Article

Discovery Medicine 2024; 36(191): 2421–2432
https://doi.org/10.24976/Discov.Med.202436191.223

Hyperbaric Oxygen Protects Acute Lung Injury Secondary to *Deinagkistrodon Acutus* Venom Poisoning by Regulating Th17/Treg Balance

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Published: 20 December 2024

Background: To explore the mechanism of hyperbaric oxygen (HBO) intervention on acute lung injury secondary to *Deinagkistrodon acutus* snake venom poisoning and provide more toxicological and clinical evidence for *Deinagkistrodon acutus* venom poisoning.

Methods: Male Kunming mice (n = 96) were randomly divided into four groups: the control group which was not given any interventional treatments, venom group in which each mouse was injected with *Deinagkistrodon acutus* venom (1 mg/kg) through the tail vein, antivenom group in which each mouse was injected with anti-*Deinagkistrodon acutus* venom immediately after the model was successfully established, and HBO+antivenom group in which each mouse was given HBO treatment at 1 h, 5 h, 11 h and 23 h following the injection of antivenom. Lung tissues of mice were obtained and processed for the detection of the lung coefficient, the levels of inflammatory factors such as interleukin (IL)-6, IL-10 and IL-17, and the protein expression of retinoic acid receptor (RAR)-related orphan receptor gamma (RORγt) and forkhead box P3 (FOXP3). Separate lung tissue specimens were acquired for hematoxylin-eosin staining.

Results: Compared with the venom group, HBO+antivenom group exhibited (1) improved survival rate within 24 h; (2) resolution of pulmonary edema, integrity restoration of alveolar structure, and reduced number of infiltrated inflammatory cells; (3) diminished levels of pro-inflammatory factors and increased abundance of anti-inflammatory factors beginning 2 h after envenomation; and (4) balanced expression of RORγt protein and FOXP3 protein at 24 h after envenomation.

Conclusion: HBO combined with antivenom can significantly relieve secondary lung injury in mice poisoned with *Deinagkistrodon acutus* venom by immediately regulating the balance of helper T cell 17 (Th17)/regulatory T cell (Treg) related proteins.

Keywords: *Deinagkistrodon acutus* venom; inflammatory response; acute lung injury; hyperbaric oxygen; Th17/Treg balance

Introduction

Snakebite is an urgent and critical injury that can be easily overlooked [1]. About 5.5 million people in the world are subjected to venomous snake bites every year, and about 100,000 people are bitten by venomous snakes every year in China, with an annual mortality rate of 5%–10% and a disability rate of about 30% [2–4]. *Deinagkistrodon acutus* is a type of snake that mainly inhabits the southern region of China (including Hubei, Guangxi, Guizhou, *etc.*) [5,6]. According to Wang *et al*. [7], the snake bite by *Deinagkistrodon acutus* endemic in Zunyi (China) ranks second among other snake bites for causing morbidity and contributes to the highest proportion of critically ill patients. The venom of *Deinagkistrodon acutus* is mainly composed of blood toxins, which often exacerbates coagulation dysfunction. In severe cases, this venom can cause disabling conditions and is life-threatening to patients [8–10]. Acute lung injury is one of the most serious complications following snake envenomation, contributing to an extremely high mortality rate among the affected individuals [11]. At present, addressing coagulation dysfunction remains the primary focus of the research on seeking a cure for repercussions following the *Deinagkistrodon acutus* bite, but little attention has been paid to investigating the secondary lung injury caused by the envenomation.

According to the literature, cobra, *Deinagkistrodon acutus* and rattlesnake can cause acute lung injury (ALI) [12–15], but ALI caused by *Deinagkistrodon acutus* envenomation has rarely been reported. Huang *et al.* [6] reported a case of *Deinagkistrodon acutus* bite, which caused the patient to succumb to multiple organ dysfunctions, accompanied by ALI. Huang *et al.* [16] detected a range of symptoms such as dyspnea, severe convulsion, mouth and nose bleeding, and even death, in the mouse poisoning model established by intravenous injection of *Deinagkistrodon acu-*

tus venom. Histopathologic examination revealed that the integrity of lung tissue internal structure was destroyed, and the endothelial cells became apoptotic and necrotic, all of which were consistently characteristic of ALI secondary to *Deinagkistrodon acutus* envenomation and the hemorrhagic change in severe lung injury caused by envenomation described in our previous animal experiments [16]. These findings lend themselves to providing a basis for further research of related mechanisms.

Previous studies have confirmed that excessive infiltration of inflammatory cells and upregulation of inflammatory factors lead to necrosis, apoptosis and diffuse alveolar injury of bronchial endothelial cells, which is considered one of the main pathological characteristics of ALI [17]. Snake venom components, including snake venom metalloproteinases (SVMPs), snake venom serine proteases (SVPs), L-amino acid oxidase (LAAOs) and snake venom phospholipase A2 (SVPLA2), can promote the upregulation of inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-1β, which trigger a strong inflammatory reaction in the blood. Further aggravating the inflammatory reaction is considered one of the factors of tissue injury induced by venomous snakes [18]. In an animal model of *Deinagkistrodon acutus* envenomation established by intraperitoneal injection, Zhang *et al.* [19] detected an increased secretion of inflammatory factor IL-1β, suggesting that *Deinagkistrodon acutus* venom can cause systemic acute inflammatory reaction and immune imbalance, which represent one of the chief factors leading to organ dysfunction.

A growing body of evidence has shown that the imbalance of helper T cell 17 (Th17) and CD4+CD25+forkhead box P3 (FOXP3)+regulatory T cell (Treg) contributes to the occurrence and progress of immune and inflammatory reactions [20]. Among them, Treg cells secrete IL-10, which inhibits the proliferation and immune response of peripheral effector T cells, regulates immune response, enhances alveolar epithelial proliferation and tissue repair, and provides potential lung tissue protection [21]. Th17/Treg balance plays a key role in the steady state of immune function. Th17 and Treg are antagonistic to each other throughout the disease process. Xia *et al.* [22] found that increasing the number of Tregs and reducing the differentiation of Th17 cells can alleviate lung injury and improve lung function in a septic ALI mouse model. Meanwhile, Th17/Treg imbalance was observed in the ALI model induced by lipopolysaccharide [23,24], suggesting that the Th17/Treg balance plays a key role in the pathogenesis of ALI. However, whether the Th17/Treg imbalance occurs in the context of ALI following *Deinagkistrodon acutus* envenomation, and whether it plays a certain role in the pathogenesis and prognosis of ALI have not been reported in the literature.

Antivenom is recognized as the first choice for the treatment of snake bites, but its function is limited to neutralizing free venom components and not expanded to reversing the tissue damage caused by the snake bite [25,26]. Therefore, it is of great significance to explore other auxiliary treatments to cure the limitations of antivenom for the diagnosis and treatment of ALI after the *Deinagkistrodon acutus* bite. Hyperbaric oxygenation (HBO), as a non-traumatic adjuvant therapy, is widely used in various diseases. Several studies [27–29] have shown that HBO may ameliorate lung injury by regulating the toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) pathway to improve inflammatory response, reducing oxidative stress in an independent manner, and improving the permeability of lung epithelial cells through aquaporin 4 (AQP4) expression. Wang *et al.* [30] found that after HBO intervention, the proportion of Treg cells in the thymus of the glioma mouse model was increased. The same research group had also previously found that HBO adjuvant therapy possesses a protective effect on kidney and brain injury caused by *Deinagkistrodon acutus* bite [31]. However, whether the protective effect of HBO on ALI secondary to *Deinagkistrodon acutus* venom is related to the regulation of Th17/Treg balance has never been reported in the literature.

In light of the existing research and theories, we designed an animal experiment to verify that hyperbaric oxygen has a protective effect on secondary lung injury following *Deinagkistrodon acutus* envenomation by regulating Th17/Treg balance and shedding light on novel therapeutic strategies for snake bite.

