Original Article

Pycnogenol, a compound isolated from the bark of Pinus maritime mill, attenuates ventilator-induced lung injury through inhibiting NF-κB-mediated inflammatory response

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Abstract: Background: During mechanical ventilation, high end-inspiratory lung volume results in a permeability type pulmonary oedema, called ventilator-induced lung injury (VILI). The pathophysiology of ventilator-induced lung injury involves multiple mechanisms, such as excessive inflammation. And pycnogenol is a mixture of flavonoid compounds extracted from pine tree bark that have anti-inflammatory activity. Objective: We investigated the effects of pycnogenol on ventilator-induced lung injury in rats. Methods: Rats were orally administrated with pycnogenol once (30 mg/kg) 2 days before lung injury induction with mechanical ventilation, then the rats were divided into three groups: lung-protective ventilation (LV group, n = 20), injurious ventilation (HV group, n = 20), HV + pycnogenol group (HV + Pyc group, n = 20). Lung specimens and the bronchoalveolar lavage fluid (BALF) were isolated for histopathological examinations and biochemical analyses. Results: Pretreatment with pycnogenol could markedly decrease lung wet/dry ratio, lower myeloperoxidase (MPO) activity and total protein concentration and reduce the production of TNF-α, IL-6, IL-1β and MIP-2 in the BALF in ventilator-induced lung injury rats. Additionally, pycnogenol improved the histology of the lung and significantly inhibited the phosphorylation of NF-κB p65 and the degradation of IκB-α. Conclusion: Pycnogenol treatment could attenuate ventilator-induced lung injury in rats, at least in part, through its ability to reduce the production of inflammatory cytokines via inhibiting the activation of NF-κB, indicating it as a potential therapeutic candidate for ventilator-induced lung injury.

Keywords: Pycnogenol, ventilator-induced ALI, inflammation, flavonoid, NF-κB pathway

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are clinical syndromes (hypoxemia, decreased lung compliance, and bilateral pulmonary infiltrates) that have multifactorial etiologies either from direct or from indirect injury to the lung [1, 2]. Histopathologically, there is an initial acute exudative phase involving an alveolar-capillary leak in conjunction with leukocyte extravasation. This is followed by a fibro-proliferative phase involving the precipitation of the alveolar proteins with hyaline membrane formation, persistent inflammation, and proliferation of alveolar epithelia and mesenchymal cells. Its incidence is common, it is likely to exist outside the intensive care setting and therefore is a condition relevant to all clinicians.

Despite advances in critical care, the mortality rate in patients with acute respiratory distress syndrome (ARDS) remains high at values exceeding 30%. Furthermore, most patients who die do so from multiple-system organ failure (MSOF) rather than from hypoxia [3]. One hypothesis that has recently been advanced to explain this is that mechanical ventilation per se may be responsible not only for worsening the underlying ALI but, by a number of mecha-
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Mechanisms, may also lead to the development of a systemic inflammatory response syndrome (SIRS) [4-6] and MSOF [7, 8]. Additionally, mechanical ventilation has been part of basic life support for several decades. During mechanical ventilation, high end-inspiratory lung volume (whether it be because of large tidal volume (VT) and/or high levels of positive end-expiratory pressure) results in a permeability type pulmonary oedema, called ventilator-induced lung injury (VILI). The pathogenesis of ventilator-induced lung injury (VILI) relates in part to the activation and release of host inflammatory mediators and cytokines after mechanical injury to alveolar epithelial and endothelial cells [9, 10].

Previous studies reported that, macrophages are key orchestrators of the inflammatory and repair responses in the lung tissue, and the diversity of their functions indicated by their polarized states and distinct subpopulations and localization in the lung. They contribute to both the induction and resolution phases of acute lung injury in clinical. Macrophage influx into the lung tissues occurs during the induction and resolution phases of lung inflammation, and previous studies demonstrated that the increased recruitment of macrophages to lung and the enhanced production of pro-inflammatory cytokines by macrophage were associated with attenuated lung injury [11].

