Study on the protective effect and mechanism of apelin-13 in ventilator-induced acute lung injury

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Research Article

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Abstract

Background: Mechanical Ventilationventilation (MV) is an essential life support mechanism in the clinic. It may also lead to ventilator-induced acute lung injury (VILI) due to local alveolar overstretching and/or repeated alveolar collapse. However, the pathogenesis of VILI is not completely clear, and its occurrence and development may be related to inflammatory reactions, oxidative stress, apoptosis and other physiological processes. Some studies have found that the apelin/APJ pathway is an endogenous antagonistic mechanism that is activated during Acute respiratory distress syndrome(ARDS), and it can counteract the injury response and prevent uncontrolled lung injury. To prove that apelin-13 plays a protective role in VILI, in this study, we established a rat VILI model to explore whether apelin-13 can attenuate VILI in rats by inhibiting inflammation, cell apoptosis and oxidative stress.

Methods: SD rats were divided into four groups: the control group, high tidal volume group, high tidal volume+NS group, and high tidal volume+apelin-13 group. After tracheotomy, autonomous breathing was maintained in the rats. After tracheotomy, the other rats were connected to a small animal ventilator for 4 hours to establish the rat VILI model. The mRNA expression of apelin was measured by real-time quantitative polymerase chain reaction (qRT—PCR), and the protein expression levels of APJ, the apoptotic protein Bax, Bcl-2 and Akt/P-Akt were measured by Western blotting (WB) and immunofluorescence. The degree of lung injury was evaluated by pathological staining of lung tissue and measuring the wet to dry ratio of lung tissue. The expression of inflammatory factors in alveolar lavage fluid was measured by enzyme-linked immunosorbent assay (ELISA), and the activity of myeloperoxidase (MPO) in lung tissue was measured to evaluate the degree of pulmonary inflammation.

Results: The expression of apelin and the APJ receptor was upregulated under VILI conditions. After the rats were treated with apelin-13, the activation of the apelin-APJ signaling pathway, the pathological damage to lung tissues, the degree of cell apoptosis, and the levels of the inflammatory cytokines IL-1, IL-6 and TNF-α were reduced in the VILI model rats. The expression of MPO was decreased, and the activity of MPO was also decreased. Moreover, the Akt/P-Akt signaling pathway is associated with apoptosis. After treatment, the expression of Akt/P-Akt pathway-related proteins increased.

Conclusion: During VILI, the apelin/APJ axis plays an endogenous role in ameliorating injury. Overexpression of apelin can significantly reduce the inflammatory response, cell apoptosis and oxidative stress in the lung tissues of VILI model rats and slow the occurrence and development of VILI.

Introduction

Mechanical ventilation is an important advanced mechanism of life support that is used in clinical practice. However, while saving lives, maintaining respiratory function and improving oxygenation, it can also cause or exacerbate lung injury, and this condition is called mechanical ventilation-related lung injury (VILI)[1, 2]. VILI includes volume injury, air pressure injury, shear injury and biological injury, but its essence is mechanical stress-induced biological injury. Its main characteristics include the following:

mechanical stress causes deformation of the lung cell membrane and its receptors, and organelles sense external abnormal mechanical stimuli, which are converted into biochemical signals and transmitted to cells, activating the intracellular signal transduction system, increasing the numbers of inflammatory cells and secreted inflammatory factors, and generating an inflammatory storm. Additionally, mechanical stress can directly damage alveolar epithelial cells, induce apoptosis, damage the structure and function of alveolar epithelial cells and capillary endothelial cells, damage the integrity of the basement membrane, cause alveolar hemorrhage, form a transparent membrane, increase lung permeability, and cause pulmonary edema, thus damaging the integrity of the alveolar barrier and impairing lung barrier function[3, 4]. In addition, mechanical stretching can lead to the release of mediators related to the activation of the immune response, further increasing damage, and it may lead to damage to other distant organs[5]. In recent years, many innovative and protective lung ventilation strategies have been proposed, which mainly avoid VILI by limiting tidal volume and/or platform pressure and by maintaining lung recruitment in alveolar areas with sufficient positive end expiratory pressure (PEEP). Relevant clinical trials have shown that ventilator management can effectively reduce the mortality of patients with acute respiratory distress syndrome (ARDS)[6, 7]. However, a large number of ARDS patients still die of multiple system organ failure (MSOF). Therefore, the prevention and treatment of biological injury is particularly important. In previous research by our research group, it was proven that VILI can be reduced by inhibiting inflammation and apoptosis in rats[8]. However, the most critical factors and signaling pathways that cause VILI have not been identified, and the pathogenesis still needs to be further studied.

