was most serious, while apoptosis cells in the sham group were almost undetectable, with an average No. of apoptosis cells of 1.4/1000. Only sporadic apoptosis cells could be detected in the LPS + HRS, the LPS + HBO and the LPS + HRS + HBO groups.

In Fig. 7, Cell apoptosis index was calculated by Image-pro plus 6.0 software. Statistical analysis indicated that there were statistical differences when comparisons were made between the LPS and the sham groups ($**P < 0.05$). As compared with the LPS group, significant differences could be noted in the simple H₂ group, the simple HBO group and the HRS combined with HBO group ($*P < 0.05$). There was

also difference when comparisons were made between the LPS + HRS group and the LPS + HRS + HBO group ($*P < 0.05$), indicating that HRS and HBO could produce a synergistic effect.

Discussion

As a treatment method, oxygen breathing, with a history of over a hundred years, was initially used for the treatment of such diseases as pneumonia, atelectasis, tuberculosis, asthma and so on. Now, oxygen breathing is also a routine treatment method used more and more extensively in emergency care of trauma or casualty patients, and surgical patients as well during perioperative period. By depending on the concentration of oxygen, oxygen breathing could further be divided into low or pure oxygen breathing, as well as atmospheric and hyperbaric oxygen breathing. Research reveals that HBO could increase oxygen level in blood and tissues, enhance oxygen diffusion, and it can produce better therapeutic effects than atmospheric oxygen. The distance and velocity of oxygen diffusion depend largely on the partial pressure of oxygen. In the patients with inflammation, trauma and burns, edema will develop in pulmonary interstitium and tissues, thus extending oxygen diffusion distance, consequently conventional oxygen breathing could not meet oxygen demand of cells, and in this case, HBO will help to solve the problem encountered. HBO therapy is now extensively used in clinical treatment of ischemia-reperfusion injury, shock, sepsis, multi-organ functional disorder, and various cardio-pulmonary vascular diseases^[20]. Studies by Jindal^[21] have demonstrated that patients with hepatic and pulmonary disorders or cardio-cerebral vascular diseases, periodic oxygen breathing could decrease frequency of sickness onset, improve functions of internal organs and enhance resistance to diseases. Prolonged and or persistent oxygen therapy has been extensively applied to disease recovery and proves to be highly beneficial to complete recovery of diseases. Oxygen therapy could also prevent complications caused by hypoxia, such as anoxic mental symptoms, encephalopathy, arrhythmia, lactic acidosis, tissue necrosis and so on. Medical research has also revealed that there exist such adverse reactions as oxygen toxicity, which may result in acute lung injury, with similar symptoms as acute respiratory distress syndrome (ARDS), and affect central nervous system with such symptoms as syncope and visual blurring due to retinal damage. In addition, excessive oxygen concentration and over-exposure to oxygen could also damage pulmonary and central nervous systems and retina^[22]. At present, the mechanism of oxygen toxicity remains largely unknown, and there are several syntheses, among which the synthesis of reactive oxygen species (ROS) or oxygen radical is the most acceptable.

Cells obtain oxygen from blood, and the glucose in the cell reacts with triglyceride to trigger oxidation reduction reaction and form energy required for the body. However, there is still a small amount of oxygen that is converted into ROS or free radicals. Under homeostatic conditions, ROS will not damage normal cells, which is attributed to the existence of a large amount of antioxidase in cells, and a balance is maintained between ROS and antioxidase within the body. However, when the tissue is in a state of hypoxia, disorder of energy metabolism will occur in cells, and oxygen will not be able to be covered into water by cytochrome oxydase, and the normally harmless oxygen will be converted into cytotoxic ROS. The main trouble of ROS is to cause damage to the structure and function of cells, mitochondrion, cut off

energy supply to cells and destruct lysosome, resulting in cell disruption. At the same time, it will also destruct vascular endotheliocyte and vascular walls, and consequently blood cell and serum leakage might be brought about, causing edema and cyanosis of surrounding tissues. On the contrary, excessively high partial pressure of oxygen will also cause damage to cells, the mechanism of which might be associated with the production of ROS^[23, 24].