Pycnogenol, a compound isolated from the bark of Pinus maritime Mill (Pinaceae), was found to be effective in reducing both the production of IL-1β and the expression of IL-1β mRNA in RAW264.7 cells, an effect dependent on interference with the transcription factors NFκB [12]. These data suggested that pycnogenol might have a therapeutic effect on ventilator-induced lung injury through inhibiting the production of inflammatory cytokines during lung injury.

Based on the potent anti-inflammatory effect of pycnogenol, we hypothesized that whether pycnogenol could protect the rat from ventilator-induced lung injury via affecting the production...
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of inflammatory cytokines. So we pre-treated ventilator-induced lung injury rats with pycnogenol or vehicles via oral administration. Our finding indicated that the treatment of pycnogenol attenuated the lung injury induced by ventilation. Additionally, the administration of pycnogenol improved the histology of the damaged lung tissue and reduced the wet/dry ratio, protein concentration in BALF, compared with the vehicle control group. Meanwhile, we also found that pycnogenol treatment reduced the infiltration of neutrophils and MPO activity, in addition, the expression of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β and MIP-2, was suppressed in pycnogenol treated ALI rats, compared with control group. In summary, our data indicated that pycnogenol has a therapeutic effect on ventilator-induced lung injury through its ability to reduce the production of inflammatory cytokines via inhibiting the activation of NF-κB.

Materials and methods

Animals

Sixty specific pathogen-free female Sprague-Dawley rats weighing 240-290 g were purchased from the SLRC Laboratory (Shanghai, China). The animals were housed under barrier conditions and kept at 22-25°C with a 12-hour light/dark cycle. The rats were randomized into three groups: lung-protective ventilation group (LV group, n = 20), lung injurious ventilation group (HV group, n = 20), HV + pycnogenol-treated group (HV + Pyc group, n = 20). Rats were sacrificed at indicated time points after ALI induction and different administration.

Mechanical ventilation

Rats received pycnogenol (2.5 mg/kg, dissolved in phosphate buffered saline (PBS)) via oral treatment 2 days before ALI induction. Then, rats were anesthetized by intraperitoneal injection of thiopental (37 mg/kg). The dose that we used ensures a profound anesthesia for at least 4 h. A tracheostomy was performed, and each animal was injected with succinylcholine (5 mg/kg) via the dorsal penile vein, after which the animal was ventilated with a rodent volume ventilator (Harvard Apparatus, Ealing, Les Ulis, France). Two ventilation modalities were used, for 2 h each, as follows: (1) lung-protective control, with low VT ventilation (7 ml/kg VT and 3 cm H2O positive end-expiratory pressure) and PEEP 5 cm H2O; (2) injurious ventilation, with high VT ventilation (18 ml/kg VT and 10 cm H2O positive end-expiratory pressure) and PEEP 10 cm H2O. Ventilation settings were maintained for 2 h. At the end of ventilation, the rats were sacrificed, and the lungs were harvested for further analysis.

Figure 2. Pycnogenol treatment reduced the MPO activity and neutrophils infiltration in differentially treated ALI rats. The myeloperoxidase (MPO) activity (A) in the lung tissues and the neutrophils count (B) in BALF of differentially treated ALI rats were determined. Data are expressed as mean ± SEM of the values of 10 rats of each group. *P < 0.05 compared with the vehicle-treated group rats (HV group). ALI: acute lung injury; LV: lung-protective ventilation; HV: injurious ventilation; HV + Pyc: HV + pycnogenol-treated group; BALF: bronchoalveolar lavage fluid; MPO: myeloperoxidase.
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Figure 3. The production of pro-inflammatory cytokines in BALF of differentially treated ALI rats. A. The level of TNF-α in BALF of rats with indicated treatment was measured by ELISA. B. The level of IL-6 in BALF of rats with indicated treatment was measured by ELISA. C. The level of IL-1β in BALF of rats with indicated treatment was measured by ELISA. D. The level of MIP-2 in BALF of rats with indicated treatment was measured by ELISA. Data are expressed as mean ± SEM of the values of 10 rats of each group. *P < 0.05 compared with the vehicle-treated group rats (HV group). ALI: acute lung injury; LV: lung-protective ventilation; HV: injurious ventilation; HV + Pyc: HV + pycnogenol-treated group; BALF: bronchoalveolar lavage fluid.

pressure [PEEP], 40 breaths/min) (LV group) and (2) an lung injurious strategy, using a high VT and no PEEP (42 ml/kg VT, zero end-expiratory volume [ZEEP], 40 breaths/min) (HV group). Then rats were killed by an intravenous injection of thiopental at the end of the mechanical ventilation period, the thorax was opened, and the blood was sampled by cardiac puncture. Simultaneously, three BAL procedures were performed, each with 2 ml of normal saline. The retrieved fluid and the blood were centrifuged (2,000 g, for 10 min), and the supernatant and plasma were stored for further processing. The survival after mechanical ventilation was assessed and the cumulative survival curve was depicted using the Kaplan-Meier method.