Apelin, a newly discovered small molecule active polypeptide, is a natural ligand of orphan G protein coupled receptor angiotensin receptor AT-1 related protein (APJ). At present, active apelin peptides, including apelin-36, apelin-17, apelin-13 and apelin-12, have been described. Among these subtypes, apelin-13 and apelin-36 are the main subtypes[9, 10]. Increasing evidence shows that the apelin/APJ system is widely expressed in the heart, lung and liver[11–14]. It is involved in regulating cardiovascular and endocrine systems[12, 15]. In recent years, the importance of the apelin/APJ system in respiratory diseases has been gradually recognized[16]. Some studies have verified that in an oleic acid (OA)-induced ARDS rat model, OA-induced ARDS is coupled with the upregulation and activation of the apelin/APJ pathway, and enhanced apelin/APJ signaling plays a functional role. In the OA- and LPS-induced ARDS model, posttraumatic treatment with apelin-13 reduced lung injury and improved oxygenation. It is suggested that the apelin/APJ system can be used as an endogenous anti-injury mechanism to protect lung tissue[17]. In addition, apelin-13 can inhibit the NF-κB pathway, and the NLRP3 inflammasome is activated to protect against LPS-induced acute lung injury. Regulation of the gene expression of various inflammatory cytokines, apoptotic proteins and antioxidant stress molecules may be the mechanism underlying the protective effect of apelin[13].

However, the effects of VILI on the expression of apelin and APJ receptors and the regulation of VILI via the apelin/AJP signaling pathway have not been studied. Based on the above research, we hypothesized that apelin-13 delayed the occurrence and development of VILI, and it may be a new treatment strategy for VILI.

Materials And Methods

Animal experiment

The Changsha Tianqin Biotechnology Co. ltd. (Changsha, China) provided 24 specific pathogen-free male Sprague-Dawley (SD) rats, (age, 6–8 weeks and weight, 200–250 g). The animal model was established as described above, ventilation parameters were set as follows: TV, 40 ml/kg; respiratory rate, 60 breaths/min; positive endexpiratory pressure, 0 cmH2O.

Six rats per group were randomly assigned to four groups: Control (spontaneous breathing), high tidal volume group (HV), high tidal volume + normal saline group (NS), high tidal volume + apelin-13 group (apelin-13). In the group Control, spontaneous respiration was retained after tracheotomy and intubation without mechanical ventilation. The rats in other groups were cut open and connected with small animal ventilator. 30 minutes before the experiment, the endogenous apelin receptor agonist [Pyr1] - Apelin-13 (MedChemExpress, USA) was injected into the apelin-13 (10 nmol/kg, intraperitoneal injection); The group HV + NS received intraperitoneal injection of the same amount of normal saline as the control; Observe mechanical ventilation or spontaneous breathing for 4 hours. Increase initial anesthesia by about 1/3 per hour. For follow-up experiments, rats were euthanized after 4 h of MV or spontaneous breathing. Their bronchoalveolar lavage fluid (BALF) and lung tissue were collected. According to the National Institutes of Health's (National Institutes of Health) (NIH publication No. 85–23, 2011) Guidelines for the Care and Use of Experimental Animals, this experiment was conducted. Animal experimental protocols were approved by Guizhou Medical University's Animal Experimental Ethics Checklist (approval number: 2001132) and followed AVMA guidelines for animal euthanasia and ARRIVE guidelines.

Hematoxylin-eosin (HE) staining

Following the establishment of the model, the lung tissue was fixed in formalin solution, stored at 4 ◦C for 24 h, dehydrated with gradient ethanol, and paraffin embedded. The sample was sliced into 4 um thick sections, and stained with Hematoxylin-eosin (Solarbio, China).After soaking with gradient ethanol, the section was mounted with a neutral balsam and cover glass. The alveolar morphology was considered under optical microscope and scored according to the pulmonary lesion scoring system[18].

Immunofluorescence staining

The expression levels of APJ(1:800;Proteintech, China) proteins were determined using immunofluorescence. After drying at 60 ◦C for 60 min, dewaxing and hydrating the 4-um thick paraffin sections, sealing them with goat serum at 37 ◦C for 30 min, and incubating them with a specific primary antibody overnight. Rewarming at 37 ◦C for 30 min, and incubated with a fluorescent secondary antibody (1:500, Proteintech, Beijing, China) for 1 h in a wet box. After the addition of DAPI (Solarbio, Beijing, China) in the dark the tablets were re-dyed for 10 min. Finally, the slides were sealed with an anti-fluorescence quenching agent, and images were obtained using a positive fluorescence microscope.

Lung Wet/dry (W/d) Weight Ratio Detection

After euthanization, rats were cleaned with PBS to separate the right upper lung and then the lung weighed. Wet lungs were dried at 65 ∘C for 48 h and referred to as dry lungs. We calculated the lung wet weight /dry weight ratio.

Enzyme-linked Immunosorbent Assay (Elisa)

After euthanization, BALF was collected. The BALF was subjected to centrifugation at 1500 rpm for 10 min and the supernatant collected. In order to determine the expression of interleukin-1β(IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in BALF, we used ELISA reagents (Cusabio Biotech, Wuhan, China), as instructed by the manufacturer IL-1β(CSB-E08053h), IL-6 (Ab100712) and TNF-α (CSB-E11987r). In less than five minutes, the optical density value of each sample was determined with a spectrophotometer at 450 nm.