The generation of ROS might be associated with oxygen partial pressure in cells. Either excessively high or low oxygen partial pressure will generate the production of large quantities of ROS, and the amount of its production is closely related with the persistence of the state. It is held that oxygen therapy could recover the function of ischemic cells, enhance oxidation reduction reaction (ORR) and activity of endogenous SOD and increase the capacity of SOD to eliminate ROS. Proper oxygen breathing could decrease the production of inflammatory factors and alleviate tissue damage. However, excessively high oxygen concentration and excessive oxygen exposure will increase ROS level in the body, causing severer tissue damage^[25]. Prolonged and high concentration of oxygen intake will result in excessive production of ROS, which will activate the expression of NF- κ B, causing release of TNF- α , IL-1 β and IL-6, and serious inflammatory reaction will be resulted. On the contrary, proper oxygen therapy could decrease the level of inflammatory factors, which will alleviate damage to tissues^[26]. In the hyperbaric oxygen treatment of diabetic foot research shows that twice daily hyperbaric oxygen treatment is more effective than once daily treatment, and no adverse reactions were found.^[27] Our experiment has also revealed that HBO therapy could decrease the expression of inflammatory factors in rats and alleviate pulmonary lesion, demonstrating that HBO could produce positive effects on ALI, HBO could not only be applied to the treatment of ALI, but was extensively used in the treatment of serious trauma injuries. Following research on the mechanism of HBO in serious trauma injury, Sourabh^[28] held that HBO could promote such synergistic effects as vascular contraction, fibroblast and capillary proliferation, toxin inhibition, as well as the synergistic effect with antibiotics. These effects are obviously beneficial to tissue edema after injury, recovery of tissue damage. In addition, combined use with antibiotics could reduce the occurrence of sepsis. Therefore, HBO could produce positive effects on serious lung injury. Our experiment has also indicated that HBO could alleviate inflammatory reactions after lung injury and reduce cell apoptosis.

Ohsawa et al reported that inhalation of 2% hydrogen could effectively scavenger hydroxyl radicals (-OH) and peroxytrite (ONOO-), obviously improve injury induced by ischemia reperfusion, and hydrogen dissolved in fluid could selectively neutralize or compromise the most important substance that cause cell oxidative damage. Hydrogen is an active, highly inflammable and explosive gas, which makes it difficult for clinical application. Hydrogen-rich saline, or simply called hydrogenised water, is a saturated, safe and effective agent dissolved in physiological saline. Hydrogen-rich saline with the hydrogen concentration as high as 0.6mmol/L could be injected into body safely and effectively. It could alleviate inflammatory reaction induced by acute injury of organs, inhibit cell apoptosis, decrease damage to target organs by oxygen radicals, and could produce good therapeutic effects on inflammatory diseases, neural retrograde degeneration and lesions induced by organ ischemia reperfusion as well, the

mechanism of which might be mainly attributed to the selective anti-oxidation of hydrogen, which selectively compromise $-OH$ and $ONOO^-$ and thus the stability of biological membrane is maintained.

Hydrogen can change signal conduction or transmission within cells, and reduce the generation of inflammatory mediators through controlled release of $NF-\kappa B$. Our experiment has revealed that the levels of $TNF-\alpha$ and $IL-1\beta$ in the hydrogen-rich saline group were significantly lower than those of the control group, and the number of apoptosis cells in the HRS group was also reduced to some extent, while the number of apoptosis cells in the control group was the highest. Statistical analysis indicated that the number of apoptosis cells in the LPS + HRS group was significantly less than that of the LPS group, implying that HRS could inhibit cell apoptosis ($P < 0.001$). The mechanism involved with cell apoptosis might be associated with the blockage of cytochrome C to activate proCaspase-9, further inducing cascade reaction. Other experiments also demonstrated that HRS could obviously decrease apoptosis index and the expression of caspase-3 in the damaged brain tissue^[29]. In cell apoptosis detection, it is found that HRS and HBO could decrease cell apoptosis, and there is also difference in the rate of cell apoptosis between the hydrogen-rich saline combined with HBO and simple HRS treatment, indicating that combined treatment could produce a certain synergistic effect and decrease cell apoptosis. This might provide us a new approach to an in-depth research on the mechanism of combined treatment with hydrogen and oxygen for acute lung injury.