Histopathology

Lung specimens were fixed in 4% paraformaldehyde (PH 7.6, Sigma Chemicals) overnight at room temperature [13]. After wash with tap water for 10 min, specimens were dehydrated through a graded series of ethanol (75% for 60 min, 80% for 60 min, 95% for 60 min, and
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100% for 2 hr.) and embedded in paraffin, sliced at 5 μm. Paraffin sections were stained with hematoxylin-eosin (H&E) for histopathological analysis.

In order to evaluate the severity of the lung injury, we used a semi-quantitative histological index of quantitative assessment (IQA) for lung injury. The assessments were done in a single blinded fashion. 10 sections were randomly selected from each group of rats, and 10 fields from each section were examined by microscopy (40 ×). A pathologist who was blinded to this study evaluated all of the sections. The average value of the lung injury obtained were considered a semi-quantitative histological IQA of lung injury.

Measurement of pulmonary edema

The right lungs were removed and the wet weights were obtained. Then, lung tissues were weighted again 3 days after drying at 80°C. The wet/dry ratio was calculated as follows: W/D ratio  (wet weight-dry weight)/dry weight [14].

Protein concentration in BALF

Bronchoalveolar lavage was performed as previously described [15]. In short, after euthanasia, trachea was exposed and intubated with a tracheal cannula. Bronchoalveolar lavage was performed by flushing the airways and lungs repeatedly with 1 mL cold saline for three times. The pooled BALF was collected on ice and centrifuged at 500 g for 5 min at 4°C. Afterwards, the supernatant was stored at -20°C for further assay. Protein concentrations in the cell-free BALF were determined using Bio-Rad protein assay reagents. A standard curve was generated in the same fashion using bovine serum albumin.

Neutrophil count in BALF and measurement of MPO activity

BALF was collected from the mice in differentially treated groups 24 h after injury to obtain total cell count and percentage of neutrophils. MPO activity in the homogenized lung tissue was measured as described by Gray et al [16]. The MPO concentration was determined using Figure 4. Effect of pycnogenol treatment on IκB-α degradation and NF-κB phosphorylation in lung tissues of ventilator-induced ALI rats. Whole tissue extracts were subjected to SDS-PAGE Western blot analysis using antibodies for phosphorylated NF-κB P65, IκB-α and β-actin. The ratio of immunointensity between the phosphorylation of NF-κB P65, IκB-α and β-actin were calculated. Values are expressed as means + SEM (n = 3 in each group). *P < 0.05 compared with the vehicle-treated group rats (HV group). ALI: acute lung injury; LV: lung-protective ventilation; HV: injurious ventilation; HV + Pyc: HV + pycnogenol-treated group; BALF: bronchoalveolar lavage fluid.

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a MPO ELISA kit (Bluegene, China). Briefly, the lung tissue was homogenized and centrifuged at 15000 rpm for 20 min at 4°C. The supernatants and standard sample were added into a microtiter plate (100 ul/well) precoated with a murine anti-MPO mAb. After incubation for 1 h at 37°C, the plate was washed for 6 times followed by addition of the substrate and stop solution, and the optical density (OD) at 450 nm was measured using a microplate reader. All the samples were assayed in triplicate.