Materials and Methods

Experimental Animals

Male Kunming mice (aged 6–8 weeks, n = 96) were purchased from China Chongqing Laite Biotechnology Co., Ltd. (experimental animal license number: SYXK(Qian)2022-0004).

The experimental mice were kept in a suitably humid and well-ventilated environment, with a room temperature of 23 $\pm$ 2 °C. The day-night cycle was set at 12–12 hours. The mice were given a standard diet and water *ad libitum* and subjected to experiments at least for 3 days after being kept and fed as described. The mice were randomly divided into four groups: control group (n = 24), venom group (n = 24), antivenom group (n = 24), and HBO+antivenom group (n = 24). In the HBO+antivenom group, HBO treatment was administered at 1 h, 5 h, 11 h and 23 h after the administration of antivenom. Each group of mice was further divided into four subgroups (n = 6).

Snake Venom and Antivenom

The freeze-dried *Deinagkistrodon acutus* venom powder was obtained from Qimen Snake Injury Research Institute, Huangshan, China, whereas the antivenom against the *Deinagkistrodon acutus* venom was acquired from Seren Biotechnology Co., Ltd. (Shanghai, China).

Animal Grouping and Model Establishment

(1) The mice in the control group were not given any treatments. (2) The venom group consisted of mice envenomated with *Deinagkistrodon acutus* venom. The mouse model was established by injecting *Deinagkistrodon acutus* venom (concentration of 0.15 mg/mL, dosage of 1.0 mg/kg) into the tail vein of mice, according to the protocols described by Li *et al.* [31] for establishing an animal model envenomated by *Deinagkistrodon acutus* venom. After the injection, the mice showed symptoms such as listlessness, rapid breathing, reduced appetite, slow response to external stimuli, and decreased mobility—all of these manifestations indicate that the modeling was successful. (3) In the antivenom group, every envenomated mouse model, established using the same method for the venom group, was given an injection of antivenom (78 U/kg [31]) into the tail vein immediately. (4) In the HBO+antivenom group, the envenomated mice were placed in the HBO cabin at 1 h, 5 h, 11 h, and 23 h after antivenom administration (78 U/kg). The time point of HBO intervention was set according to protocols by Li *et al.* [31]. In brief, the cabin was pressurized for 5 min to 0.12 MPa (1.2 ATA) and washed with pure oxygen. The cabin pressure was then raised to 0.2 MPa (2 ATA) within 5 min and then stabilized for 40 min. Decompression was subsequently performed for 10 min, with the oxygen concentration in the cabin maintained above 95%.

Sample Collection

At the corresponding time points (2 h, 6 h, 12 h and 24 h after administration of antivenom), the mice were placed in a state of deep irreversible anesthesia and were euthanized by cervical dislocation after 2% isoflurane inhalation. Then the mice were fixed in the supine position on the ultra-clean workbench. The chest of each mouse was opened, and the tissues such as the trachea and heart were separated and cut, and the lung tissue was obtained. The lung tissue was weighed for measurement of lung coefficient, and fixed with 4% paraformaldehyde (Beijing Chinese fir Biological Technology Co., Ltd., Beijing, China) for hematoxylin-eosin (HE) staining. The lung tissue was stored at –80 °C for further experiments such as enzyme-linked immunosorbent assays (ELISA) and Western blotting.

Determination of Lung Coefficient

The weight of each mouse was measured before cervical dislocation and recorded as body weight (BW). The whole lung tissue of each mouse was separated from the chest, and the residual blood was washed away. The water and blood on the surface of lung tissue were drained and dried using filter papers, and the weight was measured afterward and recorded as lung wet weight (LWW). The lung coefficient of lung tissue in each group was calculated by determining the ratio of LWW to BW (mg/g), which is a measure of pulmonary edema [32].

Histopathology

Lung tissues were dehydrated in a gradient of alcohol solutions and immersed in xylene solution for clearance. After a series of procedures such as embedding, slicing, dewaxing, rehydration, and staining with hematoxylin (Shanghai Biyuntian Biotechnology Co., Ltd., Shanghai, China) for 3 min, the tissue section slides were washed for 5 min, destained in hydrochloric acid alcohol, washed for 5 min, and fast-stained with eosin (Shanghai Biyuntian Biotechnology Co., Ltd., Shanghai, China). Finally, they were subjected to dehydration and washing, prior to sealing with neutral gum. The pathological changes in lung tissue were observed under an optical microscope (DM500+ICC50, Lecia, Wetzlar, Germany), and images were captured. Lung injury presented in the slides was scored by three experienced pathologists according to pathological changes. The pathological changes were chiefly classified as alveolar congestion, alveolar hemorrhage, inflammatory cell infiltration, and alveolar wall thickening or membrane formation, in accordance with specific scoring criteria proposed by Wang *et al.* [32]. At the same time, in order to observe collagen fiber deposition, the lung tissue sections were subjected to Masson's trichrome staining performed using a staining kit (Shanghai Biyuntian Biotechnology Co., Ltd., China). Briefly, paraffin-embedded slices were dewaxed and rehydrated, then stained with Weigert iron hematoxylin solution for 8 min, and then differentiated using acidic ethanol. Afterward, the slices were washed and then stained with Masson blue solution for 5 min and with Lichun red fuchsin staining solution for 5 min. Aniline blue dye was used for staining for 2 min, followed by weak pickling for 1 min. Subsequently, the slices were dehydrated, cleaned, and sealed with neutral gum. Following Masson's trichrome staining, collagen is stained blue and nuclei are stained dark brown. The content percentage of collagen fiber was determined: Firstly, at least three 200-fold fields of view were randomly selected for each section in each group. Image-Pro Plus 6.0 software (GraphPad, San Diego, CA, USA) was then utilized to pinpoint the blue color of the same hue, which was selected as the unified standard for identifying collagen fibers in all photos. The percentage of collagen fibers (%) was determined by calculating the ratio of the area occupied by collagen fibers to the area of whole tissue in each photo.

Enzyme-Linked Immunosorbent Assay

Fresh lung tissue specimens were ground and centrifuged at 12,000 rpm and 4 °C for 5 min. Following centrifugation, the supernatant was collected. The levels of IL-17, IL-6 and IL-10 in the lung tissue supernatant were measured using ELISA kits (Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China). The optical density (OD) value was detected at 450 nm wavelength.

Western Blotting

Lung tissue specimens were ground by using a grinder. The resultant lung tissue homogenate was added with Radio Immunoprecipitation Assay (RIPA) lysis buffer (Shanghai Biyuntian Biotechnology Co., Ltd., Shanghai, China), and was left on ice for more than 10 min, prior to centrifugation at 12,000 rpm and 4 °C for 5 min. Lung tissue supernatant was collected after centrifugation. The protein concentration of tissue specimens was detected using a bicinchoninic acid (BCA) protein kit (Shanghai Yase Biotechnology Co., Ltd., Shanghai, China). The samples were electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Shanghai Yase Biotechnology Co., Ltd., China) and transferred to a polyvinylidene fluoride (PVDF) membrane (0.45 µm; Millipore, Burlington, MA, USA). Protein-free quick sealing solution was utilized for sealing for 1 h (Shanghai Yase Biotechnology Co., Ltd., China). Specific proteins on the membrane were probed with anti-FOXP3 rabbit monoclonal antibodies (1:1000; AB215206, Abcam, Cambridge, UK), anti-retinoic acid receptor (RAR)-related orphan receptor gamma (RORγt) rabbit monoclonal antibodies (1:1000; AB190145, Abcam, UK), and anti-β-actin rabbit monoclonal antibodies (1:100,000; 81115-1-RR, Proteintech Group Biotechnology Co., Ltd., Wuhan, China) overnight at 4 °C. Subsequently, the membrane was incubated with goat anti-rabbit HRP antibodies (1:200,000; RGAR001, Proteintech Group Biotechnology Co., Wuhan, Ltd., China) for 2 h at room temperature. Then, the membrane was developed using an enhanced chemiluminescence kit by Bio-Rad system (ChemiDoc MP, Hercules, CA, USA), and finally, the band intensity was measured and analyzed by the Image-Pro software (1.51j8, Media Cybernetics, Rockville, MD, USA).