Western Blot Analysis

The pre-cryolysis solution containing PMSF and phosphatase inhibitor was added to the tissue, ground, and cleaved thoroughly using an ultrasonic crusher. Thereafter, 5 × loading buffer was added to the protein sample. Proteins were estranged via SDS-PAGE and moved onto a polyvinylidene fluoride (PVDF) membrane, which was stuck down with 5% skim milk at 25 ℃ for 1 h and incubated overnight with a particular primary antibody APJ (1:800;Proteintech, China) or Bax (1:10, 000 Proteintech, China) or Bcl-2(1:3,000 Proteintech, China)or AKT 1:7, 000 Proteintech, China P-AKT 1:3, 000 Proteintech, China at 4 ℃. The membrane was then incubated with a secondary antibody (IgG, 1:5000 Proteintech, Chicago, USA) at 25 ℃ for 1 h. Finally, imprinting was observed using enhanced chemiluminescence (ECL, P0018AM, Shanghai, China).

Real-time Quantitative Polymerase Chain Reaction (Qrt-pcr)

Sum RNA was obtained from rat lung tissue using the TRIzol kit (Thermo Fisher, USA). By using the PrimeScriptTM RT reagent kit (RR047A, Takara, Dalian, China), complementary DNA (cDNA) was synthesized from the extracted RNA. mRNA expression levels were measured in strict compliance with TB Green® Premix Ex TaqTM II instructions from Takara (RR820A, Takara, Dalian, China). GAPDH served as the internal control for APJ. All primers (Table 1) were synthesized by Shenggong Bioengineering Co. ltd. (Shanghai, China). The relation expression of the genes was deliberated using the 2-Δ ΔCT manner.

Table 1
Primer sequence for quantitative reverse transcription ploymerase chain reaction.

Name Primer sequence(5’–3’)
Apelin F:TGCTGCTCTGGCTCTCCTTGAC R:GTTGCCTTCTTCTAGCCCTTTCCC
GAPDH F:ATGGAGAAGGCTGGGGCTC R:AAGTTGTCATGGATGACCTTG
Note: F, forward; R, reverse; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Mpo Activity

MPO activity in supernatant of lung tissue was determined using MPO kit (Nanjing Institute of Bioengineering, Nanjing, China). The absorbance was measured at 460 nm.

Statistical analysis

All experiments were conducted with different batches of rats at least thrice. Data are expressed as mean ± standard deviation (X ± SD). Plots and statistical analyses were performed with GraphPadPrism8.3 and ImageJ1.51. Comparing two groups was done via a T test. Comparing more than two groups was done via an ANOVA followed by a Tukey post hoc test. $P < 0.05$ was considered statistically significant.

Result

The expression of apelin/APJ was increased by apelin-13

We measured the mRNA expression of apelin in the lung tissue of rats in each group by RT—qPCR. The results showed that apelin mRNA in the group HV and the group NS increased compared with that in group C (Fig. 1. a). We also studied the effect of VILI on the level of the APJ receptor protein. Western blotting (Fig. 1. b, c) and immunofluorescence staining showed that the level of APJ receptor protein in damaged lungs was increased compared with that in control lungs. After apeilin-13 intervention, the mRNA expression of apelin and APJ receptor protein expression were further significantly upregulated in the group apelin-13. Our results indicate that apelin is expressed in normal lung tissue. MV leads to injury; thus, the expression of apelin is upregulated. The use of the receptor agonist apelin-13 further upregulated the mRNA expression of apelin and the protein expression of the APJ receptor in lung tissue, and apelin exerts its effect by binding to the APJ receptor.

Apelin-13 Ameliorates Vili

We next aimed to prove that apelin-13 can alleviate VILI. The protein levels in the BALF from the group HV and the group NS were significantly higher than those from the lung tissue W/D ratio, and the changes in lung tissue were observed with HE staining. The lung W/D ratio and BALF protein levels in the group HV and NS were significantly higher than those in the group C and apelin-13 (Fig. 2. a, b). After HE staining of all tissues, the alveolar structure in group C appeared normal under a light microscope, and edema of the perivascular space, infiltration of neutrophils in the alveoli and interstitium, alveolar hemorrhage and formation of a hyaline membrane were observed in the group HV and NS (Fig. 2. c). The severity of inflammatory cell infiltration and hemorrhage in the group apelin-13 was significantly lower than that in tthe group HV and NS, and the pathological morphology of lung tissue was significantly improved (Fig. 2. d).

Apelin-13 Alleviates Lung Inflammation In Vili Rats

We used ELISA to measure the IL-1β, IL-6 and TNF-α levels in BALF (Fig. 3. a, b, c) to determine the degree of inflammation in the lung tissues. Compared with the group C, the IL-1 β, IL-6 and TNF-α levels in bronchoalveolar lavage fluid of the group HV and NS were significantly increased. After apelin-13 treatment, the IL-1 β, IL-6 and TNF-α levels were significantly decreased, and the difference was statistically significant. In summary, these data indicate that apelin-13 can reduce the pulmonary inflammatory response of VILI rats.