Our experiment has further revealed that HBO and the hydrogen-rich saline are all effective in the treatment of lung injury induced by LPS. Through reduced generation of ROS, HBO could decrease the expression level of $NF-\kappa B$, and further decrease the release of $TNF-\alpha$, $IL-1\beta$ and $IL-6$, thus reducing inflammatory reaction and cell apoptosis. Through inactivation of ROS, the maintenance of cell membrane could be attained and the controlled release of $NF-\kappa B$, the hydrogen-rich saline could reduce the generation of inflammatory factors. Therefore, HRS and HBO in the treatment of lung injury induced by LPS could decrease inflammatory reactions, reduce the release of inflammatory factors and decrease the levels of inflammatory products. In addition, through decreased expression of $NF-\kappa B$, HBO and HRS could alleviate cell apoptosis. The target organs of HBO and the HRS are the same, the mechanism of which might be overlapping, as there is no significant difference in efficacy between HBO combined with HRS and simple HBO therapy or simple HRS in the treatment of lung injury induced by LPS.

To sum up, the combined use of HBO and HRS produces a synergistic effect on the decrease of cell apoptosis in the treatment of ALI. In the expression of inflammatory factors and the generation of inflammatory products, the combined treatment of HBO and HRS showed a decreasing trend, as compared with single treatment. Hydrogen and oxygen, being low in price and having with anti-inflammatory, anti-oxidative and cell apoptosis inhibition effects as well as accurate therapeutic effects, are suitable for the treatment of patients with serious ALI. However, the mechanism involved remains further in-depth research, and we are sure that the combined use of HBO and HRS promises a good future in its clinical application.

Conclusion

HRS and HBO could all decrease the release of inflammatory cytokines in lung tissue, reduce accumulation of oxidative products and alleviate apoptosis of pulmonary cells, and could produce good therapeutic effects on ALI induced by LPS. HBO combined with HRS seems to have a synergistic effect on the decrease of cell apoptosis, and in the expression of inflammatory cytokines and the generation of related inflammatory products, the combined use of HBO and HRS showed a decreasing trend as compared with simple application.

Abbreviations

HBO: Hyperbaric oxygen

HRS: Hydrogen-rich saline

LPS: Lipopolysaccharide

ALI: Acute lung injury

MDA: Malondialdehyde

SOD: Superoxide dismutase

ROS: Reactive oxygen species

ARDS: Acute respiratory distress syndrome

ORR: Oxidation reduction reaction

TNF- α : Tumor Necrosis Factor

IL-1: Interleukin-1

NF- κ B: Nuclear factor kappa-B

Declarations

Availability of data and materials

The datasets used during the current study are available from the corresponding author upon reasonable request.

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Authors' Contributions

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Ethics declarations

Ethics approval and consent to participate

The experimental protocols were approved by Animal experimental ethics committee of the Second Military Medical University and conducted in compliance to the standards of Experimental Animal Regulation Ordinances defined by the China National Science and Technology Commission.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