Statistical Analysis

SPSS 29.0 (IBM Corp., Armonk, NY, USA, Version 29.0) and GraphPad 9.0 (GraphPad Inc., San Diego, CA, USA) were used for statistical analyses. Quantitative data are expressed as mean ± standard error ($\bar{x} \pm s$). Data conforming to the normal distribution, as tested by the normality test, were analyzed using *t*-tests or multiple *t*-tests, which compared the data between any two groups. One-way analysis of variance (ANOVA) was used for comparison of data between any two groups. The difference in survival rate between groups was analyzed by the log-rank method. The difference was considered statistically significant at $p < 0.05$.

Results

HBO Improves Early Survival Rate after Snake Envenomation

Within 24 h after the establishment of the model, the survival rate of the mice in the venom group was significantly lower compared with the control group (87.5% vs 100%; $p < 0.05$; Fig. 1). Dead mice or mice that did not meet the standards were excluded from the experiments, and eligible mice were supplemented accordingly at specified time points. None of the mice in the antivenom group and HBO+antivenom group were found dead within 24 h following successful modeling.

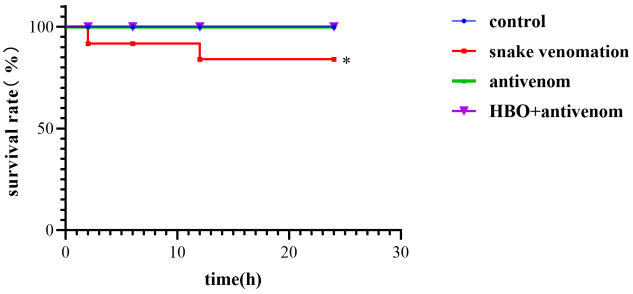

Fig. 1. The survival rate of mice among the four groups within 24 h following successful modeling. Note: *$p < 0.05$ compared with the control group. HBO, hyperbaric oxygen.

Behavioral Results

After being injected with *Deinagkistrodon acutus* venom, the mice in the venom group gradually manifested signs of listlessness, decreased activity, decreased food and water intake, shortness of breath, and slow response to external stimuli (Fig. 2a) compared with the control group (Fig. 2d). In severe cases, mouth and nose bleeding, shortness of breath and even death were observed. An autopsy showed that their lungs were congested and edematous (Fig. 2a). These symptoms and pathological manifestations could be improved by administering antivenom (Fig. 2b) and HBO+antivenom (Fig. 2c).

Determination of Lung Coefficient

As shown in Fig. 3, compared with the control mice, the envenomated mice showed significantly higher lung coefficients ($p < 0.05$). Compared with the venom group, the venom group and HBO+antivenom group experienced a reduction in lung coefficient ($p < 0.05$). The lung coefficient of the HBO+antivenom group was significantly lower than that of the antivenom group ($p < 0.05$).

HBO Ameliorates Pathological Changes of Lung Tissue Following Snake Envenomation

As shown in Fig. 4, HE staining results showed that the lung tissue morphology of the control mice was normal, the alveolar wall structure was intact, the pulmonary septum was not thickened, and there was no obvious inflammatory reaction around the trachea. Compared with the control group, the mice in the venom group showed impaired pulmonary alveolar structure, alveolar congestion, pulmonary

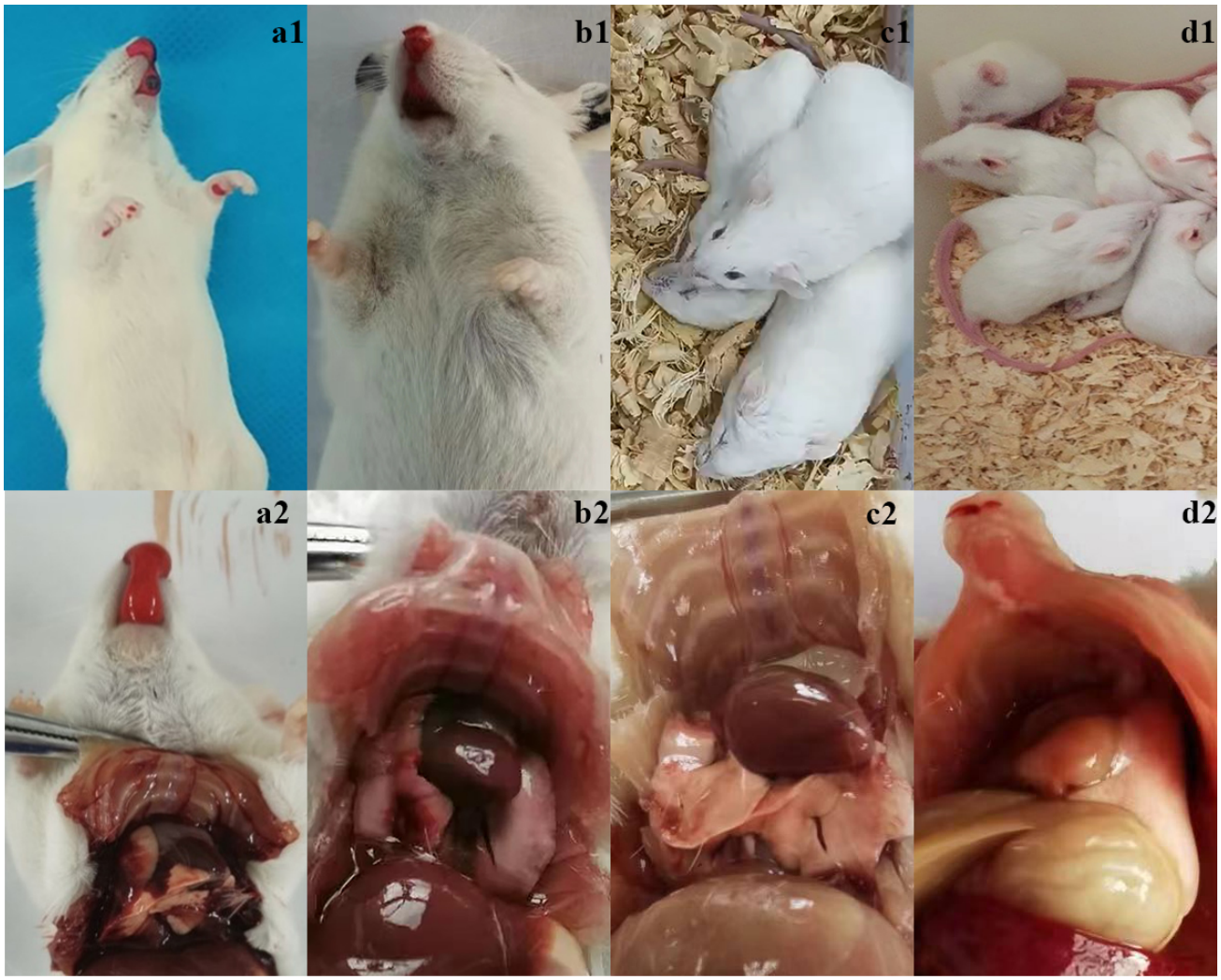

Fig. 2. Behavioral manifestation and autopsy examination of mice in every group. (a1,b1,c1) Behavioral manifestations of mice in venom, antivenom, HBO+antivenom groups, respectively. (a2,b2,c2) Autopsy examinations on mice that succumbed to a large dose of *Deinagkistrodon acutus* venom. (d1,d2) Behavioral manifestation and autopsy examination of control mice.

