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Original Article

Role of NLRP3 inflammasome in the obesity paradox of rats with ventilator-induced lung injury

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Abstract: The objective of this research is to determine the action of the nucleotide oligomerization domain-like receptors containing pyrin domain 3 (NLRP3) inflammasome in the obesity paradox of rats with ventilator-induced lung injury (VILI). Twenty-four (average weight: 250 ± 20 g) pathogen-free adult male Sprague Dawley (SD) rats were randomly classified as group A and group B, while twelve obese (420 ± 20 g) pathogen-free adult male SD rats were classified as group C. Three groups received open tracheotomies after anesthesia. Group A then underwent spontaneous breathing for 4 h after endotracheal intubation, and group B and group C were then connected to a ventilator to administer high tidal volume ventilation (V_T=30 mL/kg) for 4 h; All groups then underwent open tracheotomy. Blood, bronchoalveolar lavage fluid (BALF), and lung tissue were obtained for related testing at the end of ventilation. The lung injury score, the wet/dry weight (W/D) ratio, inflammatory content, and cytokine level in BALF and serum were lower for group C than for group B. For instance, compared with group B, the level of PaO_2 in serum separated from blood was higher than in group C, while the lungs of group B were highly enriched with NLRP3, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and cysteinyl aspartate specific proteinase 1 (caspase-1). Causal mechanisms for the obesity paradox in VILI might partly be related to the NLRP3 inflammasome.

Keywords: NLRP3 inflammasome, ventilator-induced lung injury, obesity paradox, inflammation

Introduction

Obesity is a risk factor for adverse prognoses for many diseases [1]. However, the inflammation level brought about by mechanical ventilation in the lungs is not significantly higher for obese patients than for patients with normal weight. Instead, such injuries show a good prognosis, so the phenomenon of the "obesity paradox" may exist in lung injury from mechanical ventilation [2, 3]. This clinical phenomenon existed in various diseases, such as sepsis [4], renal tumors [5], and chronic heart failure [6].

But, there is controversy about these theories whether these findings imply that obesity actually has a beneficial effect or if they are due to some confounding factors. Some researchers observed an impaired host neutrophil CXCR2 expression in obese mice which significantly attenuated respiratory infection [7]. Similarly, fewer lung impairments and anti-inflammatory effect have been appeared in obese rats [8]. As far as we know, the exact relationship between obesity paradox and ventilator-induced lung injury (VILI) is unclear. The nucleotide oligomerization domain-like receptors containing pyrin domain 3 (NLRP3) inflammasome plays a role in regulating the occurrence of lung injury in a variety of complex ways [9]; however, whether the influences of obesity on markers of NLRP3 inflammasome could explain the theory obesity paradox in VILI has not been determined.

Materials and methods

Experimental animals

Adult Wistar rats aged 6-8 weeks weighing 250 ± 20 g from the Pengyue Experimental Animals Center, Shandong, China were used for this experiment. The operating procedures and processing methods for animals have been adopted by the Animal Experimental Medical Ethics

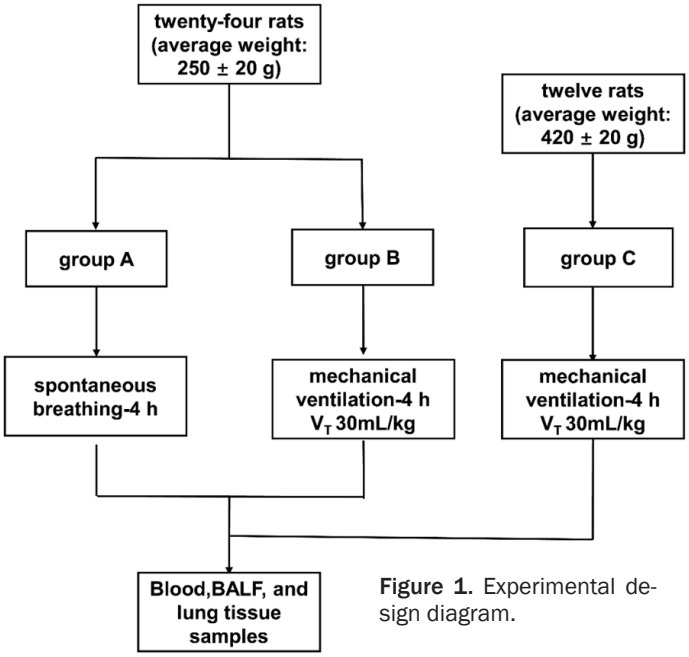

Figure 1. Experimental design diagram.