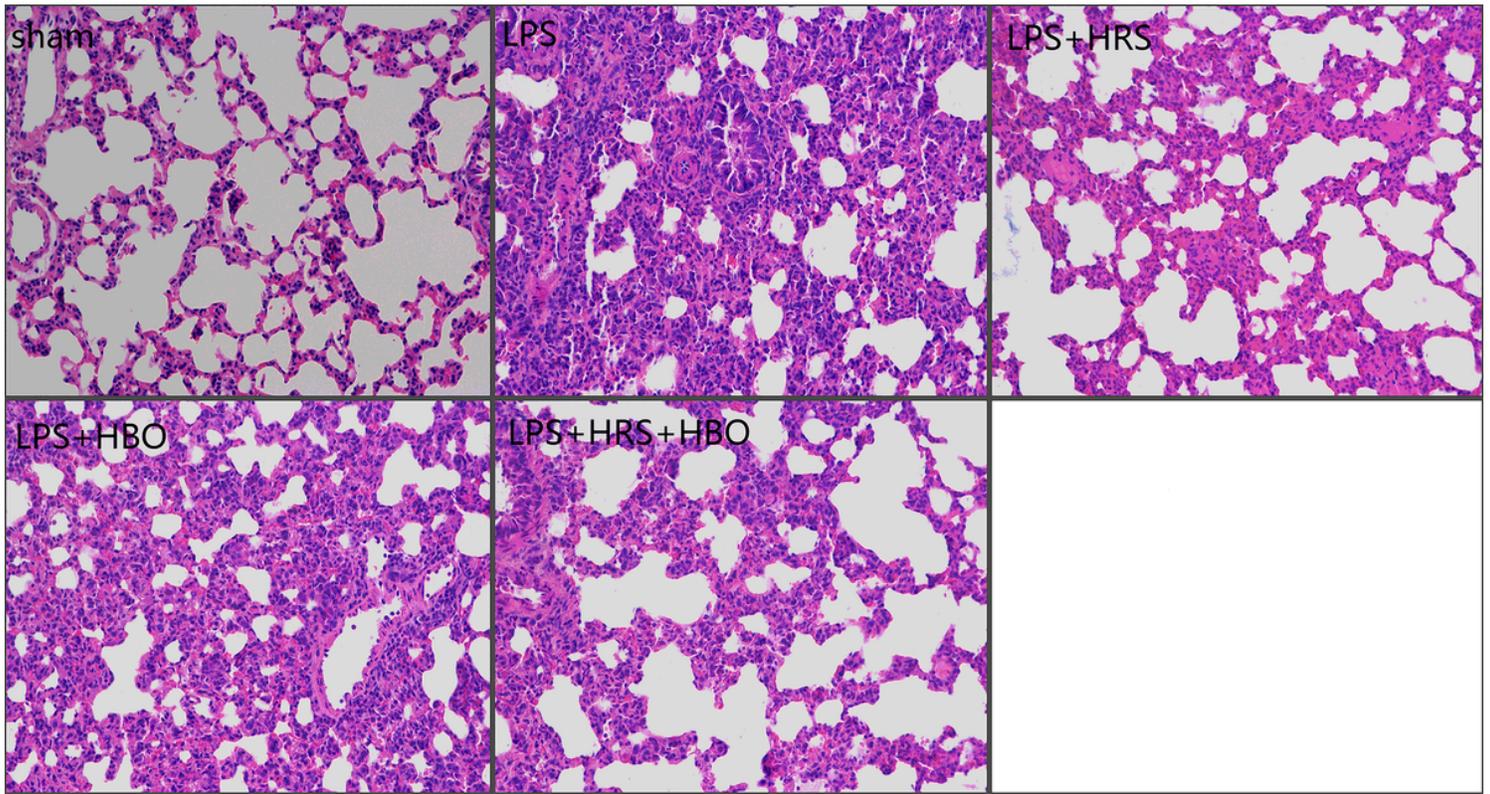


Figure 1

Pathological photos of pulmonary tissue HE staining (X 200)