septum thickening, extensive inflammatory cell infiltration, and bleeding, accompanied by increased lung injury score ($p < 0.05$). Compared with the venom group, the antivenom group and HBO+antivenom group demonstrated partially impaired pulmonary alveolar structure integrity; reduced alveolar congestion, hemorrhage, septal thickening, and inflammatory cell infiltration; and reduced lung injury score ($p < 0.05$). While narrowing down to the comparison between the antivenom and the HBO+antivenom groups, we found that the pathological changes in the lung tissues of the HBO+antivenom group were much alleviated, in addition to the decrease in lung injury score, as compared to the antivenom group ($p < 0.05$). Masson's trichrome staining showed that there was almost no collagen fiber deposition in the lung tissue of the control mice, with the alveolar structure remaining intact. Alveolar interstitial thickening and collagen fiber deposition underwent a significant augmentation in the venom group, as compared with the control group ($p < 0.05$). Compared with the venom group, the antivenom group and HBO+antivenom group suffered from milder pulmonary fibrosis ($p < 0.05$). Administration of HBO treatment contributed to a synergistic effect in therapy, marked by a lower extent of pulmonary fibrosis as seen in the HBO+antivenom group than in the antivenom group ($p < 0.05$).

HBO Regulates the Activation of Inflammatory Factors in Lung Tissue of Envenomated Mice

To investigate the expression of inflammatory factors in the lung tissue of envenomated mice, we quantified their levels at different specified time points (2 h, 6 h, 12 h, 24 h after venom administration) by using ELISA. As shown in Fig. 5, the pro-inflammatory factor IL-6 in lung tissue distinctly started increasing and peaked at 2 h after envenomation compared with that of the control group ($p < 0.05$; Fig. 5a), and decreased gradually over time and dropped

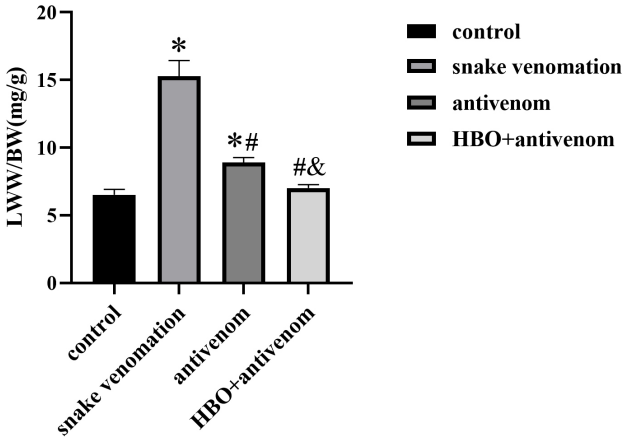

Fig. 3. Comparison of lung coefficients of mice between the four groups. Data are expressed as mean ± standard error. n = 6; *$p < 0.05$ compared with the control group; $^{\#}p < 0.05$ compared with the venom group; $^{\&}p < 0.05$ compared with the antivenom group.

to the lowest level at 24 h after envenomation. However, IL-6 level significantly decreased after antivenom injection and administration of HBO+antivenom, as compared with that of venom group ($p < 0.05$; Fig. 5a), and exhibited conspicuous reduction following the administration of HBO+antivenom at each time point ($p < 0.05$; Fig. 5a). The level of IL-17 started increasing at 2 h and peaked at 24 h after envenomation ($p < 0.05$; Fig. 5b). Compared with the venom group, the secretion of IL-17 markedly decreased after antivenom injection and administration of HBO+antivenom, respectively, at each time point and exhibited augmentation following HBO+antivenom treatment at 24 h after envenomation ($p < 0.05$; Fig. 5b). Compared with the control group, the anti-inflammatory IL-10 significantly started decreasing at 2 h after envenomation ($p < 0.05$; Fig. 5c). However, there was no difference in IL-10 level between each time points after envenomation. Compared with the venom group, the secretion of IL-10 dramatically increased after antivenom injection and treatment using HBO+antivenom, respectively, across the specified time points, and exhibited augmentation after HBO+antivenom administration at 24 h after envenomation ($p < 0.05$; Fig. 5c).

HBO Alleviates Acute Lung Injury Secondary to Snake Envenomation by Regulating Inflammatory Immune in Lung Tissue

To determine the inflammatory immune effect of HBO on ALI secondary to snake envenomation, we analyzed the expression of regulatory T cell and Th17-related proteins in lung tissue at 24 h after envenomation. As shown in Fig. 6, the protein expression of RORγt, a key transcription factor of Th17, was significantly increased at 24 h following envenomation, compared with the control group ($p < 0.05$). The expression of RORγt protein in the antivenom group and HBO+antivenom group was lower than that in the venom group ($p < 0.05$). Compared with the antivenom group, the expression of RORγt protein in the HBO+antivenom group was downregulated ($p < 0.05$). Relative to the control group, the protein expression of FOXP3—a key transcription factor of Treg—was significantly decreased in the venom group ($p < 0.05$). The expression of FOXP3 protein in the antivenom group and HBO+antivenom group was higher than that in the venom group ($p < 0.05$). Compared with the antivenom group, the expression of FOXP3 protein in the HBO+antivenom group was elevated ($p < 0.05$).

Discussion

The lungs are the primary organs that are the most vulnerable to the damages elicited by snake venom, closely followed by the kidneys and liver. Snake venom mediates the infiltration and accumulation of inflammatory cells in the lungs, leading to excessive activation and release of inflammatory mediators, which in turn triggers systemic inflammatory response and imbalance of immune inflammation, a critical factor leading to ALI, although the exact underlying mechanism remains unclear. In the mouse model of *Deinagkistrodon acutus* venom, we observed hemorrhagic changes in their lung tissues, which provided evidence that *Deinagkistrodon acutus* venom can induce secondary lung injury. At present, antivenom is widely used in clinics, which is a special treatment for snake bites, but it is unable to reverse damage to tissues and organs [33]. Hence, finding new strategies to fortify the treatment for snake bites has become an increasingly concerned issue. Some studies have shown that HBO can alleviate ALI caused by multiple factors by increasing the partial pressure of blood oxygen and inhibiting inflammatory reactions [34]. However, whether HBO has a protective effect on ALI secondary to *Deinagkistrodon acutus* bite has never been reported.

In order to further investigate the lung injury mechanism caused by *Deinagkistrodon acutus* venom, a preliminary experiment was conducted in accordance with the dosage and concentration of *Deinagkistrodon acutus* venom adopted in Li *et al.*'s study [31], in which *Deinagkistrodon acutus* venom was injected into the tail vein of mice to establish an animal model, which was then examined for behavioral changes and lung injury index. The results showed that: (1) After envenomation, the mice in the venom group showed signs of listlessness, decreased activity, decreased food and water intake, shortness of breath, and slow response to external stimuli. In severe cases, they showed signs of mouth and nose bleeding, shortness of breath, and even death. An autopsy examination showed that their lungs were congested and edematous. (2) The alveolar structures of the mice in the venom group were destroyed, accompanied by alveolar congestion, thickened

