



Atractylenolide III alleviates sepsis-mediated lung injury via inhibition of FoxO1 and VNN1 protein

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ABSTRACT

Purpose: To evaluate the influence of atractylenolide (Atr) III on sepsis-induced lung damage. **Methods:** We constructed a mouse sepsis model through cecal ligation and puncture. These mice were allocated to the normal, sepsis, sepsis + Atr III-L (2 mg/kg), as well as Atr III-H (8 mg/kg) group. Lung injury and pulmonary fibrosis were accessed via hematoxylin-eosin (HE) and Masson's staining. We used terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and flow cytometry for detecting sepsis-induced lung cell apoptosis. The contents of the inflammatory cytokines in lung tissue were measured via enzyme-linked immunosorbent assay (ELISA). **Results:** Atr III-H did not only reduce sepsis-induced lung injury and apoptosis level, but also curbed the secretion of inflammatory factors. Atr III-H substantially ameliorated lung function and raised Bcl-2 expression. Atr III-H eased the pulmonary fibrosis damage and Bax, caspase-3, Vanin-1 (VNN1), as well as Forkhead Box Protein O1 (FoxO1) expression. **Conclusion:** Atr III alleviates sepsis-mediated lung injury via inhibition of FoxO1 and VNN1 protein.

Key words: Atractylenolide. Sepsis. Forkhead Box Protein O1. Lung Injury. Mice.

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■ Introduction

Sepsis, a kind of systemic inflammatory response syndrome (SIRS), arises from infection¹⁻³. Sepsis can cause lots of damage to the lung tissue, and pulmonary dysfunction induced by sepsis can aggravate other organs dysfunction⁴⁻⁶. Inflammatory cytokines, endotoxin, and oxygen free radicals in the blood can induce apoptosis of pulmonary epithelial and pulmonary vascular endothelial cells, which in turn promotes the infiltration of edema including rich protein into the interstitium, ultimately leading to acute lung injury (ALI) or acute respiratory distress syndrome (ARDS)⁷⁻⁹. However, nowadays no complete theoretical system can explain the complex pathogenesis of ALI.

When SIRS occurs, excessive activation and recruitment of macrophages and neutrophils in the lung, caused by systemic inflammatory mediators, can promote the uncontrolled release of various proinflammatory cytokines in the lung, leading to ARDS. Excessive activation of immune cells in the lung is seen as an essential aspect in the development of ARDS¹⁰⁻¹². Therefore, new methods are needed to reduce the pulmonary inflammatory response in the future treatment of sepsis-induced lung injury.

As we know, Atractylenolide (Atr) III, as a kind of sesquiterpenoid, is isolated from *Atractylodes*. Atr III has been reported to possess extensive biological activities, containing anti-inflammatory, antioxidant, and anti-cancer activities, and neuroprotective effects¹³⁻¹⁶. Studies have shown that Atr III was able to attenuate pulmonary fibrosis and oxidative stress level¹⁷⁻¹⁹.

Nevertheless, there are still no studies about the effects of Atr III on sepsis-mediated lung injury. Besides, the molecular mechanism was blurry. Forkhead box protein O1 (FoxO1), as a momentous regulator of endothelial cell proliferation, is able to accelerate cell apoptosis. Evidence suggested that FoxO1 was memorably raised in skeletal muscle of sepsis. Vanin-1 (VNN1) is a glycosylated phosphatidylinositol-anchored ubiquitinase, which is located on chromosome 6q23-q24 and highly expressed in the lung^{20,21}.

Considering this, the purpose of this study was to probe the role of Atr III on sepsis-mediated lung injury via adjusting FoxO1 and VNN1 in mice, to supply substantial evidence for Atr III as a promising drug to preventive treatment sepsis-mediated lung injury.

■ Methods

Atr III was purchased from Shanghai Bohu Biotechnology Co. IL-6, TNF- α , and IL-1 β kits were provided from Shanghai

Kanglang Biotechnology Co. Hematoxylin-eosin (HE) kit was purchased from Beijing Kulaibo Technology Co. Masson kit was provided from Nanjing Senbeijia Biotechnology Co.

All assays carried out during the research were consistent with the United States National Institutes of Health guidelines for the use of experimental animals. We purchased male 7-week-old BALB/c mice from the institution of Shanghai SLAC Laboratory Animal. We stochastically allocated the mice to the normal, sepsis, Atr III-L (2 mg/kg) and Atr III-H (8 mg/kg) groups, with 10 mice in each group. The mice were fed at 20-22°C and under standard conditions (12:12 h light/dark cycle).