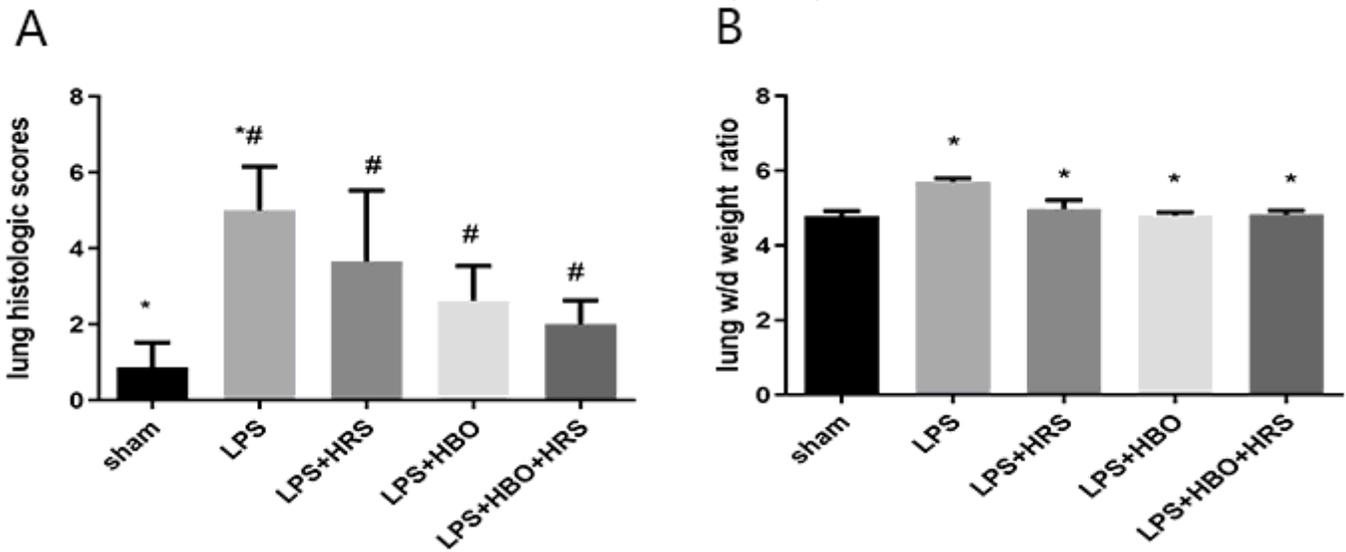


Figure 2

Pathological scores and Lung wet-dry weight ratio of the experimental groups

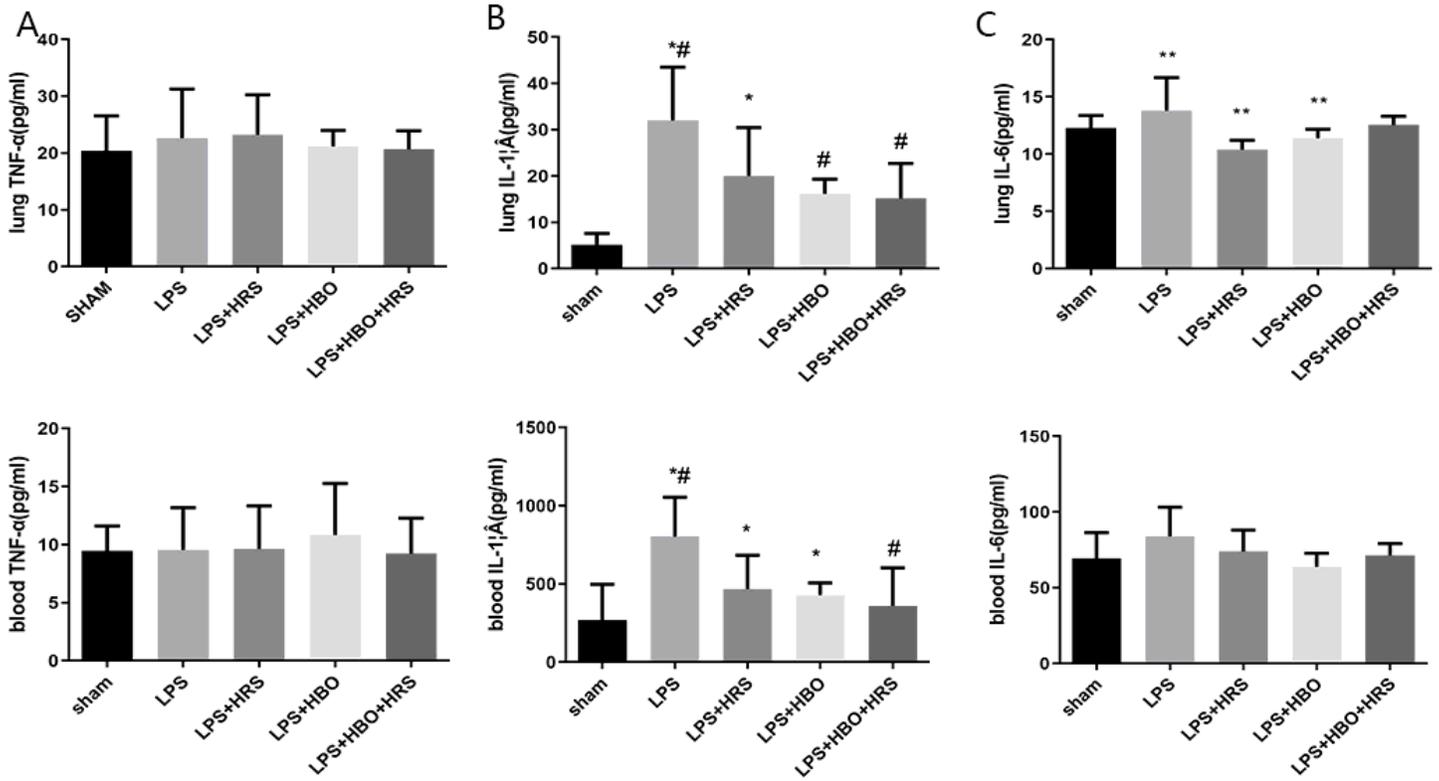


Figure 3