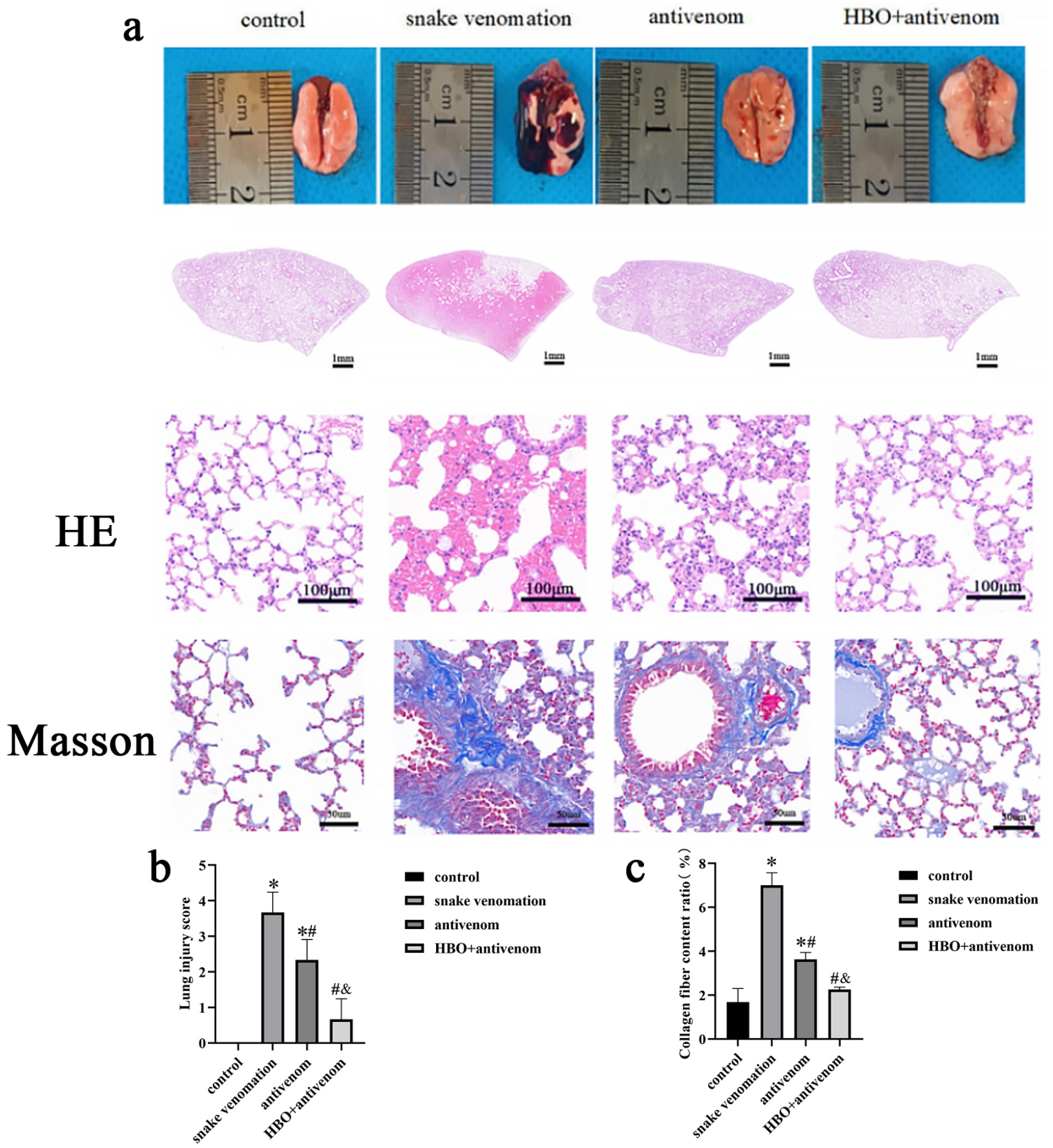

Fig. 4. HBO ameliorates pathological changes of lung tissue after snake envenomation. (a) Hematoxylin-eosin (HE) staining and Masson's trichrome staining of lung tissues were acquired from each group. (b) Lung injury score and (c) percentage of collagen fiber content of each group. Data are expressed as mean ± standard error. n = 6; $^{*}p < 0.05$ compared with the control group; $^{\#}p < 0.05$ compared with the venom group; $^{\&}p < 0.05$ compared with the antivenom group.

pulmonary septa, infiltration by abundant inflammatory cells, and bleeding. Compared with the control group, the lung injury score of the venom group was increased. (3) Compared with the control group, the envenomated mice had higher lung coefficients and exhibited apparent pulmonary edema. Combined with the experimental results by Qin *et al.* [10], the preliminary experimental results of our research demonstrated that *Deinagkistrodon acutus* venom can also elicit ALI, similar to what *Deinagkistrodon halys* venom can do. These experimental findings aligned with the criteria for judging the acute respiratory distress syndrome (ARDS)/ALI animal model issued by the American Thoracic Association in 2022, indicating the successful construction of the ALI mouse model through the induction of *Deinagkistrodon acutus* venom.

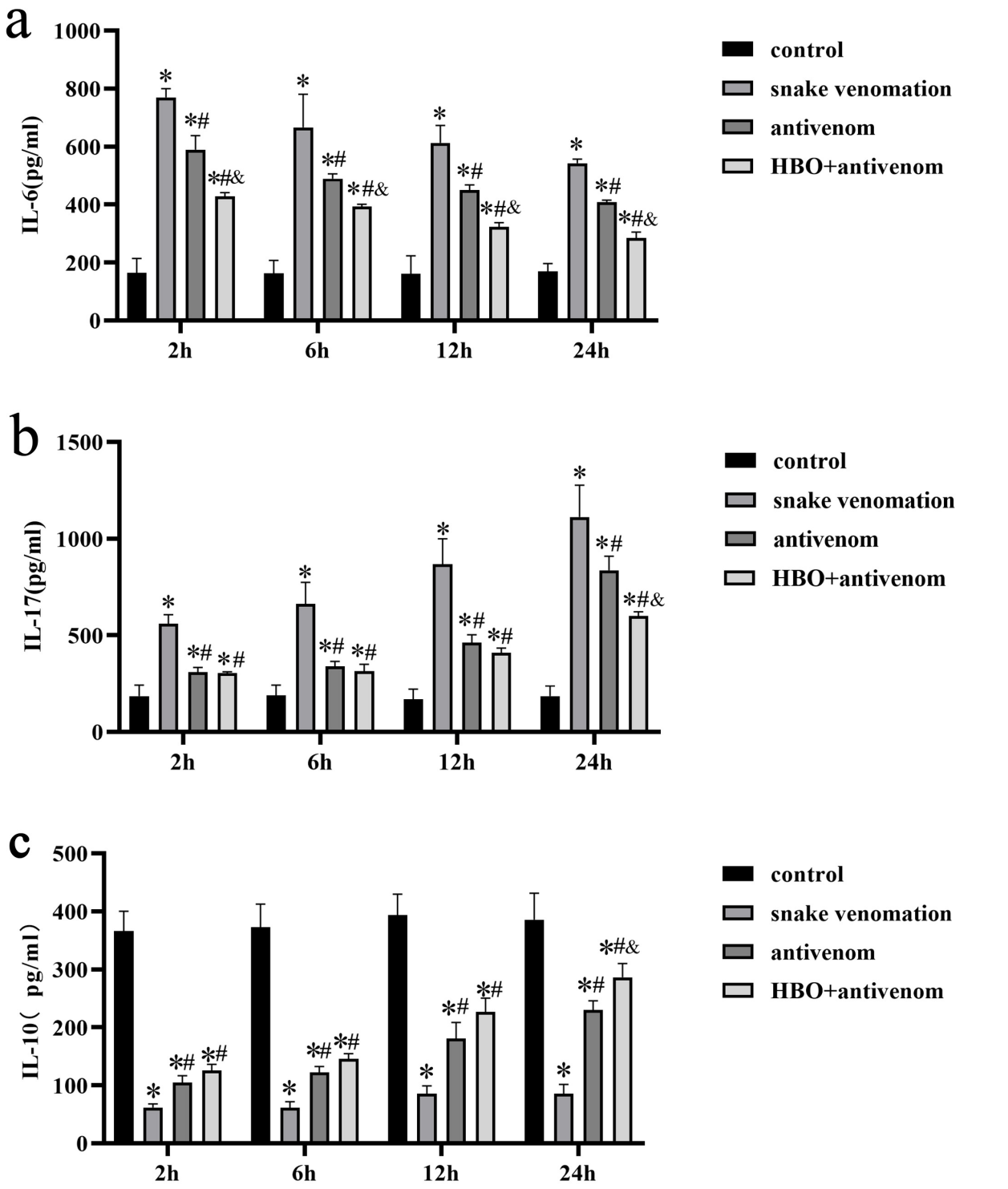

Fig. 5. The levels of inflammatory factors in lung tissue of each group at a different time point after poisoning. Levels of interleukin (IL)-6 (a), IL-17 (b) and IL-10 (c) in lung tissue of each group. Data are expressed as mean ± standard error. n = 6; *$p < 0.05$ compared with the control group; $^{\#}p < 0.05$ compared with the venom group; $^{\&}p < 0.05$ compared with the antivenom group.