Model preparation

Our crew built cecal ligation and puncture via previous researcher's operation. Briefly, mice were conducted anesthesia with pentobarbital sodium. A sterile abdominal incision was followed. After the cecum was exposed, the middle part of the cecum was ligated. Next, the cecum was punctured with 18 needles. Ultimately, the abdominal cavity is closed. The normal group was carried out the same procedure via without ligation or puncture. After surgery, the mice were managed normally.

HE and Masson's staining

After 24 h of the last administration, the rats were killed, and the lung tissue was instantly removed. Lung tissue was cut into 5-mm thin sections. Then, thin sections were immersed in paraformaldehyde for 10 min. The slices were dried in 45°C incubator and further stained with HE and Masson. The slices were decolorized with ethanol, dehydrated with xylene, and sealed with neutral glue. The histological structure and pathological changes of heart were observed under microscope, with the specific criterion predominantly formed according to previous studies.

Lung function detection

After 24-h management, the mice were placed in instruments. Furthermore, the lung function of mice was further tested via whole-body flow-through plethysmography (Beijing AMCA Shenwu Technology Co.). Our crew determined the ventilation, airway resistance, as well as lung volume in levels to assess lung function.

ELISA detection

After the last treatment, the blood was collected from the eyeballs of mice. Serum was separated by centrifugation at 12,000 r/min for 10 min and then kept at -80°C until use.

The levels of inflammatory factors, including TNF- α , IL-6, as well as IL-1 β (Shanghai Jingkang Bioengineering Co.) in serum, were tested via enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Calculation of lung wet-dry weight ratio

After the sacrifice of animals, the left lung of rats was taken, and the surface water and blood were sucked dry. The pulmonary wet-dry (W/D) weight ratio was measured to perform the evaluation of pulmonary edema. The fresh upper right lung tissue was cleaned and weighed to acquire the W, and then dried in an oven at 180°C lasting at least 24 h for examining the D. The ratio of W/D is used to express the water content of lung tissue. Lung W/D weight ratio is used as an indicator of lung injury.

TUNEL assay

Apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in each group. After rehydrating the cells, 20 mg/mL proteinase K for treatment lasting 8-10 min under room temperature condition, phosphate buffered saline (PBS) for washing, as well as 4% paraformaldehyde for refixation, were used. DNA fragments from apoptotic cells were marked with nucleotide mixture and rTdT enzyme (DeadEnd TUNEL System, Promega) for 60 min at 37°C, followed by 15-min incubation in 2SSC to stop the reaction. After rinsing with deionized water, the slide was overlapped by another one. Then, relevant images were finally captured, with the use of fluorescence microscope. The proportion of apoptotic cells was measured by TUNEL-positive cells/all cells in 10 high magnification field of view.

Flow cytometry analysis

Twenty-four hours after the last administration, the lung tissue was removed. The 1:9 homogeneous slurry was fleetly prepared with 4°C normal saline. Homogenate was separated by centrifugation at 10,000 r/min for 20 min. Next, the supernatant was absorbed, 5- μ L annexin-v-FITC was added, and 5- μ L PI was incubated in a dark room for 15 min. Then, the apoptosis was determined through flow cytometry:

Apoptosis rate = (early apoptotic cells + late apoptotic cells) / all cells \times 100%.

2.9 qRT-PCR

One hundred mg of tissue or 1×10^6 cells were used to lyse the cells and extract total RNA with Trizol reagent

(Invitrogen). The purity and concentration of RNA were determined using a 96-well plate micro-spectrophotometer, and RNA purity was considered high as the D260/D/280 value was between 1.8 and 2. After adjusting the RNA concentration to 300~500 ng/ μ L, its reverse transcription was developed with the RNA Reverse Transcription Kit (Invitrogen, California, United States), to produce cDNA, which was the first strand.

Under the instructions of SYBR Green fluorescence quantitative kit, the reaction system was configured, in which three repeat wells were set for each system. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were seen as standardized internal references, and the results were calculated employing the $2^{-\Delta\Delta CT}$ method. Each experiment was carried out repeatedly for three times. The specific primer sequences were: FoxO1 F: 5'-GCC GTG CTA CTC GTT TGC-3', R: 5'-CTT GGG TCA GGC GGT TC-3'; VNN1 F: 5'-ATA CTC CCG CCA TGC GAC TG-3', R: 5'-CTG TCA CAA CCT CAC TGT CAT-3'; Bax F: 5'-CCG GGA GAT CGT GAT GAA GT-3', R: 5'-ATC CCA GCC TCC GTT ATC CT-3'; Bcl-2 F: 5'-CCG GGA GAT CGT GAT GAA GT-3', R: 5'-ATC CCA GCC TCC GTT ATC CT-3'; caspase-3 F: 5'-TGG AAC AAA TGG ACC TGT TGA CC-3', R: 5'-AGG ACT CAA ATT CTG TTG CCA CC-3'; GAPDH: 5'-GGA GCG AGA TCC CTC CAA AAT-3', R: 5'-GGC TGT TGT CAT ACT TCT CAT GG-3'.