Cytokines in BALF

For determining the levels of inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and MIP-2, in the BALF. The levels of inflammatory cytokines were measured by ELISA according to the manufacturer’s instructions (R&D System, Minneapolis, MN, USA). Briefly, ELISA plates were coated overnight with 5 µg/ml capture antibody to either TNF-α, IL-6, IL-1β, and MIP-2. The plates were washed and blocked for 1 hour with PBS containing 1% bovine serum albumin. Various dilutions of samples with appropriate standards were added to the wells and incubated for 2 hours, followed by washing and incubation in appropriate biotinylated secondary antibody for 1 hour. Wells were washed and streptavidin peroxidase was added for 30 minutes followed by washing and incubation in OPD substrate (Sigma Aldrich) for 10 minutes. The reaction was stopped by addition of 1.5 mol/L sulfuric acid. Absorbance was measured at 490 nm using a Molecular Devices plate reader (Sunnyvale, CA). The detection limit for all cytokines ranged between 30 and 120 pg/ml. All measurements were performed in duplicate.

Western blotting analysis of lung tissue

The lung tissues were homogenized, washed with PBS, incubated in lysis buffer, and added with a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) to obtain extracts of lung proteins. The protein concentration in the supernatant was determined by Bradford assay, and the samples were loaded to 10% SDS-PAGE gels and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dried milk for 1 h at room temperature to reduce non-specific binding, washing with PBS containing 0.1% Tween-20, and then probed with the indicated antibodies including phosphorylated NF-κB P65, β-actin, IκB.

Quantitative realtime PCR assay

For RNA extraction from differentially treated mice macrophages, cells were lysed in 1 ml Trizol reagent and total RNA was isolated with an Rneasy Mini Kit (Qiagen, Valencia, CA). Then 1 mg of total RNA was reverse-transcribed into cDNA with a Takara RNA PCR kit (Takara, China). RT-PCR detection was performed with SYBR Green Premix Ex Taq (Takara, Japan) in a sequence detection system (ABI7900fast realtime detection system). The following primer pairs were used in the analysis: rat GAPDH, 5’-
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GACATGCCGCCTGGAGAAAC-3’ (forward) and 5’-AGCCCAGGATGCCCTTTAGT-3’ (reverse); rat IL-6, 5’-GCCCTTCAGGAACAGCTATGA-3’; rat IL-1β, 5’-CACCTCTCAAGCAGAGCACAG (forward) and 5’-GGGTTCCATGGTGAAGTCAAC-3’ (reverse); rat TNF-α, 5’-AAATGGGCTCCCTCTCATCAGTTC (forward) and 5’-TCTGCTTGGTGGTTTGC-TACGAC-3’ (reverse).

**Isolation of peritoneal macrophages**

Macrophages were isolated from the peritoneal cavity of differentially treated ventilator-induced ALI rats. Briefly, rats were sacrificed by neck break after CO₂ euthanasia. To collect rat macrophages, 5-10 ml of pre-cold PBS was injected into peritoneal cavity and flushed twice. The cell suspension was centrifuged and washed once with ice-cold PBS at 4°C. Then the cells were lysed for further experiments.

**Statistical analysis**

All the data were analyzed using SPSS13.0 software and expressed as Means ± SD. Significant differences were assessed by one-way analysis of variance (ANOVA) followed by Fisher protected least significant difference test. A probability value of less than 0.05 was considered to indicate a statistical significance.

**Results**

**Pycnogenol treatment ameliorated ventilator-induced morphologic lesions in lung tissue**

Ventilator-induced lung injury animal model is a commonly used experimental model for investigating the molecular mechanisms and seeking new therapeutic drugs in acute lung injury during mechanical ventilation in clinical. As we know, inflammatory responses play an essential role in the lung injury caused by mechanical ventilation. In addition, previous studies demonstrated that pycnogenol could effectively reduce the production of IL-1β and inhibit the expression of IL-1β mRNA in vitro [12]. So, we hypothesized that if pycnogenol could alleviate the ventilator-induced lung injury by inhibiting the inflammatory responses. To determine the effect of pycnogenol treatment on ventilator-induced ALI, we administrated ventilator-induced ALI rats with pycnogenol via oral treatment 2 days before ALI induction, PBS as the control. H&E staining of the lung sections showed increased thickness of alveolar walls and neutrophil infiltration in ventilator-induced ALI rats lungs. Compared with HV + vehicle (Figure 1A) or LV (Figure 1B) treated lungs, pycnogenol treated lungs (Figure 1C) exhibited less structural damage and neutrophil sequestration.