The excessive release and activation of inflammatory mediators and relative reduction of anti-inflammatory factors after snake envenomation leads to systemic inflammatory response imbalance, which is considered to be an important factor causing ALI [35,36]. The current study showed that compared with the control group, the levels of pro-inflammatory factors IL-6 and IL-17 in the venom group were significantly increased, and anti-inflammatory

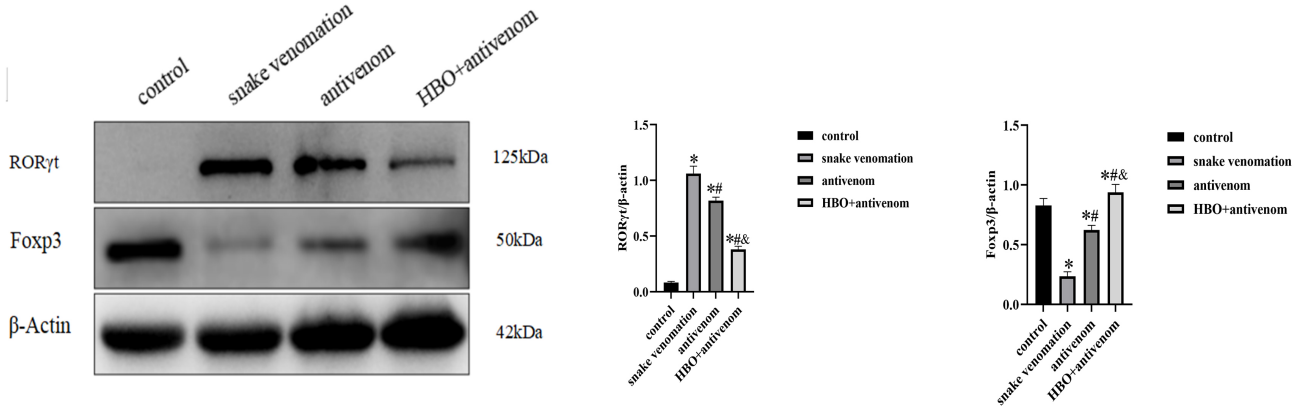

Fig. 6. The expression of retinoic acid receptor (RAR)-related orphan receptor gamma (RORγt) and forkhead box P3 (FOXP3) proteins in lung tissue of each group at 24 h after envenomation. $^{*}p < 0.05$ compared with the control group; $^{\#}p < 0.05$ compared with the venom group; $^{\&}p < 0.05$ compared with the antivenom group.

factor IL-10 level was significantly suppressed, with the significant change registered at 2 h after envenomation. This suggests that *Deinagkistrodon acutus* venom could instantly induce the imbalance of inflammatory response in ALI onset as early as within 2 h following snake envenomation. Thus, clinically, individuals who have just suffered from a bite by *Deinagkistrodon acutus* need to be treated as soon as possible to avoid inflammatory cascade and secondary tissue injury. The elevation of IL-17 as demonstrated in our research is consistent with the report by Cavalcante *et al.* [37], pointing to the ability of *Deinagkistrodon acutus* venom to induce systemic inflammatory response through IL-17 secretion mediated by Th17. At the same time, this study found that the level of IL-6 reached its peak at 2 h after envenomation, and gradually decreased with time, indicating that pro-inflammatory cytokines play a key role in acute inflammation, a postulation consistent with the view of Alsolaiss *et al.* [38] that the body would experience a rapid inflammatory reaction within 2 h following a venomous snake bite, and also consistent with the animal experimental results by Yacoub *et al.* [39]. Different from the peak of IL-6 secretion in the early stage, the level of IL-17 continued to increase 24 h after envenomation. It is speculated that IL-17-mediated immune inflammatory reaction may persist after ALI secondary to *Deinagkistrodon acutus* envenomation. Thus, further explorations of the temporal trends of IL-17 level alterations and the mechanisms behind this phenomenon are warranted. The Th17 and Tregs are considered the key regulatory factors that maintain inflammatory response in the systemic inflammatory response, and Th17/Treg imbalance is identified as the risk index of early ARDS/ALI [40–43]. In this experiment, the protein expression of RORγt and FOXP3 in the lung tissue of mice experiencing ALI secondary to *Deinagkistrodon acutus* envenomation was quantified. The results showed that compared with the control group, the RORγt protein was significantly upregulated, while the expression of FOXP3 protein was decreased, at 24 h following envenomation. The above results provided important evidence that the imbalance of the Th17/Treg ratio represents a pathogenic factor of ALI secondary to *Deinagkistrodon acutus* envenomation. It was consistent with those reports that showed a critical factor of the Th17/Treg imbalance in the ALI [43–46]. Thus, further research into the venom's effect on the mechanism that regulates Th17/Treg ratio *in vitro* is needed to shed light on new research directions in immune treatment for snake bites.

Antivenom is broadly applied in clinical settings as a treatment approach for snake bites, but technically, it cannot reverse the tissue damages that are already induced. HBO is widely used in the treatment of carbon monoxide poisoning complicated with aspiration pneumonia, tumor radiation-induced lung injury, acute respiratory distress syndrome, and related complications of snake bite [44,47]. It is believed that HBO may alleviate the inflammatory reaction and injury in tissues by increasing the production of Treg cells and regulating the balance of Th17/Treg [48]. The results of the current study showed that compared with the antivenom group, the combination of HBO and antivenom could significantly improve the pathological damage in lung tissue and the lung edema starting at 2 h after envenomation, which is consistent with the experimental results of Kang *et al.* [48]. Compared with the sole usage of antivenom, HBO combined with antiserum could conspicuously attenuate the production of pro-inflammatory factors IL-6 and IL-17 and promote the synthesis of the anti-inflammatory IL-10 in the lung tissue starting at 2 h after envenomation. Furthermore, HBO plus antivenom could markedly improve the balance of the protein expression of RORγt and FOXP3 at 24 h following envenomation. It is suggested that HBO combination therapy may further participate in immune inflammatory reaction by regulating Th17/Treg balance, thus playing a protective role in ALI secondary to *Deinagkistrodon acutus* envenomation. Thus, the proposed mechanism through which HBO+antivenom

imparts protective effects offers us an immunotherapeutic strategy against the deleterious implications of snake bites. It is worth noting that at the three different time points (*i.e.*, 2 h, 6 h, and 12 h), the HBO+antivenom group, relative to the antivenom group, manifested a decreasing, but statistically insignificant, trend in the IL-17 level. It was not until 24 h later that the IL-17 level in the HBO+antivenom group began to exhibit a statistically significant reduction trend. These findings indicate that the therapeutic effect of HBO is not immediate but instead takes some time after administration.