Western blotting

Immunoprecipitation (RIPA) plus protease inhibitor cocktail (Roche) was used to collect cells. Forty μ g of protein lysate was first solubilized with 12% SDS-PAGE, and then its transfer to nitrocellulose membranes was completed. Subsequently, the membranes were blocked applying 5% non-fat milk lasting 1 h under room temperature condition, conducted incubation employing primary antibodies (1:1,000) overnight at 4°C, then washed adopting phosphate buffered saline-Tween (PBST) the next day, and finally incubated with secondary antibodies (1:5,000) lasting 1 h under room temperature environment. Eventually, the target protein was detected utilizing SuperSignal West Pico PLUS chemiluminescence kit. FoxO1, Bax, VNN1, caspase-3, Bcl-2, as well as GAPDH (SantaCruz), were the antibodies used in the experiments.

Statistical analysis

We displayed data in the form of mean \pm standard deviation (SD). Statistical assessment was done with t-test for inter-group comparison or one-way variance analysis (ANOVA) for multi-group comparisons, adopting GraphPad Prism 5 software and $P < 0.05$.

Results

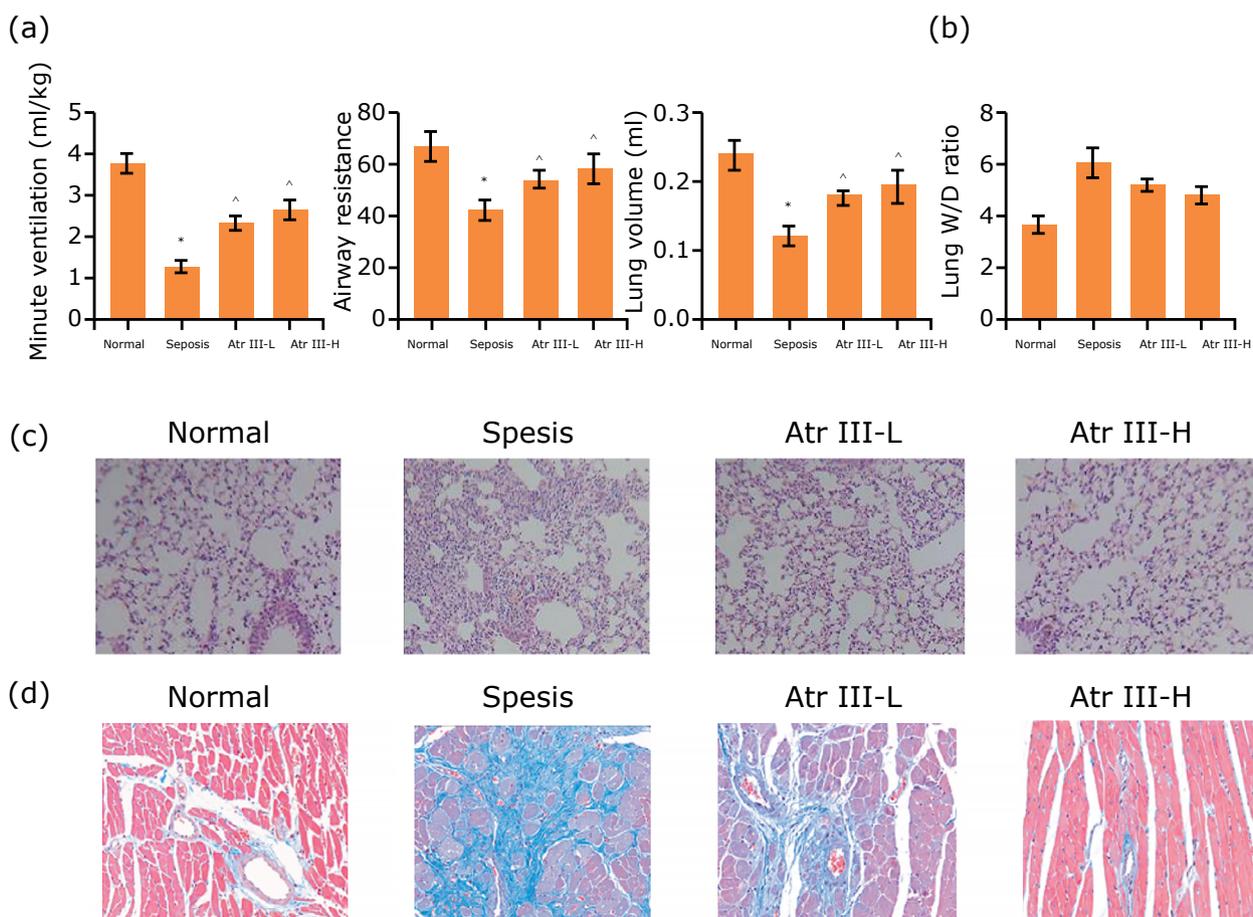
Atr III ameliorated sepsis-mediated lung injury