Next, to further assess the therapeutic effect of pycnogenol on ventilator-induced lung injury, we scored the injury in the lung of rats from differentially treated group (Figure 1D). We found that the average index of the pycnogenol treated group was dramatically lower than that of the HV + vehicle treated group rats (P < 0.05). Afterwards, we determined the effect of pycnogenol on the lung wet/dry ratio and protein concentration in the BALF of differentially treated rats. Our results showed that the pycnogenol treated ALI rats had a significantly lower wet/dry ratio (Figure 1E) and protein concentration (Figure 1F) compared to the vehicle-treated group. These results indicated that pycnogenol could alleviate ventilator-induced lung injury.

**Pycnogenol treatment reduced the activity of MPO and the infiltration of neutrophils during ventilator-induced lung injury**

As we know, infiltration of activated neutrophils into the lung tissue is a critical component of
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the inflammatory response in acute lung injury. To further examine the effect of pycnogenol on the inflammatory cells infiltration, we determined the level of the MPO activity, a reliable marker of neutrophil infiltration, in lung homogenates of the differentially treated rats. The pycnogenol treatment reduced the activity of MPO in the lung homogenates, compared with the vehicle treated group (Figure 2A). Then we measured the counts of neutrophils in the BALF of differentially treated rats, in consistent with the MPO activity, the pycnogenol treatment also reduced the number of neutrophils in the BALF compared with the vehicle treated group (Figure 2B). These results demonstrated that pycnogenol could suppress the ventilator-induced lung injury in rats.

Pycnogenol treatment inhibited the production of pro-inflammatory cytokines in ventilator-induced ALI rats BALF

After ALI was induced in rats, pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β, and MIP-2, will be elevated [17]. Therefore, neutrophils, monocytes and lymphocytes will be recruited into alveolar space and cause severe lung inflammation. So we detected the production of pro-inflammatory cytokines in differentially treated rats BALF. As Figure 3 shows, the elevation of pro-inflammatory cytokines caused by ventilator-induced ALI was inhibited by the administration of pycnogenol, such as TNF-α (Figure 3A), IL-6 (Figure 3B), IL-1β (Figure 3C), and MIP-2 (Figure 3D). These data were in agreement with the above results concerning the therapeutic effect and degree of lung injury.

Pycnogenol treatment inhibited degradation of IκB-α and activation of NF-κB in ventilator-induced ALI rats

As we know, NF-κB signaling play a vital role in the initiation and regulation of cellular inflammatory response to multiple stimuli. But, in normal conditions, IκB-α could inhibit the activation of NF-κB through binding to NF-κB. In inflammatory conditions, the IκB-α is phosphorylated and degraded, NF-κB is released, phosphorylated and translocated into the nucleus, resulting in the increased expressions of pro-inflammatory cytokines [18]. So, to illuminate the detail mechanisms in which pycnogenol reduced lung inflammation resolution, we examined the effect of pycnogenol on the degradation of IκB-α and the activation of NF-κB in differentially treated ALI rats lungs by western blot analysis. As shown in Figure 4, we found that pycnogenol treatment dramatically inhibited the degradation of IκB-α, compared with the vehicle treated group rats (Figure 4A). Meanwhile, the phosphorylation of NF-κB was also suppressed in pycnogenol treated ALI rats lungs compared with vehicle treated rats (Figure 4B). These data was consistent with the production of inflammatory cytokines in differentially treated rats BALF.

Pycnogenol treatment inhibited the up-regulation of inflammatory cytokines in macrophages of ventilator-induced ALI rats

Inflammation is propagated from the initial site of tissue injury or infection by the diffusion of small molecules that act locally and more distantly. Previous studies demonstrated that macrophages are a major source of inflammatory cytokines in the air spaces, such as IL-1β [19]. To assess the effect of pycnogenol on the expression of inflammatory cytokines in macrophages, we isolated the macrophages from the differentially treated ALI rats, and then we detected the expression of inflammatory cytokines with RT-PCR. We found that, pycnogenol treatment could inhibit the up-regulation of inflammatory cytokines, including IL-1β (Figure 5A), IL-6 (Figure 5B), and TNF-α (Figure 5C), in macrophages from ALI rats. These results indicated that pycnogenol could attenuate ventilator-induced lung injury through inhibiting the expression of inflammatory cytokines in macrophages.