Effect Of Apelin-13 On Apoptosis In Rat Lung Tissue

To study whether apelin-13 plays a role in VILI apoptosis, we used Western blotting to measure the expression of the antiapoptotic protein Bcl-2 and proapoptotic protein Bax and analyzed their expression ratio (Fig. 4. a). The results showed that compared with the group HV and NS, the intraperitoneal injection of apelin-13 significantly increased the ratio of Bcl-2/Bax in lung tissues. This shows that apelin-13 activates Bcl-2, promotes its binding to Bax, and thus inhibits the apoptosis process. In addition, the ratio of p-Akt/Akt in rat lung tissue was determined by Western blotting (Fig. 4. b). The results showed that compared with the group C, the ratio of p-Akt/Akt was significantly reduced. After apelin-13 intervention, the Akt pathway was significantly activated, and the ratio of p-Akt/Akt was increased. This indicates that activation of the Akt pathway is involved in the inhibition of apoptosis by apelin-13.

Apelin-13 Alleviates Oxidative Stress In The Lungs Of Vili Rats

MPO, as a marker of neutrophil infiltration, is also an index of enzymes related to oxidative damage. The change in its activity is positively related to the survival of neutrophils. In our experimental results, we found that the MPO activity in the group HV and NS was significantly higher than that in the group C. The results showed that oxidative stress injury occurred during VILI, and the antioxidant system was imbalanced. After apelin-13 intervention, the MPO activity in the group apelin-13 was significantly lower

than that in the group HV and NS, indicating that apelin-13 could slow the progression of oxidative stress (Fig. 5).

Discussion

VILI triggers a wide range of biological reactions, namely, biological injury, in which proinflammatory cytokines and injury-related signaling cascades are activated. TNF-α plays a very important role in the occurrence and development of VILI and is a key link in the activation and expansion of the inflammatory response[19]. The release of inflammatory factors, such as IL-1β and IL-6 is promoted by increasing capillary permeability, which leads to the cascading of inflammatory responses and exacerbates tissue damage and necrosis[20]. Increasing evidence shows that the apelin/APJ signaling pathway is closely related to the development of respiratory diseases. High concentrations of apelin mRNA and APJ mRNA were observed in rat lung tissues[21, 22]. Early studies reported that apelin and/or APJ receptors were upregulated by 2-fold during tissue injury[23–25]. Fan[17] found that the ARDS induced by OA was related to the upregulation and activation of the apelin/APJ pathway. In the ARDS model induced by OA and LPS, treatment with the receptor agonist apelin-13 reduced lung injury and improved oxygenation. The apelin/APJ signaling pathway is an endogenous anti-injury mechanism, and the receptor agonist apelin-13 can reduce the stimulation of apelin/APJ signaling, further playing a functional role and alleviating inflammation and the injury response. In this study, we found that in the rat VILI model with large tidal volume ventilation, after 4 hours of ventilation, the inflammatory response in rat lung tissues eventually led to pathological damage to lungs and the formation of pulmonary edema. After intraperitoneal administration of the receptor agonist apelin-13, the experimental data showed that in the intervention group, the expression of apelin and APJ receptor in lung tissue was significantly increased compared with that in group C, the group HV and NS. The levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α in bronchoalveolar lavage fluid during VILI was significantly decreased, and the inflammatory response of the rat VILI model was reduced or inhibited, indicating that the severity of VILI in rats was alleviated after treatment with apelin-13. The mechanism may be related to the anti-inflammatory effect. It has been verified that apelin and/or APJ receptors are upregulated during VILI tissue injury, but further activation of apelin-13 results in tissue protection. In summary, the apelin/APJ signaling pathway is also an important endogenous mechanism that protects against injury during VILI. The anti-inflammatory mechanism of apelin-13 may also be related to the inhibition of the classical NF-κB signaling pathway and NF-κB activation. Zhang[13] found that in an LPS-induced acute lung injury animal model, the administration of apelin-13 inhibited ROS formation, the NF-κB pathway and the activation of NLRP3 inflammasome in the lung. In addition to the NF- κB pathway, apelin-13 can activate the ERK1/2 pathway through PTX-sensitive G protein and inhibit forskolin-induced intracellular cAMP production, which may be involved in regulating different biological responses[26].

After mechanical ventilation, the expression of p-Akt in the lung tissue of rats decreased, suggesting that the Akt protein plays an important role in VILI and that apelin-13 promotes the phosphorylation of Akt in the lung tissue of VILI rats. It has been reported that after Akt is activated by apelin, it can inhibit the apoptosis of ischemic myocardial cells by regulating the protein expression of Bcl-2 and Bax[27], and