Lung function was determined. These indexes of lung function, including the ventilation, airway resistance, as well as lung volume in levels, were memorably abated in sepsis group. In sepsis group, ventilation, airway resistance, as well as lung volume in levels, were dramatically lessened. Interestingly, Atr III ameliorated lung function, which was modified in a dose-dependent manner ($P < 0.01$, Fig. 1a). Compared with the normal group, sepsis raised the lung W/D specific gravity value, while Atr III treatment decreased the lung W/D weight ratio at 12 or 24 h after sepsis-mediated in contrast with the normal group ($P < 0.05$, Fig. 1b). Atr III performed best. HE staining was utilized for observation of the impact on Atr III on lung damage 24 h after sepsis-mediated. As it can be seen in Fig. 1c, the inflammatory

cell infiltration, along with necrosis, developed in sepsis group. However, no obvious difference was revealed in lung histomorphology between the normal group, as well as Atr III one. The results suggested that Atr III relieved sepsis-induced lung injury. Figure 1d displayed that in Atr III group the myocardial fibers were relieved, and the cells were stained uniformly. Atr III-H effect was better than Atr III-L. It can be inferred that Atr III may be a protective agent in sepsis-mediated lung injury.

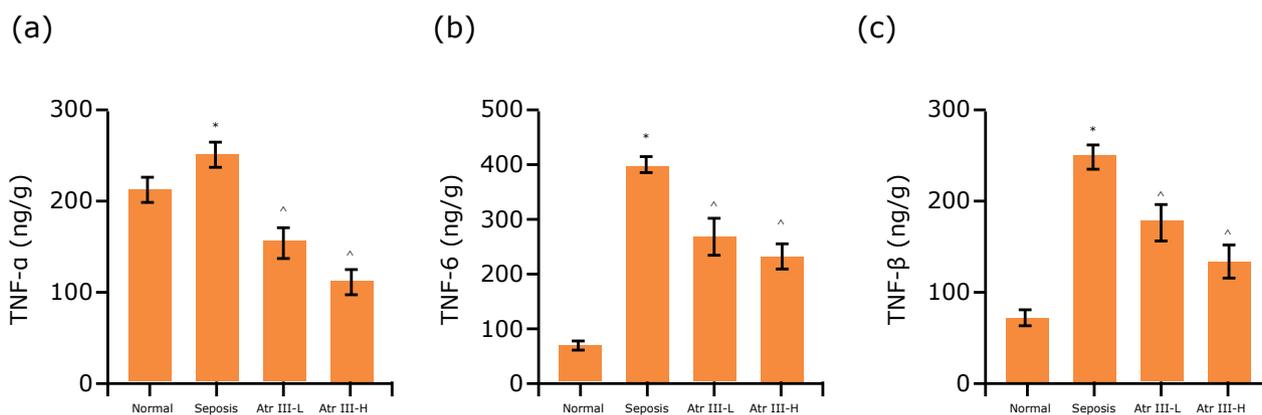
Impact of Atr III on inflammatory cytokine release

For evaluating the inflammatory response caused by sepsis-induced pulmonary damage, the release of IL-1 β , TNF- α , as well as IL-6, in the supernatant of lung homogenates was monitored via ELISA. The expression of these inflammatory factors was lower in the normal group. According to Fig. 2, sepsis can induce the expression



Atr III: atractylenolide III; W/D: wet/dry; ELISA: enzyme-linked immunosorbent assay; HE: hematoxylin-eosin.

Figure 1 - Atr III ameliorated sepsis-mediated lung injury. **(a)** Measurement of lung function indicators. **(b)** ELISA kits to assay the lung W/D weight ratio after sepsis. **(c)** HE staining in rat lung tissues. **(d)** Changes of myocardial fibrosis in rats. Values were expressed as N=10, * $p < 0.05$ contrast to normal, ^ $p < 0.05$, contrast to sepsis.