Taken together, this study confirmed that the *Deinagkistrodon acutus* venom can cause ALI, and an envenomated animal model can be established by injecting *Deinagkistrodon acutus* venom into the tail vein of the animal. We also showed that immune inflammatory imbalance plays an important pathogenic role in ALI secondary to *Deinagkistrodon acutus* envenomation. In addition, HBO confers protection against envenomation by *Deinagkistrodon acutus* by regulating immune inflammation, alleviating pulmonary edema, relieving lung injury, inhibiting inflammatory reaction, and regulating Th17/Treg balance, providing a new direction for further research and development of the relevant treatment. Aside from that, the current research also provides stronger evidence and guidance for the prevention and treatment of ALI caused by *Deinagkistrodon acutus* envenomation. Despite the promising therapeutic potential of HBO in combination with antivenom, the public at large needs to be aware of the gravity of snake bite complications and ALI secondary to snake bites. Furthermore, relevant immune inflammatory treatment guidelines should be formulated.

This study is not without limitations. Firstly, Treg-related proteins were only detected and analyzed in the initial experiment and during the first 24 h of the modeling process and were not observed at other time points. Secondly, this study did not further track the continued elevation of IL-17 release, which had persisted beyond 24 h time point. The relatively short time span for monitoring IL-17 level rendered the determination of the peak of IL-17 secretion impossible, necessitating further confirmation in future experiments. Lastly, the main focus of this study is to observe the effect of HBO on Th17/Treg balance in ALI caused by *Deinagkistrodon acutus* envenomation, and thus other plausible mechanisms were not explored in this investigation.

Conclusion

Through this study, we found that *Deinagkistrodon acutus* venom can cause secondary injury to the murine lung tissues, which can be ameliorated by HBO combined with antivenom by regulating the balance of Th17/Treg-related proteins and controlling the level of inflammatory factors.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

AYY conceived the thesis of this paper, TXZ finalized the manuscript and contributed to investigating, ML designed this experiment and reviewed this manuscript critically and CY drafted the manuscript, ML and CY are major contributors in modeling and animal experiments, and XHJ contributed to examining the data and the formal analysis, XFH contributed to the validation, FJG contributed to the methodology and TJS contributed to the data curation. SSL contributed to the visualization, artical layout and formal check. All authors have been involved in the drafting or critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental protocols for this study were approved by the Animal Experimental Ethics Committee of Zunyi Medical University in accordance with the National Institutes of Health guidelines for the care and use of experimental animals. And the approval number is ZMU21-2301-046.

Acknowledgment

Not applicable.

Funding

The study was supported by the Technology Project of Guizhou Province (No.2021446).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Méndez-Molina R, Villela-Oriza AK, Espinosa-Couoh AA, Huchim-Lara O. Snakebites epidemiology in Mexico: a 13-year ecological analysis. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2024; 118: 118–126.

[2] Waiddyanatha S, Silva A, Weerakoon K, Siribaddana S, Isbister GK. Long-term health effects perceived by snakebite patients in rural Sri Lanka: A cohort study. PLoS Neglected Tropical Diseases. 2022; 16: e0010723.

[3] Gong XC, Yang WF. Current epidemiological research on snakebite in China. Chinese Journal of Emergency Medicine. 2012; 21: 778–780.

[4] Longbottom J, Shearer FM, Devine M, Alcoba G, Chappuis F, Weiss DJ, *et al*. Vulnerability to snakebite envenoming: a global

mapping of hotspots. Lancet (London, England). 2018; 392: 673–684.

[5] Wang X, Liu L, Zhu W, Wang S, Shi M, Yang S, *et al*. Genome assembly and annotation of the Sharp-nosed Pit Viper *Deinagkistrodon acutus* based on next-generation sequencing data. GigaByte (Hong Kong, China). 2023; 2023: gigabyte88.

[6] Huang F, Zhao S, Tong F, Liang Y, Le Grange JM, Kuang W, *et al*. Unexpected death in a young man associated with a unilateral swollen leg: Pathological and toxicological findings in a fatal snakebite from Deinagkistrodon acutus (Chinese moccasin). Journal of Forensic Sciences. 2021; 66: 786–792.

[7] Wang S, Yang G, An YL, Lin K, Yu AY. Epidemiological analysis of 716 cases of snakebite in Zunyi City, Guizhou Province. Chinese Journal of Emergency Medicine. 2019; 39: 1095–1097. (In Chinese)

[8] Sarkar S, Sinha R, Chaudhury AR, Maduwage K, Abeyagunawardena A, Bose N, *et al*. Snake bite associated with acute kidney injury. Pediatric Nephrology (Berlin, Germany). 2021; 36: 3829–3840.

[9] Bhikane AU, Jadhav RK, Masare PS, Chavhan SG. Clinical, hematobiochemical, and pathological findings and therapeutic management of viperine snake envenomation in zebu cattle. Tropical Animal Health and Production. 2020; 52: 3425–3437.

[10] Qin WG, Zhuo ZP, Hu H, Lay M, Li QQ, Huang JT, *et al*. Proteomic characteristics of six snake venoms from the Viperidae and Elapidae families in China and their relation to local tissue necrosis. Toxicon: Official Journal of the International Society on Toxinology. 2023; 235: 107317.

[11] Chen K, Deng L. Research progress on snake venom and acute lung injury. Journal of Snake. 2010; 22: 255–257.

[12] Guo J, Li M, Yang Y, Zhang L, Zhang LW, Sun QY. Pretreatment with atorvastatin ameliorates cobra venom factor-induced acute lung inflammation in mice. BMC Pulmonary Medicine. 2020; 20: 263.

[13] Azevedo E, Figueiredo RG, Pinto RV, Ramos TDCF, Sampaio GP, Bulhosa Santos RP, *et al*. Evaluation of systemic inflammatory response and lung injury induced by Crotalus durissus cascavella venom. PloS One. 2020; 15: e0224584.

[14] Srirangan A, Pushpakumara J, Wanigasuriya K. A life-threatening complication due to pulmonary haemorrhage following hump-nosed viper bite. BMC Pulmonary Medicine. 2020; 20: 35.

[15] Sampley S, Sakhuja V, Bhasin D, Singh K, Singh H. Plasmapheresis for Pulmonary Hemorrhage Following Viperine Snakebite: A Case Report with Review of Literature. Indian Journal of Critical Care Medicine: Peer-reviewed, Official Publication of Indian Society of Critical Care Medicine. 2020; 24: 986–990.

[16] Huang J, Zhao M, Xue C, Liang J, Huang F. Analysis of the Composition of *Deinagkistrodon acutus* Snake Venom Based on Proteomics, and Its Antithrombotic Activity and Toxicity Studies. Molecules (Basel, Switzerland). 2022; 27: 2229.

[17] Price DR, Garcia JGN. A Razor's Edge: Vascular Responses to Acute Inflammatory Lung Injury/Acute Respiratory Distress Syndrome. Annual Review of Physiology. 2024; 86: 505–529.

[18] Chen PC, Huang MN, Chang JF, Liu CC, Chen CK, Hsieh CH. Snake venom proteome and immuno-profiling of the hundred-pace viper, Deinagkistrodon acutus, in Taiwan. Acta Tropica. 2019; 189: 137–144.

[19] Zhang L, Chen C, Cao Y, Xie B, Chen X, Zeng F, *et al*. Purification and Immunoprotection Evaluation of AaHIV from Complex Venom Metalloproteinases of Deinagkistrodon acutus. Journal of Biochemical and Molecular Toxicology. 2016; 30: 470–476.

[20] Wang L, Wang X, Tong L, Wang J, Dou M, Ji S, *et al*. Recovery from acute lung injury can be regulated via modulation of regulatory T cells and Th17 cells. Scandinavian Journal of Immunology. 2018; 88: e12715.

[21] Mock JR, Dial CF, Tune MK, Norton DL, Martin JR, Gomez JC, *et al*. Transcriptional analysis of Foxp3+ Tregs and functions of two identified molecules during resolution of ALI. JCI Insight. 2019; 4: e124958.