Committee of Qingdao Municipal Hospital. All rats were given free access to tap water and animal food are housed in a regular environment (room temperature: 20-25°C, room humidity: 40-60%).

Experimental groups

Thirty-six pathogen-free adult male SD rats were used in the experiment. Twenty-four rats with average weight were randomly allocated into two groups, A and B; group C was comprised of twelve obese rats. The groups were treated as follows: (1) group A was anesthetized with an injection of 40 mg/kg sodium pentobarbital and then was intubated endotracheally, all group members were then able to breathe spontaneously without mechanical ventilation; (2) group B was anesthetized with an injection of 40 mg/kg sodium pentobarbital and was then intubated endotracheally, all group members received ventilation for 4 h with a tidal volume of 30 mL/kg and a ventilatory frequency of 60 times/min; and (3) group C was given the same treatment as group B. In all groups, the anesthetic, sodium pentobarbital, was additionally administered as needed (**Figure 1**).

Tissue preparation

Blood samples were taken from the femoral artery for blood gas analysis from rats on mechanical ventilation, while blood samples from the other rats were collected and instantly placed in a pre-cooled (4°C) centrifuge run at 1500 rpm/min for 10 min. ELISA kits purchased from eBiosciences in Vienna, Austria were performed to detect serum inflammatory factors interleukin-18 (IL-18) and interleukin-1β (IL-1β).

The rats were subsequently sacrificed by arterial bleeding, and their lungs were speedily isolated. Subsequently, 2 mL of phosphate-buffered saline was injected into each left lung to collect bronchoalveolar lavage fluid (BALF) through an endotracheal tube, and the process was repeated three times. About 4 mL of BALF was centrifuged at 3000 r/min for 10 min to collect the plasma for detecting inflammatory factors IL-18 and IL-1β. These were measured by ELISA kits purchased from eBiosciences in Vienna, Austria according to the manufacturer's instructions.

The upper lung on the right side was removed for testing the wet/dry weight (W/D) ratio. The right middle lung was collected for routine hematoxylin and eosin (H&E) staining, then the lung injury score was obtained based on microscopic changes. Then, the right lower lung was homogenized for the expression of NLRP3, recruitment domain (ASC), and cysteinyl aspartate specific proteinase 1 (caspase-1).

H&E staining

In conclusion, the right upper lung samples were placed in a 10% formalin solution and then treated with paraffin embedding. The embedded lung was subsequently sliced into a 5 μm segment and observed under a light microscope at 200× magnification. We examined the tissues, assessing the pathologic changes using defined criteria from previous studies [10]. A score of 0 to 4 was used to describe the severity of lung injury, with 0 representing minimal damage; 1 mild damage; 2 moderate damage; 3 severe damage; and 4 maximum damage.

ELISA assays

According to the procedures that accompanied the ELISA kits, the IL-1β, IL-18 concentrations in