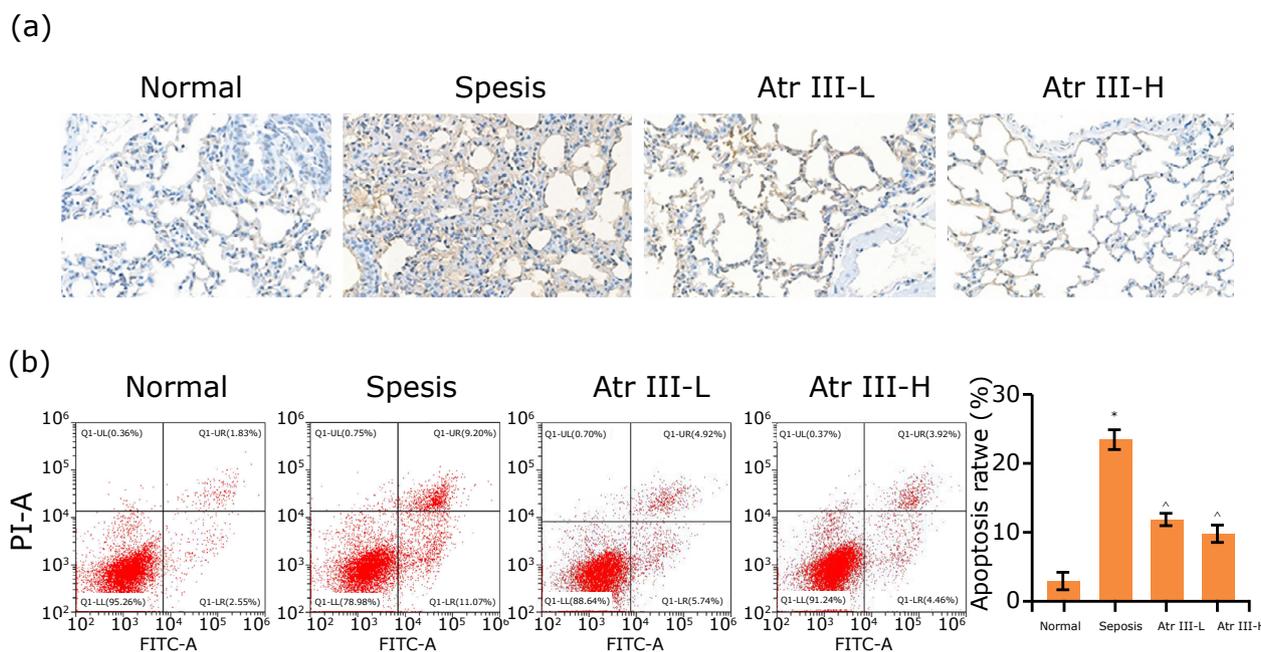
Atr III: atractylenolide III.

Figure 2 - Effects of Atr III on inflammatory cytokine release. **(a)** TNF- α . **(b)** IL-6. **(c)** IL-1 β . Values were expressed as N=10, * $p < 0.01$ contrast to normal, ^ $p < 0.05$, contrast to sepsis.

of lung inflammatory factors. After sepsis, the secretions of IL-6, TNF- α , as well as IL-1 β in sepsis group, were substantially enhanced ($P < 0.01$). After Atr III treatment, the numbers of these inflammatory factors were further lessened. In addition, Atr III-H represented the best effect. The findings implicated that Atr III may inhibit the inflammatory response induced by septic lung injury via modulating the secretion of inflammatory cytokines.

Effects of Atr III on apoptosis induced by sepsis-mediated lung damage

For evaluating the impact of Atr III on apoptosis caused by sepsis-mediated lung injury, sepsis-induced apoptosis in lung tissue via TUNEL staining was examined. Figure 3a manifested that sepsis treatment could memorably boost apoptosis. Even more impressively, Atr III could dramatically lessen the apoptosis rate. More importantly, Atr III-H



Atr III: atractylenolide III; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.

Figure 3 - Effects of Atr III on apoptosis induced via sepsis-mediated lung damage. **(a)** TUNEL staining. **(b)** Flow cytometry detection. Values were expressed as N=10, * $p < 0.05$ contrast to normal, ^ $p < 0.05$, contrast to sepsis.

displayed the best effect. Figure 3b displayed the same trend. Contrasted to sham operation group, the apoptosis rate of sepsis group was memorably raised. Contrasted to sepsis group, Atr III was able to lessen apoptosis proportion. The mentioned results indicated that ATR III was able to restrain sepsis mediated apoptosis of lung cells.