Pycnogenol treatment improved survival after ventilator-induced lung injury

To evaluate the long-term effect of pycnogenol in our ventilator-induced ALI rats, we compared the survival rate between differentially treated rats. As Figure 6 shows, the survival rate was dramatically improved by pycnogenol treatment, compared with these vehicle treatment (Figure 6).

Discussion

The results of this study demonstrate a suppressive role for pycnogenol in mediating the lung inflammatory response induced by ventilator-induced lung injury. Pycnogenol could reduce the production of pro-inflammatory
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cytokines in ventilator-induced ALI rats, through inhibiting the degradation of IκB-α and the activation of NF-κB in macrophages.

Acute lung injury (ALI) is a clinical disease marked by respiratory failure due to disruption of the epithelial and endothelial barrier, flooding of the alveolar compartment with protein-rich fluid and recruitment of inflammatory cells into the alveolar space. Mechanical ventilation, while necessary to support patients with ALI or ARDS, has been implicated as a mediator of lung injury (VILI) during ALI and ARDS [20]. In fact, more recent studies have shown that patients with ALI or ARDS who were ventilated with a lung-protective strategy (i.e., low peak pressure/stretch and tidal volume) had a reduced mortality [21-23]. Like early ALI and ARDS, VILI is characterized by non-cardiogenic edema, neutrophil sequestration, alveolar hemorrhage, and increased alveolar wall thickness [24-26]. Traditional high-peak pressure/stretch mechanical ventilation may cause overdistension and shearing of the alveolar units, leading to deformation and injury of the cellular components of the alveolar-capillary wall, and thus the release of inflammatory cytokines.

As we know, within the inflammatory process, a delicate balance exists between the potential for tissue destruction and mechanisms of protective immune defense and tissue repair. The acute lung injury (ALI) or adult respiratory distress syndrome (ARDS) has been characterized as a disease state, in which the inflammatory balance is shifted towards tissue injury. The excessive inflammatory reactions and, in particular, the activation of NF-κB in inflammatory cells have been implicated in ALI/ARDS pathogenesis [27, 28].

In our study, we found the treatment of pycnogenol improved the histology of the lung compared with the vehicle-treated control group (Figure 1B). Meanwhile, we also observed that pycnogenol treatment reduced the lung wet/dry ratio (Figure 1E), protein concentration in BALF (Figure 1F), MPO activity in lung homogenates (Figure 2A) and the neutrophils count in BALF (Figure 2B) in ventilator-induced lung injury rats. Moreover, the production of inflammatory cytokines in the BALF of differentially treated ALI rats, including TNF-α (Figure 3A), IL-6 (Figure 3B), IL-1β (Figure 3C), and MIP-2 (Figure 3D), were decreased compared with the vehicle control treated group rats. This results indicated that pycnogenol have the a therapeutic effect on ventilator-induced lung injury in rats.

Previous data reported that pycnogenol could inhibit the degradation of IκB-α and suppressed the activation of NF-κB in macrophages in vitro [12]. So we hypothesized that if pycnogenol could attenuate ventilator-induced ALI through inhibiting the activation of NF-κB in macrophages in vivo. Then we isolated macrophages from differentially treated ALI rats and determined the degradation of IκB-α and the activation of NF-κB using western blot assay, and measured the expression of inflammatory cytokines in macrophages. We found pycnogenol treatment could inhibit the degradation of IκB-α (Figure 4A) and the phosphorylation of NF-κB p65 (Figure 4B), we also observed the expression of inflammatory cytokines was decreased by pycnogenol administration (Figure 5). These suggested pycnogenol suppress the inflammatory responses via mediating the activation of NF-κB signaling pathway.

In summary, our study demonstrated the pycnogenol could inhibit the ventilator-induced ALI. As a inflammatory mediated factor, pycnogenol administration prolonged the survival time of injurious ventilator-induced ALI rats (Figure 6). Pycnogenol might use as a drug for treatment of ventilator-induced ALI through inhibiting NF-κB mediated inflammatory reactions in macrophages in ALI rats.

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Disclosure of conflict of interest

None.

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