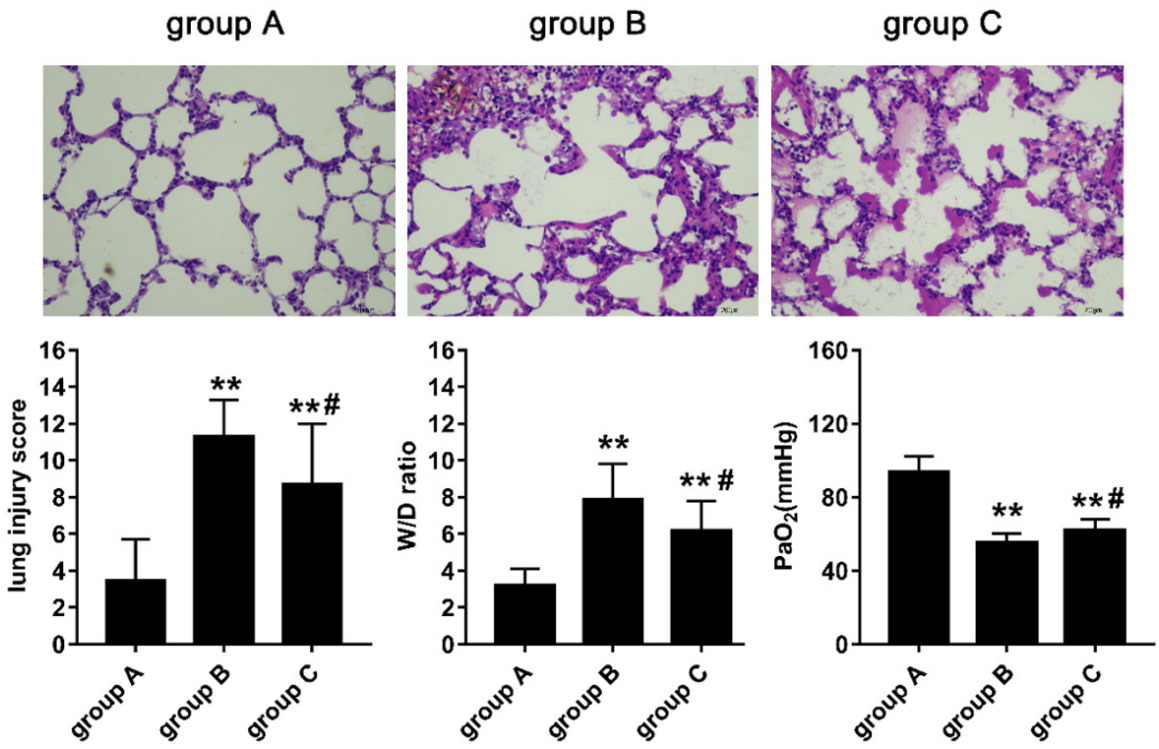

Figure 2. Representative pathologic and histologic analysis of the lungs (200×), the lung injury score, the W/D ratio, and level of PaO_2 in blood gas analysis. $^{**}P<0.01$ group B and group C versus group A; $^{\#}P<0.05$ group C versus group B.

serum and IL-1β and IL-18 concentrations in BALF were analyzed.

Western blotting

Membranes were blocked with a solution of 5% skim milk and phosphate-buffered saline/Tween20 for forty minutes with an equal amount of protein (50-60 μg) in each group, which was separated onto 10% SDS-PAGE gels, then were transferred onto PVDF membranes. The membranes were treated with proper antibodies: anti-NLRP3 (1:1000; Abcam, Cambridge, UK), anti-ASC (1:1000; Abcam, Cambridge, UK), anti-Caspase-1 (1:1000; Abcam, Cambridge, UK), and β-actin (1:5000; Easybio, Shenzhen, China). We prepared bound antibodies by using labeled secondary antibodies (goat anti-rabbit, bioeasy, Shenzhen, China). Eventually, each protein was used as ECL chromogenic substrate (Millipore, USA). At the same time, antibodies for NLRP3, caspase-1, and ASC were utilized in the present study.

NLRP3 mRNA expression

The Trnzol reagent prepared by Tiangen Biotech, Beijing, was purchased, and reverse transcription was performed with the Prime Script™ RT reagent kit to extract RNA from the right lower lung tissues of the rats. The cDNA was utilized for real-time PCR used with the QuantStudio3 software made by Genecopoeia, USA. A SYBR Green master mix was bought from TaKaRa, Japan for the experiment. The process of PCR as follows: 95°C for 30 s, 40 cycles at 95°C for 5 s and 60°C for 34 s. According to the $2^{-\Delta\Delta CT}$ method, the melting curve is determined by the thermal denaturation and the relative amount of mRNA. The relative mRNA levels were normalized to β-actin control. The following primers were applied: NLRP3 forward primer, 5'-AGAAGGACCAGCCAGAGTGGAATGA-3'; reverse primer 5'-TTTTTACAAATCGAGATGCGGGAGA-3'; β-actin forward primer, 5'-TCCTGTGGCATCCATGAAACT-3'; reverse primer; 5'-GAAGCATTTGCGGTGCACGAT-3'.

Statistical analyses

Statistical analyses were performed using SPSS 19.0. The experiment revealed the mean ± standard deviation. Multiple groups applied One-way ANOVA procedures to make one comparison, and multiple comparisons with the