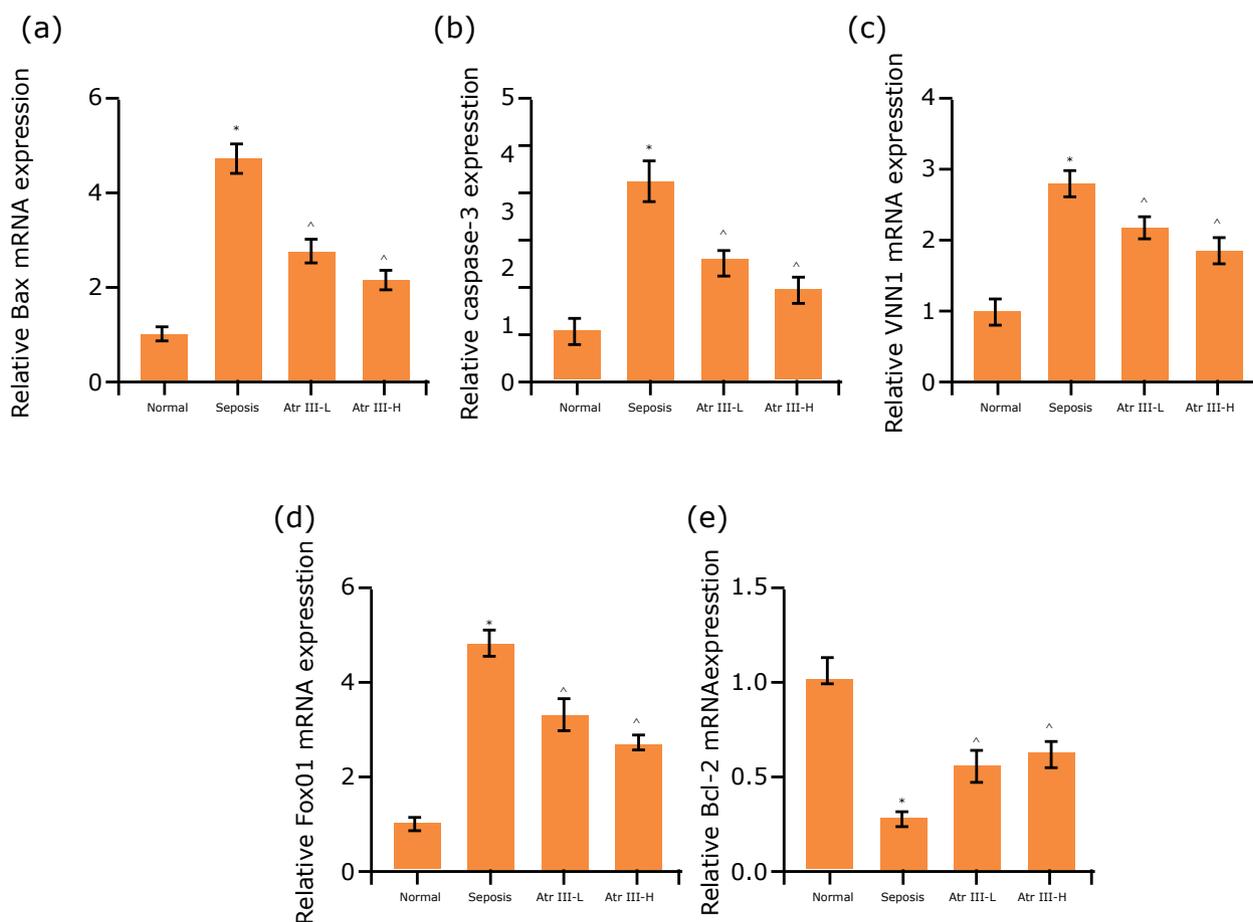
qRT-PCR determined the expression of relation gene

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was applied to measure gene expression in sepsis-mediated lung injury. Figure 4 demonstrated that sepsis group was able to reduce Bcl-2 mRNA expression and promote Bax, caspase-3, VNN1, as well as FoxO1 mRNA expression. By contrast, Atr III could enhance Bcl-2 mRNA expression ($P < 0.05/0.01$).

Concurrently, Atr III was able to restrain Bax, caspase-3, VNN1, as well as FoxO1 mRNA expression, in sepsis-mediated lung injury ($P < 0.05/0.01$). Furthermore, Atr III-H effects were the most remarkable.