effectively reduce the infarct volume and brain edema caused by cerebral ischemia reperfusion injury[28]. Our research is consistent with previous research results, which prove that VILI significantly upregulates the level of the apoptosis-promoting protein Bax and downregulates the level of the antiapoptotic protein Bcl-2 in lung tissue, and this result is reversed after treatment with the receptor agonist apelin-13. Therefore, activation of the Akt signaling pathway may be involved in the antiapoptotic mechanism of apelin-13. In addition to anti-inflammatory effects and inhibition of apoptosis, the protective mechanism of apelin may also be related to antioxidant stress responses. MPO is a marker of neutrophil infiltration. After acute lung injury[17] and early brain injury[29] in subarachnoid hemorrhage (SAH), neutrophil infiltration increases, and the MPO activity level is high. Apelin-13 activation of APJ can reduce the degree of oxidative stress. Apelin significantly inhibits MPO activity and reverses the imbalance of the antioxidant system. In this study, we observed that the level of MPO activity after VILI was consistent with that after ALI and early SAH brain injury, and exogenous 13 could significantly reduce the level of MPO. This mechanism may also play a role in inhibiting apoptosis and oxidative stress through the PI3K and p38MAPK signaling pathways. Apolin overexpression inhibits apoptosis and oxidative stress in diabetic rats with myocardial ischemia—reperfusion injury (D-IRI) through the PI3K and p38MAPK signaling pathways, thereby protecting against D-IRI[30].

This study shows that apelin-13 (receptor agonist) plays a protective role in VILI. However, due to experimental limitations, this study did not explore the effects of a concentration gradient of apelin-13 or conduct further in vitro cell experiments. These two limitations need to be addressed in future research.

Conclusion

In summary, we believe that during VILI, the apelin/APJ axis plays an endogenous anti-injury role, and overexpression of apelin can significantly reduce the inflammatory response, apoptosis and oxidative stress in the lung tissues of VILI model rats. Therefore, apelin-13 may become a potential target for the treatment of VILI in the future.

Abbreviations

VILI: Ventilator-induced acute lung injury; MV: Mechanical ventilation; SD: Sprague–Dawley; SPF: Specific-pathogen-free; HE: Hematoxylin and eosin; IHC: Immunohistochemistry; TNF-α: Necrosis factor-α; ELISA: Enzyme-linked immunosorbent assay; IL-1β: interleukin-1β; IL-6: Interleukin-6; W/D: Wet/dry.

Declarations

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Not applicable.

Author's information

Not applicable.

Authors' contributions

S. L., S. H. and Z C., X. Z. provided experimental design and supervision, S L., L. Z. and Y S. wrote the main manuscript text, and W. J. prepared the resources and software. X. Z. provided capital acquisition and project management. All the authors reviewed the manuscript. The authors read and approved the manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Animal Protection and Use Committee of Guizhou Medical University (No. 2001132). All procedures involving animals comply with the National Institutes of Health's (National Institutes of Health) (NIH publication No. 85–23, 2011) Guidelines for the Care and Use of Experimental Animals. The euthanasia method conforms to the recognized veterinary best practice. All methods were carried out in accordance with AVMA guidelines for animal euthanasia and ARRIVE guidelines.

Consent for publication

Not applicable (animal study).

Competing interest

The authors declare no competing interests.