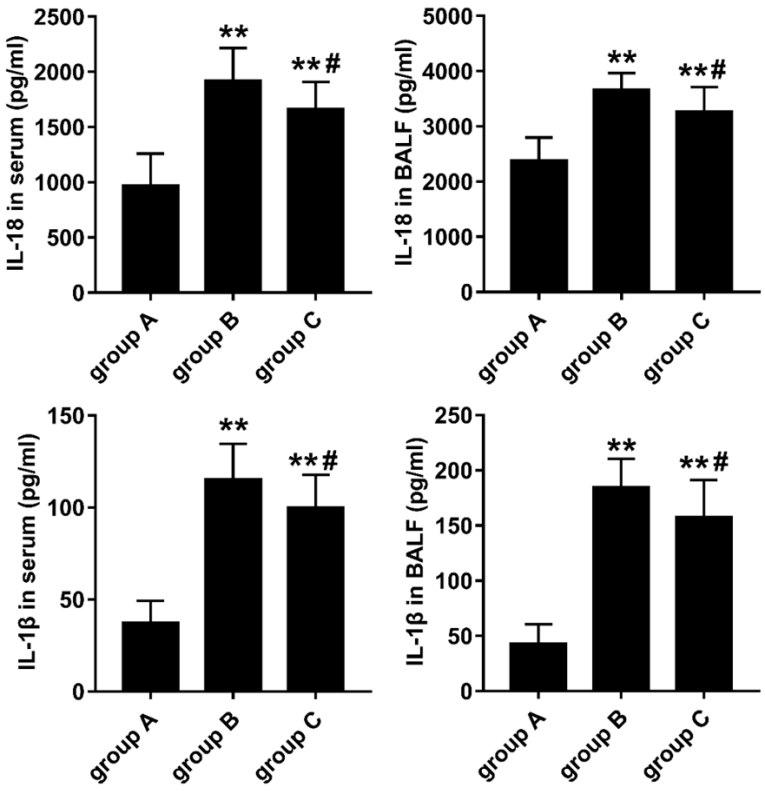

Figure 3. The concentration of IL-18 and IL-1β in serum and BALF. $^{**}P<0.01$ for group B and group C versus group A; $^{\#}P<0.05$ for group C versus group B.

Bonferroni test. Differences were considered significant at $P<0.05$.

Results

H&E staining, lung injury score, W/D ratio, and PaO_2

The conspicuous features of the lung tissues in group B included severe alveolar edema, to include: destroyed pulmonary architecture, thickened alveolar septa, hyaline membrane formation, alveolar hemorrhage, and infiltration of inflammatory cells. In contrast, the features mentioned above were less obvious in group C. Besides, different lung injury score, the W/D ratio, and PaO_2 from different groups were in keeping with their histopathologic results (**Figure 2**).

IL-18 and IL-1β levels in serum and BALF

To determine whether lung inflammation was altered in group C as compared to group B, IL-18 and IL-1β in serum and BALF were measured. IL-18 and IL-1β levels in serum and BALF were all increased, with a decrease in group C (**Figure 3**).

NLRP3, ASC, and caspase-1 levels in lung tissue

NLRP3, ASC, and caspase-1 protein levels were tested in all groups. Compared with group A, all of these experimental indicators in groups B and C were increased after 4 h of ventilation, implying that the NLRP3 inflammasome could be triggered in this procedure. Results showed that group B exhibited a higher protein expression of NLRP3, ASC, and caspase-1 in comparison with group C by the end of the ventilation period. The mRNA level of NLRP3, the core protein in NLRP3 inflammasome, was lower in group C, compared with group A and group B. (**Figure 4**).

Signaling pathway diagram

This study also illustrated the model of NLRP3 inflammasome activation in the obesity paradox of rats with VILI (**Figure 5**).

Discussion

In this study, an attenuating effect of obesity on ventilator-induced lung injury (VILI) as well as pathologic changes was observed. Levels of IL-18 and IL-1β in serum and BALF, and PaO_2 support this diminished obesity-related response. Moreover, involvement of the NLRP3 inflammasome in the regulation of the obesity paradox occurring in VILI was demonstrated. First, it was found that circulating levels of NLRP3, caspase-1, and ASC were raised in group B, in that of the non-obese rats. Second, obese rats developed lighter VILI with reduced expression level of NLRP3, caspase-1, and ASC. Third, it was proposed that changes in NLRP3, caspase-1, and ASC protein expression explained the altered vulnerability of obese subjects to VILI. In short, these findings hinted that the NLRP3 inflammasome was essential to the development of VILI in obese rats and that