Inflammatory factors in lung tissue and serum of the experimental groups

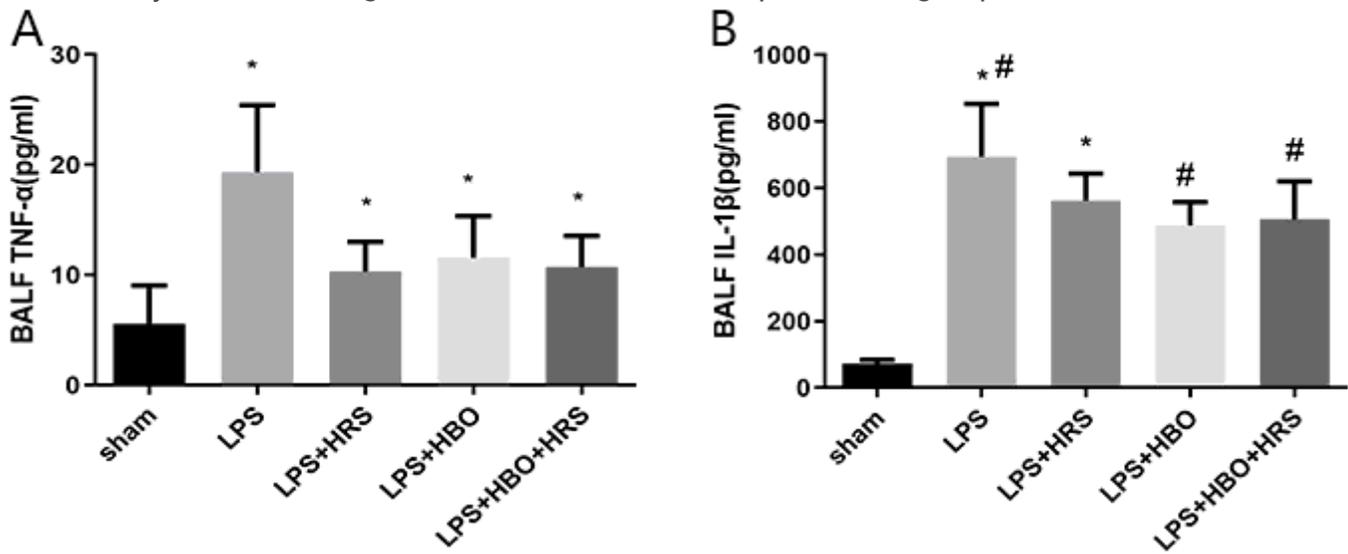


Figure 4

TNF-α and IL-1β levels in pulmonary lavage fluid

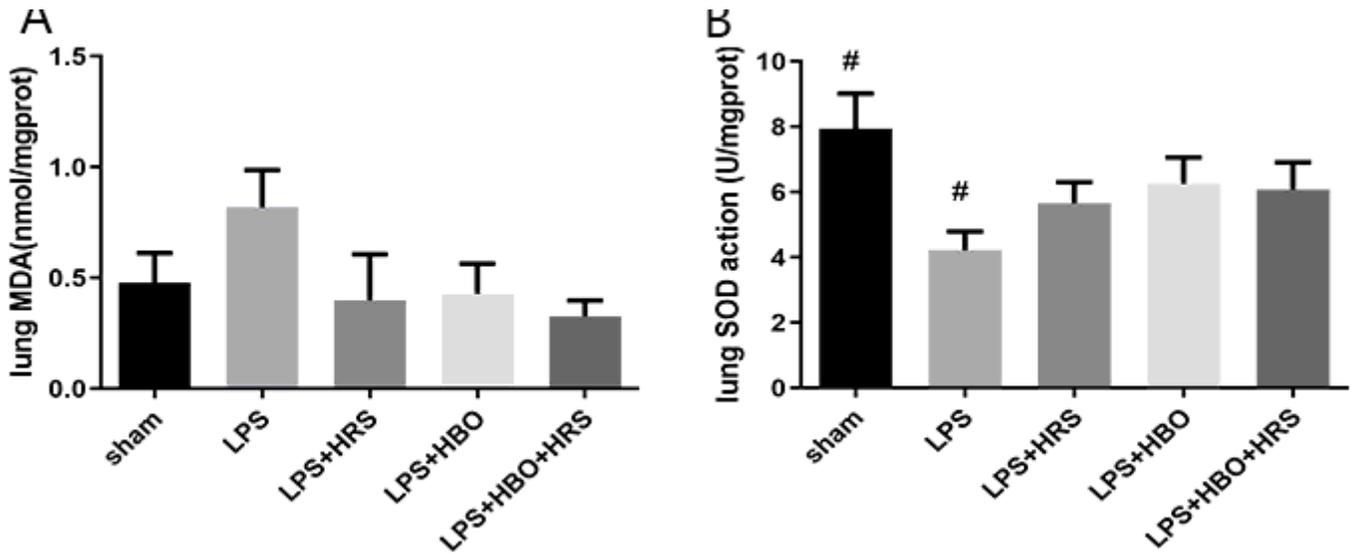