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Figures

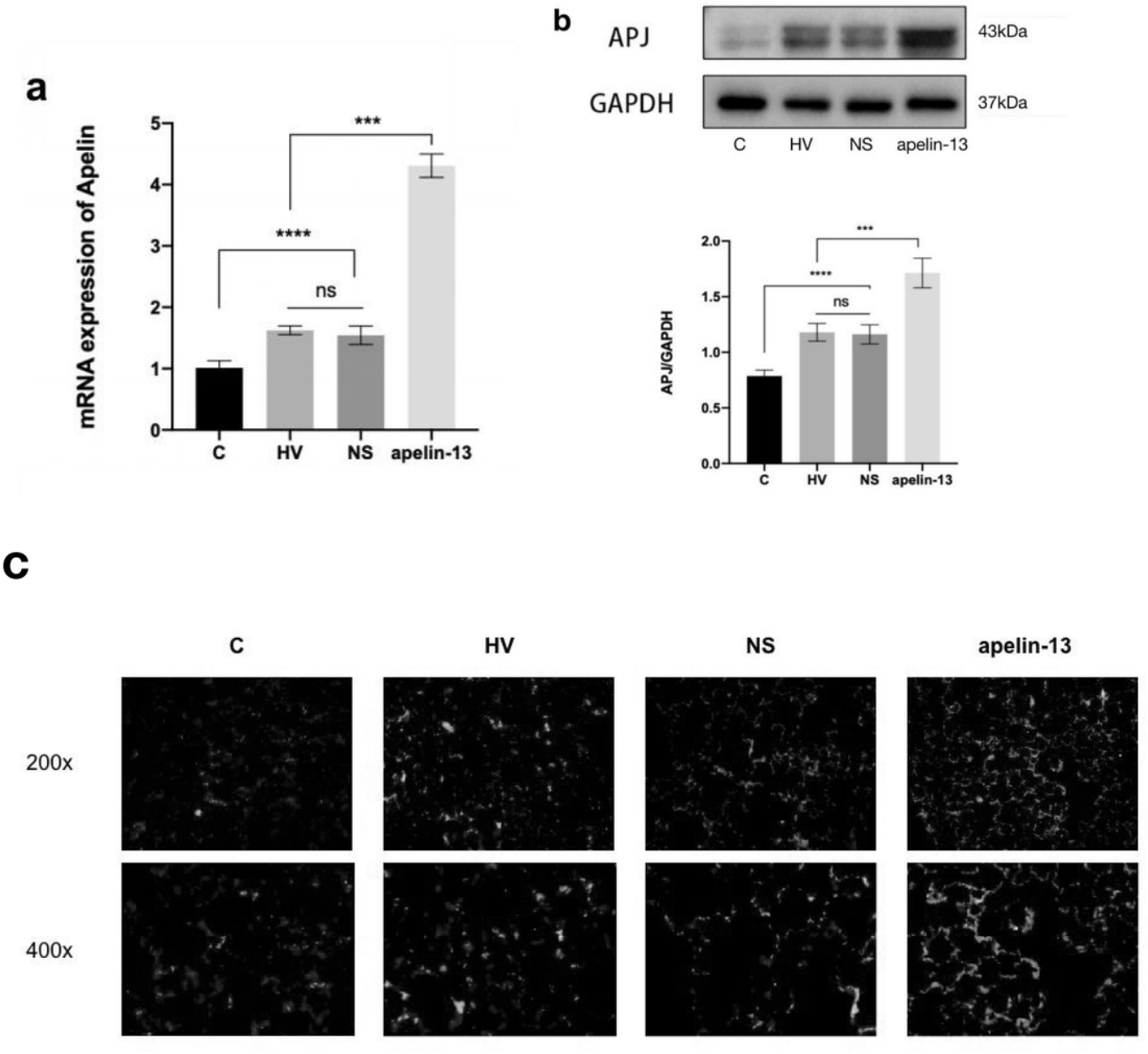

Figure 1

VILI is associated with increased the mRNA expression of apelin and APJ receptors. (a) RT-qPCR results showed that VILI resulted in increased expression of the mRNA expression of apelin, and the expression

of the group apelin-13 was significantly increased, ****P<0.05 ***P<0.0001 ns>0.05. (b, c) Western blot and Immunofluorescence staining showed that VILI increased the expression of APJ receptor protein, while the expression of APJ receptor protein in the group apelin-13 was further up-regulated. The ratio of APJ/GAPDH, ****P<0.05 ***P<0.05 ns>0.05.