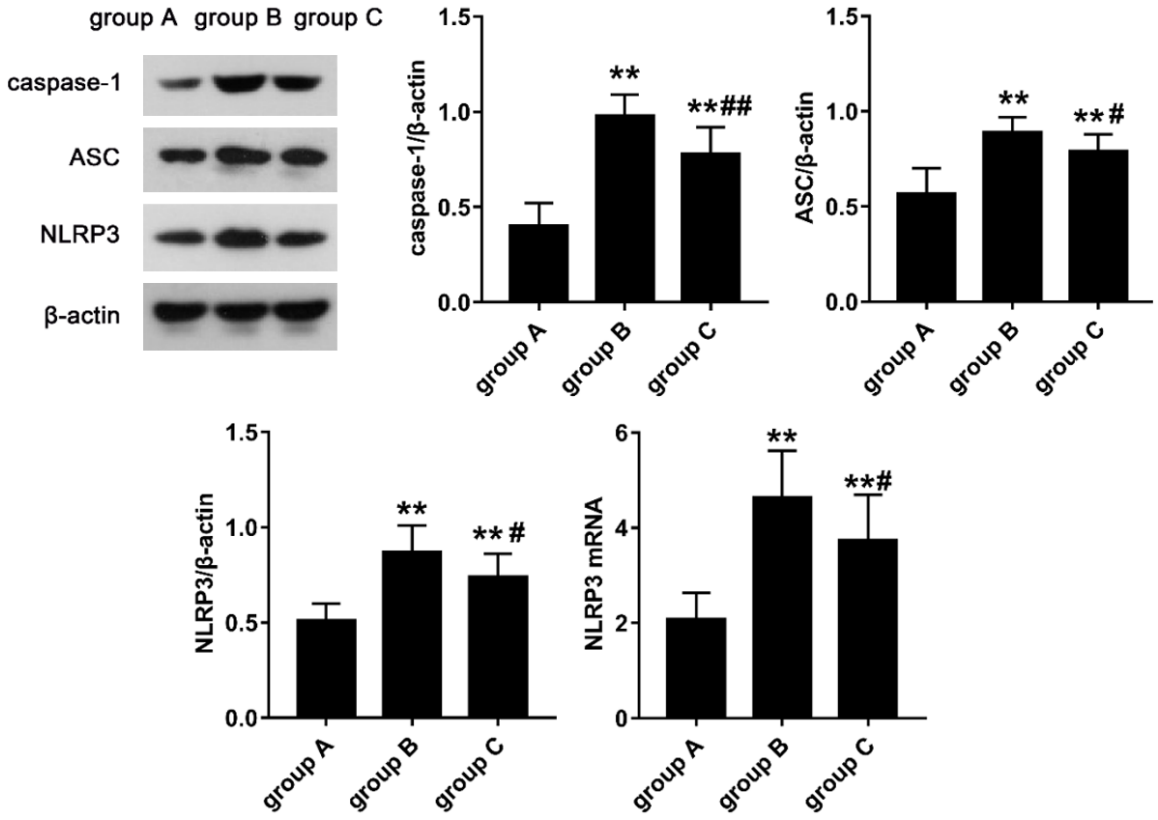

Figure 4. Protein levels of caspase-1, ASC, and NLRP3 in lung tissue and the mRNA level of NLRP3. Data were expressed as the ratio of caspase-1, ASC, and NLRP3 protein to β-actin. To precisely assess NLRP3 mRNA level, β-actin gene expression level was used as an internal reference gene. $^{**}P<0.01$ for group B and group C versus group A; $^{\#}P<0.05$ for group C versus group B, $^{\#\#}P<0.01$ for group C versus group B.

reduced NLRP3, caspase-1, and ASC might mediate the process of the obesity paradox theory specific to VILI.

Lung pathologic damage including inflammatory cells infiltration, alveolar edema, and pulmonary interstitial rupture 4 h after high tidal volume ventilation (V_T=30 mL/kg) was consistent with a previous report related to this model [10]. Research has shown that increases of IL-18 and IL-1β with other inflammatory cytokines were determined in lung tissue after 4 h mechanical ventilation in rats [11], suggesting that the model of VILI was successful.

The increased expression of NLRP3, caspase-1, and ASC response in VILI suggested the promotion of further inflammatory cytokine production in VILI. Yang et al. [12] and Liu et al. [13] reported that the NLRP3 inflammasome could stimulate LPS-induced IL-18 and IL-1β transcription through the NF-κB pathway. Notably, involvement of the NLRP3 inflammasome, linked to inflammation regulation, is associated with many other diseases [14]. In the role of the cytosolic pattern recognition receptor, the NLRP3 inflammasome can activate downstream caspase-1, accompanied by subsequent processing of the cleaved inactive IL-1β and IL-18 precursors, which is essential for some acute lung injury models [15-17]. In this study, after ventilation, group C showed suppressed spontaneous NLRP3 inflammasome activation with decreased caspase-1, IL-1β, and IL-18 levels. Therefore, the NLRP3 inflammasome pathway might represent a novel candidate marker to understand the theory of the "obesity paradox".