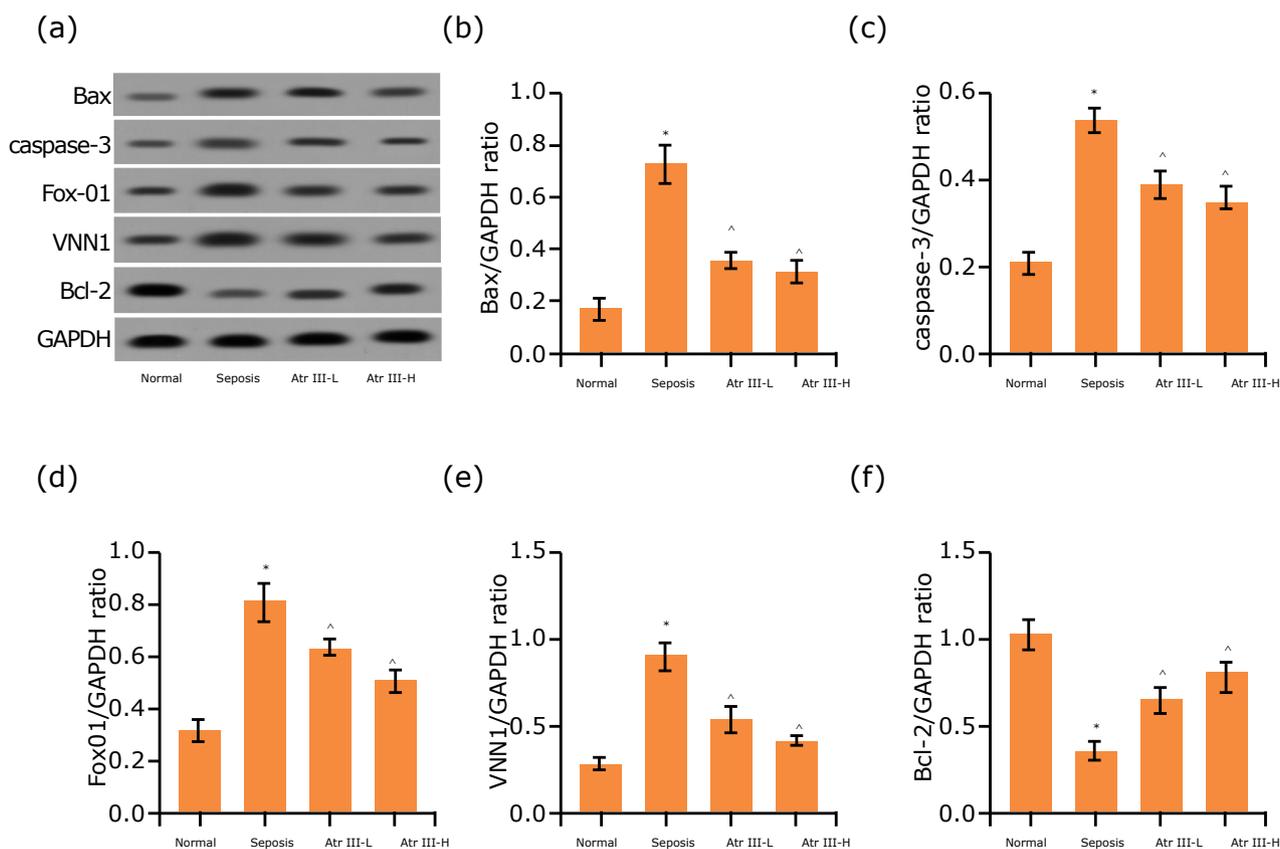
Western blot measured protein expression

Just like the qRT-PCR results, western blot displayed that sepsis group could reduce Bcl-2 expression and enhance Bax, caspase-3, VNN1, as well as FoxO1 expression. By contrary, Atr III could enhance Bcl-2 protein expression in sepsis-mediated lung injury ($P < 0.05/0.01$). On the other side, Atr III further restrained Bax, caspase-3, VNN1, as well as FoxO1 protein expression ($P < 0.05/0.01$). More than that, Atr III-H effects were noticeably superior to Atr III-L, which was consistent with the qRT-PCR outcome ($P < 0.05$, Fig. 5).



Atr III: atractylenolide III; VNN1: Vanin-1; FoxO1: Forkhead Box Protein O1; qRT-PCR: real-time quantitative reverse transcription polymerase chain reaction.

Figure 4 - qRT-PCR assay the expression of relation gene. **(a)** Bax. **(b)** Caspase-3. **(c)** VNN1. **(d)** FoxO1 **(e)** Bcl-2. Values were expressed as N=10, * $p < 0.05$ contrast to normal, ^ $p < 0.05$, contrast to sepsis.



Atr III: atractylenolide III; FoxO1: Forkhead Box Protein O1; VNN1: Vanin-1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

Figure 5 - Associated protein was detected by western blot. (a) Bax. (b) caspase-3. (c) VNN1. (d) FoxO1 (e) Bcl-2. Values were expressed as N=10, * $p < 0.05$ contrast to normal, ^ $p < 0.05$, contrast to sepsis.