Figure 5

MDA and SOD levels in the pulmonary tissue of the treatment groups

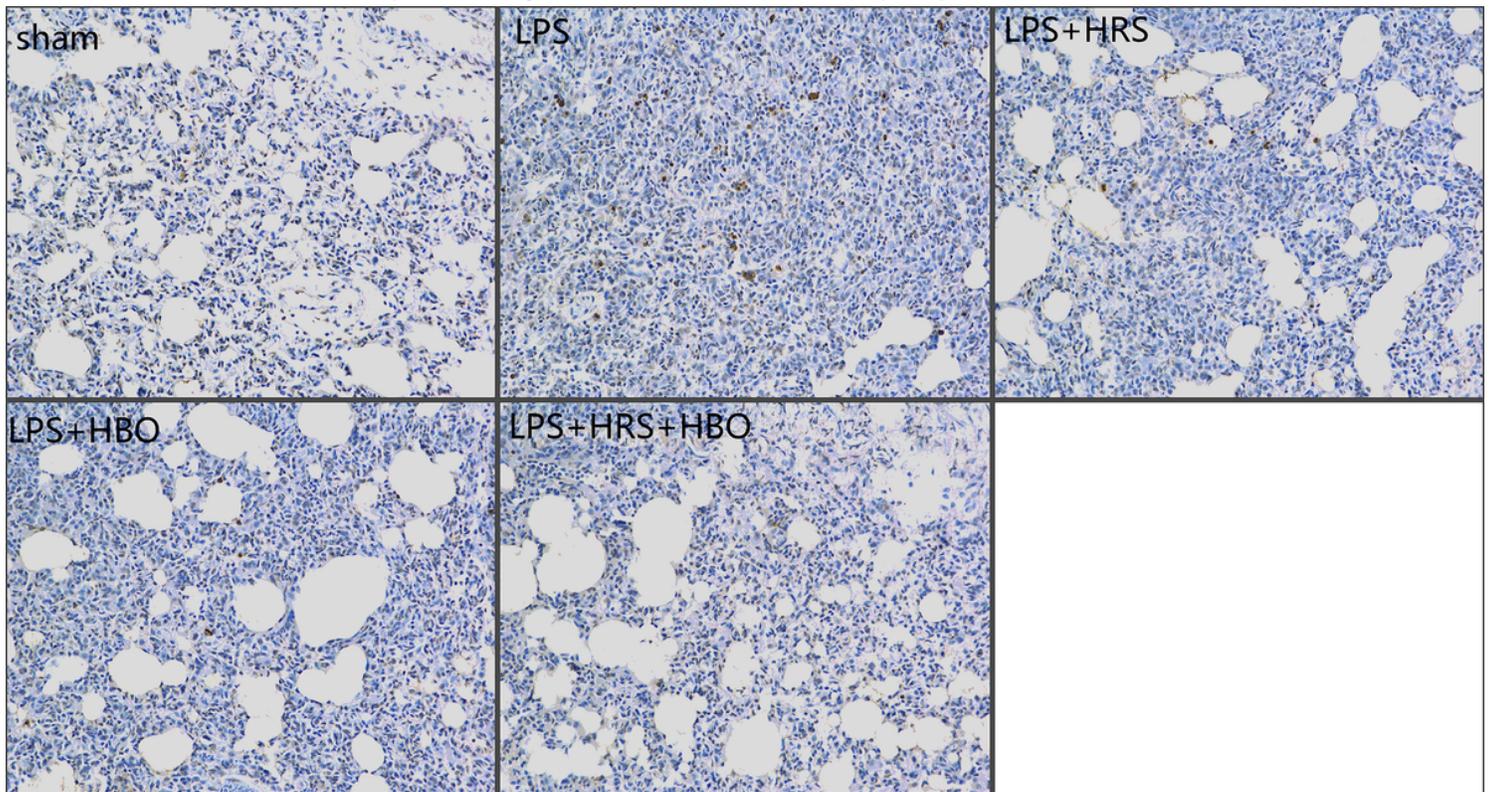


Figure 6

Detection of cell apoptosis by Tunel (×200)