Overweight acts as an important mediator of chronic low-grade inflammation-mediated atherosclerosis in response to cardiovascular diseases that can impair health. However, a study found that the incidence of acute lung injury in hospitalized patients after surgery or the probability of tracheal intubation was lower than that for non-obese patients [18]. Similarly, others showed an increase in secretory leukocyte

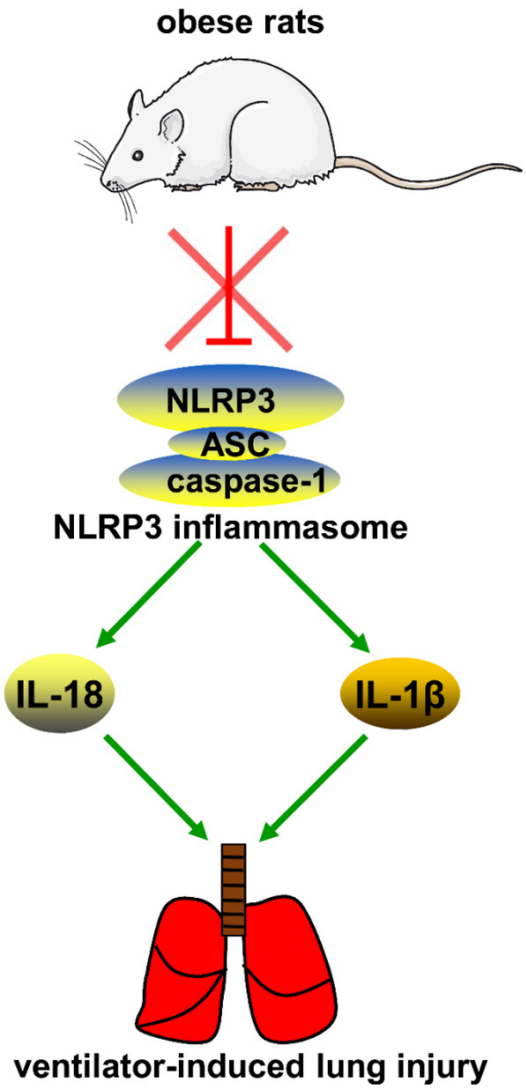

Figure 5. Model of NLRP3 inflammasome activation in obesity paradox of rats with VILI.

protease inhibitor expression levels in obese rats with a lighter inflammatory response of acute lung injury [19]. The beneficial effect of higher weight on all-cause diseases has been called "obesity paradox". Many studies have discovered that the benefits of obesity existed in different health conditions [20]. For example, the survival paradox existed in obese patients with chronic heart failure and the mechanism might be related to reduced vasopressin [21]. Obesity related clinically to lower subsequent mortality due to acute lung injury [3]. Previous work has hinted that obesity tends to diminish hyperoxic and ozone-induced lung injury [22]. In comparison with the previous-mentioned study, recent extensive cohort studies have discussed sleep apnea in general [23]. These findings proved that obesity has a protective effect, and that weight plays a critical role in altering the acute pulmonary inflammatory response. Given that obesity plays a complex part in immunity and inflammation that can have a two-sided effect on long-term or short-term results, and so, for better comprehension of the "obesity paradox", we observed activation of the NLRP3 inflammasome involved in VILI. The data examined showed that obese rats were protected against the pathologic outcome of over tidal volume ventilation and had improved lung functional respiratory and gas exchange by ameliorating oxygenation.

Increased NLRP3 inflammasome activity in obese rats in this research is consistent with the emerging conception of the "obesity paradox", which also showed the potential mechanism of NLRP3 inflammasome function in it. Our study proposed that suppression of the NLRP3 inflammasome might be a crucial mechanism that mitigates VILI in obese rats. In rats under high-calorie diet conditions, the NLRP3 inflammasome has been shown to mitigate lung injury induced by mechanical ventilation and it has an important role in the ventilation-induced "obesity paradox" which needed worth considering.

In conclusion, the NLRP3 inflammasome could be a focal mechanism of the "obesity paradox" in VILI. The NLRP3 inflammasome pathway does appear to take part in suppressing lung injury in obese rats caused by ventilation.

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Disclosure of conflict of interest

None.

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