Discussion

Lung damage, a prevalent complication of sepsis, gives rise in multiple organ dysfunction syndrome, as well as death of those who suffer from it²². The perfect target for treating sepsis-mediated lung damage is to inhibit inflammatory response, prevent apoptosis, and maintain lung function²³⁻²⁵. Nevertheless, the causative role of sepsis-mediated lung damage is still distinct, which immensely impedes the screening of diagnostic markers of sepsis-mediated lung injury.

Substantive research has stressed that Atr III displayed anti-inflammatory, antioxidant and anti-cancer activities and neuroprotective effects. Nevertheless, the physiological role of Atr III in sepsis, together with its underlying molecular mechanism, has not been reported. Therefore, this work probed into the impact of Atr III on sepsis-induced lung injury in mouse with sepsis. At the start, it was indicated that Atr III exerted a protective effect on ameliorating sepsis-mediated lung injury via raising pulmonary function

and debasing lung W/D weight ratio. More importantly, Atr III did not only relieve inflammatory cell infiltration and along with necrosis, but also renewed myocardial fiber relaxation, which hinting Atr III may be a protective agent in sepsis-mediated lung injury.

In the inflammatory response to sepsis, the expression of the key cytokine TNF- α is rapidly increased and peaks at an early stage, which induces the production of inflammatory factors like IL-6. These inflammatory cytokines trigger a series of inflammatory cascades through related signal transduction pathways, resulting in damage to capillary endothelial and alveolar epithelial cells. Increased vascular permeability and alveolar surfactant release lead to the thickening of the alveolar wall, capillary and alveolar hemorrhage, and pulmonary capillary and alveolar hemorrhage. The mentioned pathological changes further cause severe pulmonary edema, ventilation/blood flow imbalance, refractory hypoxemia, and ultimately ALI/ARDS²⁶.

Zhang's study on ALI during lipopolysaccharide-induced sepsis showed that TNF- α levels were sharply increased in the bronchoalveolar lavage fluid of septic animals²⁷. Animal experiments and clinical studies have confirmed that plasma TNF- α levels are positively correlated with the development and prognostic severity of ALI in patients with sepsis²⁸. IL-6-mediated neutrophil activation and accumulation promote the massive release of elastase and oxygen free radicals, causing damage to alveolar epithelial cells and extracellular matrix. The main cause of ARDS is pulmonary interstitial edema caused by pulmonary vascular hyperpermeability²⁹.

Therefore, it is important to investigate the mechanism of abnormal secretion of inflammatory factors for preventing and treating sepsis-mediated lung injury. The study discovered that Atr III was able to restrain release of inflammatory factors via adjusting IL-6, TNF- α and IL-1 β levels.

Studies have indicated that many apoptotic cells are involved in lung injury, thereby inducing a series of proteolytic reactions of caspase, such as caspase-3 protein³⁰⁻³². In the present study, same with the results previously reported, Bax and caspase-3 were enhanced, and Bcl-2 was declined in sepsis group. Nevertheless, Atr III completely reversed this trend, indicating that Atr III could boost apoptosis of lung cells. The results found here show that Atr III could induce apoptosis of lung cancer cells in a dose-dependent manner.

It has been concluded in the literature that VNN1 is implicated in sepsis and involved in the systemic inflammatory response^{33,34}. FoxO protein is a large class of transcription factors, which can directly participate in gene transcription and expression^{35,36}. FoxO1 exerts a momentous role in the regulation of apoptosis, oxidative stress, and inflammatory diseases³⁷⁻³⁹. At present, FoxO1 is known to improve the inflammatory response of sepsis via regulating early inflammatory factors (NF- κ B, TNF- α)⁴⁰. The results presented here show that Atr III could restrain VNN1 and FoxO1 protein expression, suggesting that Atr III may reduce tissue damage in sepsis-induced lung injury by controlling VNN1 and FoxO1 expression.

■ Conclusions

This study firstly demonstrated that Atr III could alleviate sepsis-mediated lung injury via inhibition of FoxO1 and VNN1 protein, and the mechanism might be related to down-regulation of pro-inflammatory cytokines and protection of lung function. Atr III can be developed as an effective and safe agent for treating sepsis-induced lung injury in the future.

■ Author's contribution

Conception and design the study: Fu J, Gao C, Li S and Tian Y; **Design the study:** Wei Y and Xian L; **Analysis of data:** Li S; **Manuscript writing:** Fu J and Xian L; **Critical revision:** Fu J and Xian L; **Final approval:** Fu J, Gao C, Li S, Tian Y, Wei Y and Xian L.

■ Data availability statement

Data will be available upon request.

■ Funding

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■ Acknowledgments

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