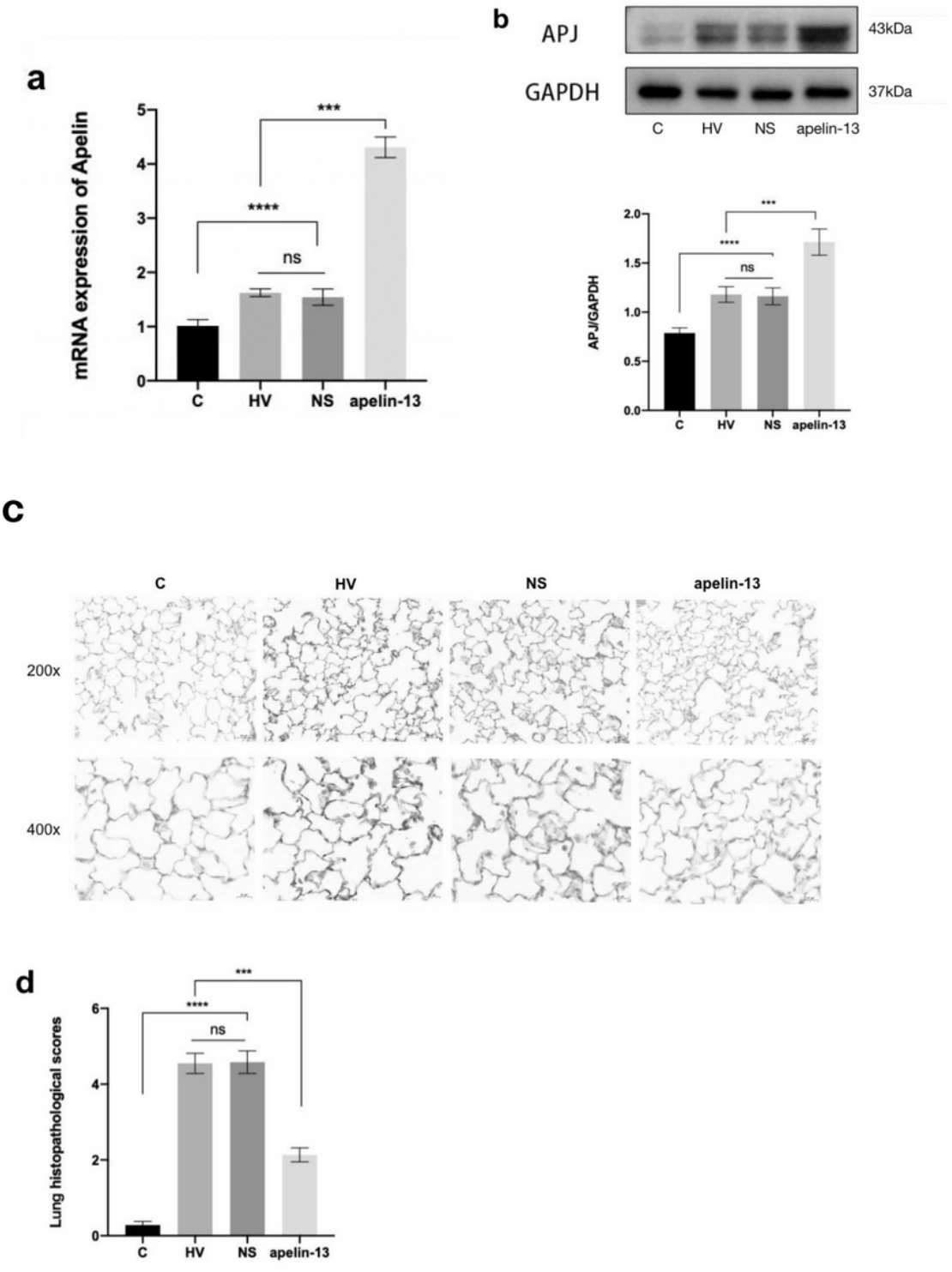

Figure 2

(a) the levels of wet to dry weight ratio (W/D), ****P<0.0001 ***P<0.005 ns>0.05 (b) the protein level in BALF, ****P<0.0001 ***P<0.0001 ns>0.05 representative appearances and photomicrographs of hematoxylineosin stained lung sections (magnification×200 and×400): severe lung injury occurred in the group HV and NS and the lung injury in the Group apeilin-13 was significantly smaller than that in the group HV and NS. Lung histology was characterized by perivascular edema, interstitial and intra-alveolar leukocyte infiltration, and marked heterogeneity in alveolar inflation. (d) histological sub-scores in experimental groups: lung injury scores were determined based on leukocyte infiltration, exudative edema, hemorrhage, and alveolar wall thickness, ****P<0.0001 ***P<0.0001 ns>0.05.

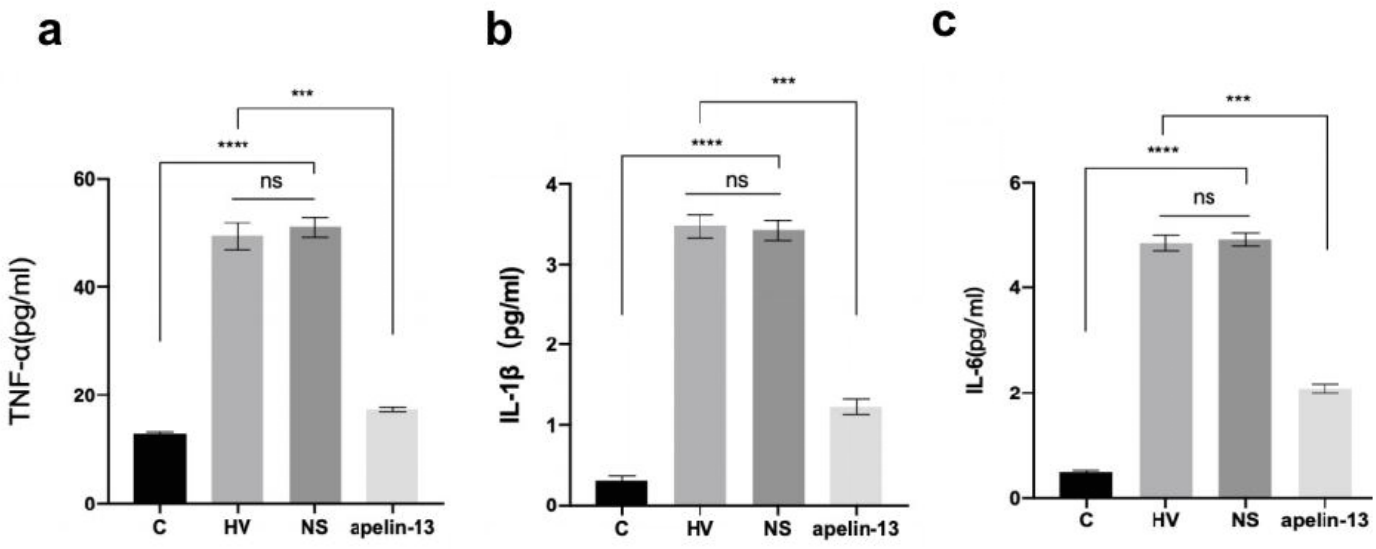

Figure 3

(a) levels of TNF-a in broncho-alveolar lavage fluid; (b) levels of IL-1β in broncho-alveolar lavage fluid; (c) levels of IL-6 in broncho-alveolar lavage fluid; the expression of TNF-a and IL-6 in the group C was significantly lower than that in the group HV and NS, after apelin-13 intervention, the expression of inflammatory factors decreased significantly. ****P<0.0001 ***P<0.0001 ns>0.05.