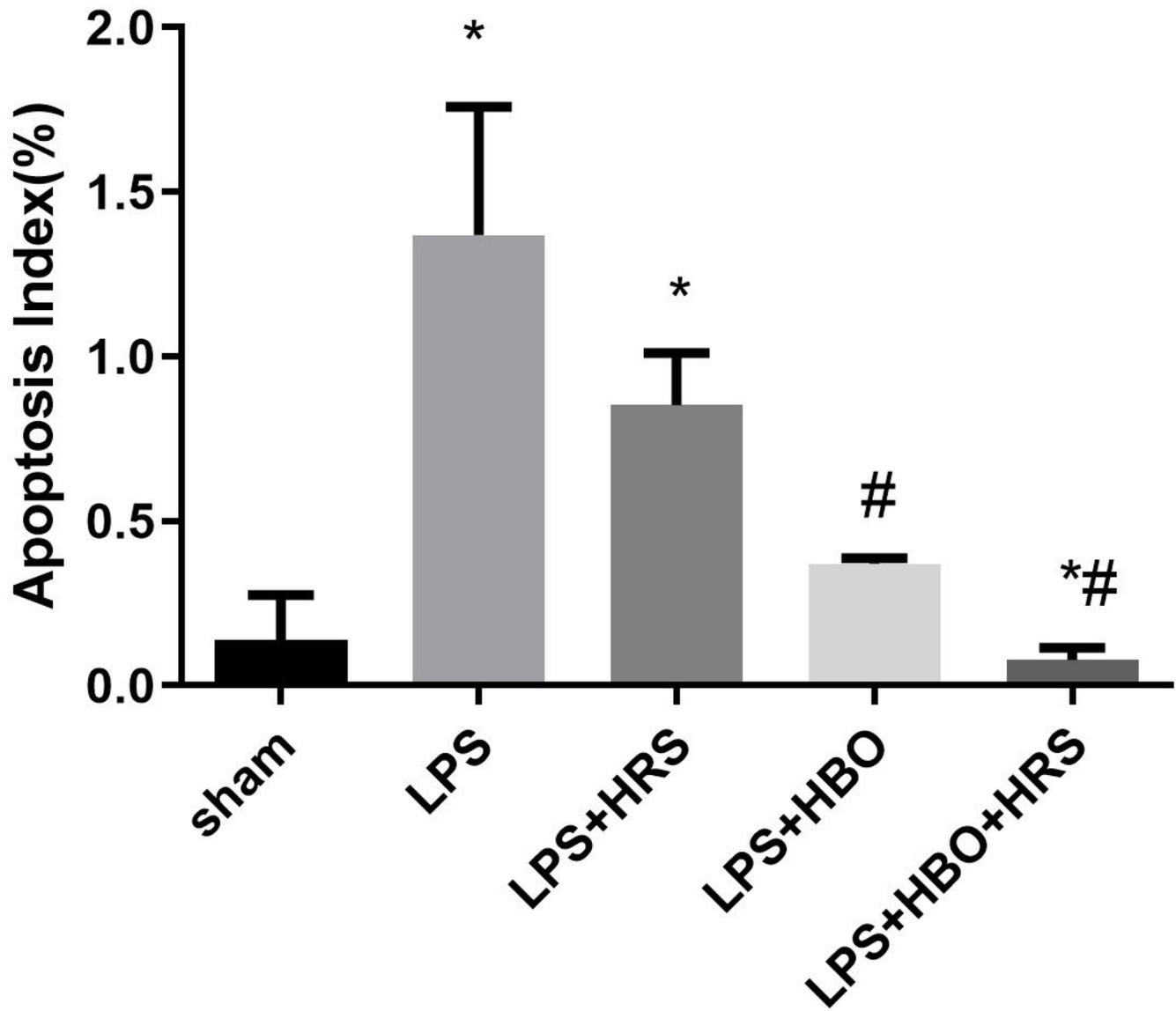


Figure 7

cell apoptosis index

Supplementary Files

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- [NC3RsARRIVEGuidelinesChecklistfillable.pdf](#)