[22] Xia H, Wang F, Wang M, Wang J, Sun S, Chen M, *et al*. Maresin1 ameliorates acute lung injury induced by sepsis through regulating Th17/Treg balance. Life Sciences. 2020; 254: 117773.

[23] Zhang S, Chen X, Devshilt I, Yun Q, Huang C, An L, *et al*. Fennel main constituent, trans anethole treatment against LPS induced acute lung injury by regulation of Th17/Treg function. Molecular Medicine Reports. 2018; 18: 1369–1376.

[24] Yu C, Li Y, Li Y, Li S, Zeng F, Yu J, *et al*. A novel mechanism for regulating lung immune homeostasis: Zukamu granules alleviated acute lung injury in mice by inhibiting NLRP3 inflammasome activation and regulating Th17/Treg cytokine balance. Journal of Ethnopharmacology. 2024; 324: 117831.

[25] Quet M. Non-standard nature. Venoms, serum and serpentariums in the uneven fabrication of global health. Social Science & Medicine (1982). 2023; 332: 116113.

[26] Nyarko E, Ameho EK, Iddi S, Asiedu L. Challenges associated with the availability, accessibility, and use of antivenoms for treating snakebite envenoming in Ghana: A MaxDiff experiment design. Toxicon: Official Journal of the International Society on Toxinology. 2024; 238: 107594.

[27] Yilmaz Y, Tumkaya L. Effects of hyperbaric oxygen and iloprost on intestinal ischemia-reperfusion induced acute lung injury. Annals of Surgical Treatment and Research. 2019; 96: 34–40.

[28] Han G, Ma L, Guo Y, Li L, Li D, Liu H. Hyperbaric oxygen therapy palliates lipopolysaccharide-induced acute lung injury in rats by upregulating AQP1 and AQP5 expression. Experimental Lung Research. 2015; 41: 444–449.

[29] Cao W, Zeng Y, Su Y, Gong H, He J, Liu Y, *et al*. The involvement of oxidative stress and the TLR4/NF-κB/NLRP3 pathway in acute lung injury induced by high-altitude hypoxia. Immunobiology. 2024; 229: 152809.

[30] Wang YG, Long J, Shao DC, Song H. Hyperbaric oxygen inhibits production of CD3+ T cells in the thymus and facilitates malignant glioma cell growth. The Journal of International Medical Research. 2018; 46: 2780–2791.

[31] Li M, Xie ZH, Yu AY, He DP. Increased Efficacy of Antivenom Combined with Hyperbaric Oxygen on *Deinagkistrodon acutus* Envenomation in Adult Rats. Chinese Medical Journal. 2018; 131: 323–329.

[32] Wang C, Jiang C, Yang Y, Xi C, Yin Y, Wu H, *et al*. Therapeutic potential of HUC-MSC-exos primed with IFN-γ against LPS-induced acute lung injury. Iranian Journal of Basic Medical Sciences. 2024; 27: 375–382.

[33] Waiddyanatha S, Silva A, Wedasingha S, Siribaddana S, Isbister GK. Incidence of serum sickness following Indian polyvalent antivenom therapy in a cohort of snake-envenomed patients in rural Sri Lanka. Clinical Toxicology (Philadelphia, Pa.). 2023; 61: 518–523.

[34] Chu SJ, Li MH, Hsu CW, Tsai SH, Lin SH, Huang KL. Influence of hyperbaric oxygen on tumor necrosis factor-alpha and nitric oxide production in endotoxin-induced acute lung injury in rats. Pulmonary Pharmacology & Therapeutics. 2007; 20: 684–690.

[35] Resiere D, Mehdaoui H, Neviere R. Inflammation and Oxidative Stress in Snakebite Envenomation: A Brief Descriptive Review and Clinical Implications. Toxins. 2022; 14: 802.

[36] Thumtecho S, Suteparuk S, Sitprija V. Pulmonary involvement from animal toxins: the cellular mechanisms. The Journal of Venomous Animals and Toxins Including Tropical Diseases. 2023; 29: e20230026.

[37] Cavalcante JS, de Almeida DEG, Santos-Filho NA, Sartim MA,

de Almeida Baldo A, Brasileiro L, *et al*. Crosstalk of Inflammation and Coagulation in *Bothrops* Snakebite Envenoming: Endogenous Signaling Pathways and Pathophysiology. International Journal of Molecular Sciences. 2023; 24: 11508.

[38] Alsolaiss J, Evans CA, Oluoch GO, Casewell NR, Harrison RA. Profiling the Murine Acute Phase and Inflammatory Responses to African Snake Venom: An Approach to Inform Acute Snakebite Pathology. Toxins. 2022; 14: 229.

[39] Yacoub T, Rima M, Sadek R, Hleihel W, Fajloun Z, Karam M. *Montivipera bornmuelleri* venom has immunomodulatory effects mainly up-regulating pro-inflammatory cytokines in the spleens of mice. Toxicology Reports. 2018; 5: 318–323.

[40] Thomas R, Qiao S, Yang X. Th17/Treg Imbalance: Implications in Lung Inflammatory Diseases. International Journal of Molecular Sciences. 2023; 24: 4865.

[41] Yu ZX, Ji MS, Yan J, Cai Y, Liu J, Yang HF, *et al*. The ratio of Th17/Treg cells as a risk indicator in early acute respiratory distress syndrome. Critical Care (London, England). 2015; 19: 82.

[42] Cheng L, Jiao Y, Jiang W, Zhang X, Zhang L, Jia G. IL-33 Deficiency Attenuates Lung Inflammation by Inducing Th17 Response and Impacting the Th17/Treg Balance in LPS-Induced ARDS Mice via Dendritic Cells. Journal of Immunology Research. 2022; 2022: 9543083.

[43] Yang X, Zhang JH, Zhang JF, Lin H, Chen W, Xiang L, *et al*. Imbalance of Th17/Treg in the Pathogenesis of Mice with Paraquat-induced Acute Lung Injury. Iranian Journal of Allergy, Asthma, and Immunology. 2017; 16: 511–519.

[44] Jie XL, Luo ZR, Yu J, Tong ZR, Li QQ, Wu JH, *et al*. Pi-Pa-Run-Fei-Tang alleviates lung injury by modulating IL-6/JAK2/STAT3/IL-17 and PI3K/AKT/NF-κB signaling pathway and balancing Th17 and Treg in murine model of OVA-induced asthma. Journal of Ethnopharmacology. 2023; 317: 116719.

[45] Wu Y, Pei C, Wang X, Wang Y, Huang D, Shi S, *et al*. Probiotics ameliorates pulmonary inflammation via modulating gut microbiota and rectifying Th17/Treg imbalance in a rat model of PM2.5 induced lung injury. Ecotoxicology and Environmental Safety. 2022; 244: 114060.

[46] Wakasugi M, Kawagishi T, Hatano T, Shibuya T, Kuwano H, Matsui K. Case Report: Treatment of a Severe Puff Adder Snakebite Without Antivenom Administration. The American Journal of Tropical Medicine and Hygiene. 2021; 105: 525–527.

[47] Zanon V, Morri A, Lonati D, Paoli A, Camporesi EM, Bosco G. HBO_2 in snake envenomation (atrox albinus rattlesnake): a case report in a human. Undersea & Hyperbaric Medicine: Journal of the Undersea and Hyperbaric Medical Society, Inc. 2016; 43: 473–476.

[48] Kang JY, Xu MM, Sun Y, Ding ZX, Wei YY, Zhang DW, *et al*. Melatonin attenuates LPS-induced pyroptosis in acute lung injury by inhibiting NLRP3-GSDMD pathway via activating Nrf2/HO-1 signaling axis. International Immunopharmacology. 2022; 109: 108782.