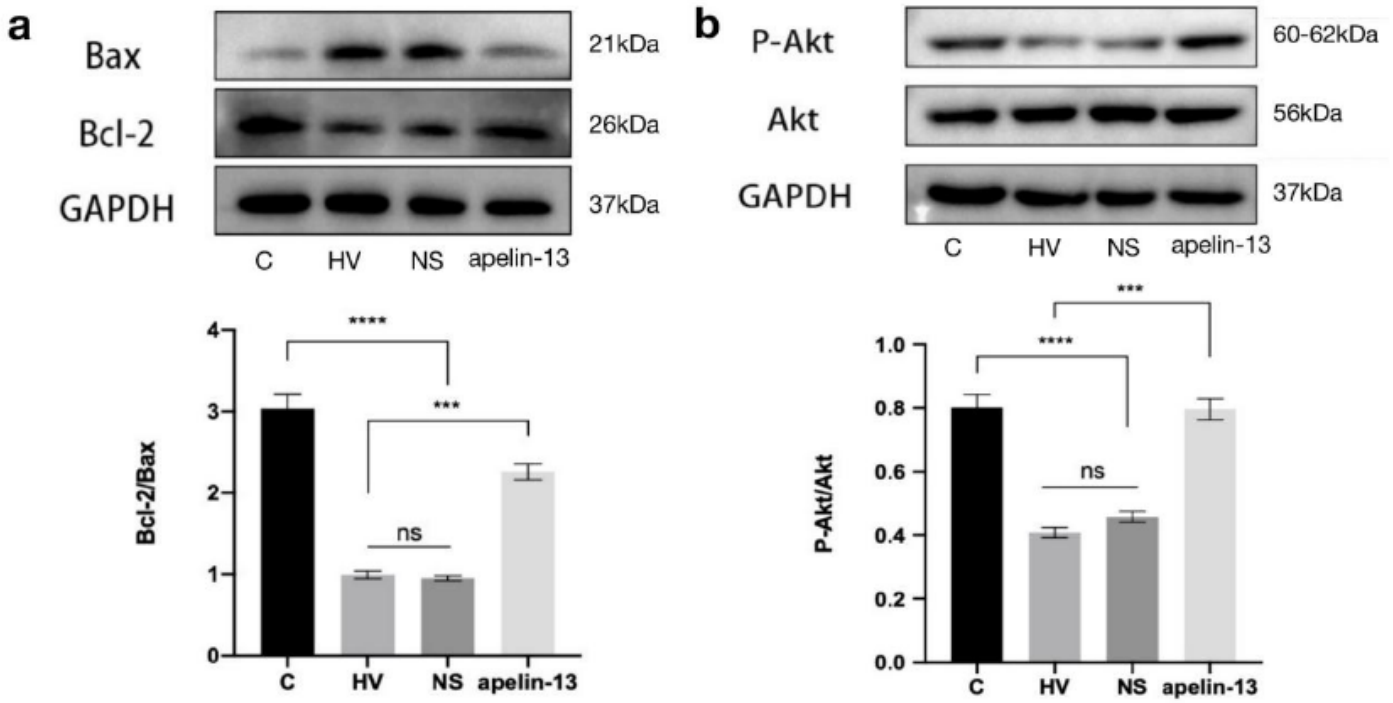

Figure 4

(a) Western blotting to measure the expression of the antiapoptotic protein Bcl-2 and proapoptotic protein Bax and their expression ratio, ****P<0.0001 ***P<0.0001 ns>0.05. (b) The expression of p-Akt and Akt in rat lung tissue, and the ratio of p-Akt/Akt in lung tissue of each group, ****P<0.0001 ***P<0.0001 ns>0.05.

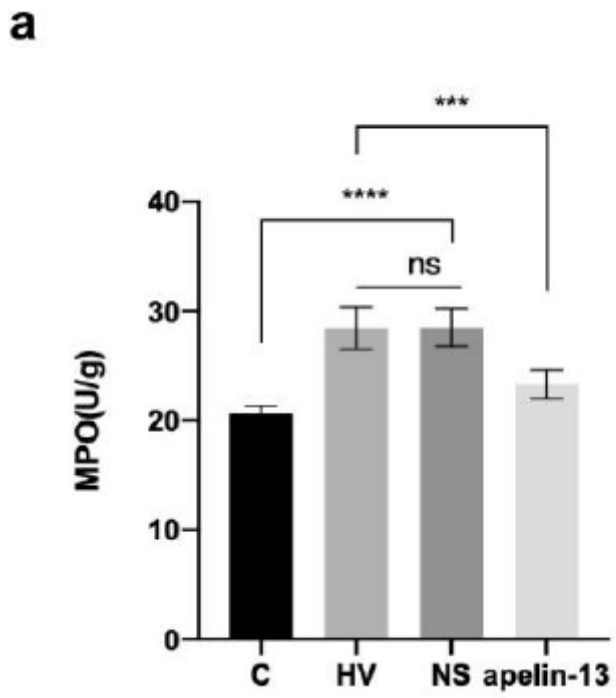

Figure 5

(a) MPO activity in lung tissues of each group, ****P<0.0001 ***P<0.0001 ns>0.05.