Melatonin alleviates acute lung injury through inhibiting the NLRP3 inflammasome

Abstract: Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are clinically severe respiratory disorders, and there are currently no Food and Drug Administration-approved drug therapies. Melatonin is a well-known anti-inflammatory molecule, which has proven to be effective in ALI induced by many conditions. Emerging studies suggest that the NLRP3 inflammasome plays a critical role during ALI. How melatonin directly blocks activation of the NLRP3 inflammasome in ALI remains unclear. In this study, using an LPS-induced ALI mouse model, we found intratracheal (i.t.) administration of melatonin markedly reduced the pulmonary injury and decreased the infiltration of macrophages and neutrophils into lung. During ALI, the NLRP3 inflammasome is significantly activated with a large amount of IL-1β and the activated caspase-1 occurring in the lung. Melatonin inhibits the activation of the NLRP3 inflammasome by both suppressing the release of extracellular histones and directly blocking histone-induced NLRP3 inflammasome activation. Notably, i.t. route of melatonin administration opens a more efficient therapeutic approach for treating ALI.

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are clinically severe respiratory disorders, each characterized by pulmonary edema, intrapulmonary hemorrhage, and severely impaired gas exchange [1]. With an annual incidence of greater than 80 per 100,000 people and a mortality of 30–40% in the USA, ALI remains a leading cause of morbidity and mortality [2]. Many conditions, such as sepsis, pancreatitis, multiple trauma, pneumonia, aspiration of gastric contents, pulmonary contusion, or inhalation of injurious gases can lead to ALI [3]. In spite of considerable effort, there is still no Food and Drug Administration-approved treatment for ALI/ARDS [3].

The NLRP3 inflammasome is a multi-protein complex of the innate immune system, consisting of the NOD-like receptor (NLR) NLRP3, the adaptor protein ASC and caspase-1 [4]. Activation of the NLRP3 inflammasome requires two signals: the first signal is mediated by microbial molecules or endogenous cytokines, which upregulate the expression of NLRP3 and pro-IL-1β through activating the NF-κB signaling pathway. The second signal is mediated by various damage associated molecular patterns (DAMPs), leading to the assembly of a multi-protein complex [5]. Once activated, caspase-1 proteolytically cleaves the cytokine precursors pro-interleukin-1β (pro-IL-1β) and pro-IL-18, resulting in the maturation and release of IL-1β and IL-18 [5]. Whereas activation of the NLRP3 inflammasome can help host defense against invading bacteria and pathogens, excessive activation of the inflammasome can lead to inflammation-associated tissue injury [6]. Emerging studies suggest that the NLRP3 inflammasome plays a critical role during ALI caused by various infectious etiologies, such as Staphylococcus aureus [7], Pseudomonas aeruginosa [8], Mycobacterium tuberculosis [9] and influenza A virus [10]. Some noninfectious etiologies such as exposure to asbestos and silica can also activate the NLRP3 inflammasome [11]. In some animal models of ALI, host-derived DAMPs such as uric acid and extracellular histones serve the second signal to activate the NLRP3 inflammasome [12, 13]. Even without the specificity to NLRP3 activity, there have been clinically available therapeutics for NLRP3-driven inflammation, which are antibodies targeting IL-1β signaling, namely anakinra, rilonacept, and canakinumab [14]. More antagonists specifically targeting NLRP3-inflammasome-associated pathways are under development [15].

Melatonin (N-acetyl-5-methoxytryptamine), is synthesized by the pineal gland in mammals, and also by other
nonendocrine organs such as the Harderian gland, skin, gut and the immune system [16]. Experimental studies have shown that melatonin is an antioxidant and anti-inflammatory molecule under the conditions of exacerbated immune responses [17, 18]. The anti-inflammatory effects of melatonin act through blocking the NF-κB signaling pathway, and have been studied at length [16, 19, 20]. Recently, Ortiz et al. and also Garcia et al. [21, 22] from the same group extensively demonstrated that melatonin could inhibit the NLRP3 inflammasome pathway in the radiation-induced oral mucusitis and cecal ligation induced-sepsis. However, whether and how melatonin can inhibit the activation signal of the NLRP3 inflammasome in ALI still need to be investigated. In this study, we demonstrate that intratracheally administration of melatonin improves LPS-induced ALI through inhibiting activation of the NLRP3 inflammasome. This inhibition is mediated not only through reduction of the release of extracellular histones in lung, but also through direct inhibition of extracellular histones-induced activation of the NLRP3 inflammasome.

Materials and methods

Animals

Seven-week-old male C57BL/6 mice were purchased from Weitonglihua, Beijing (under the license of Charles River, Hollister, CA, USA). The mice were maintained on a 12 hr light/dark cycle under specific pathogen-free conditions. All procedures were approved by the Ethics Committee for Animal Experimentation of the China Agricultural University (SKLAB-2014-15).

Experimental design: in vivo mouse studies

At the age of 8 wk, the animals were divided into four groups: the control group, the ALI + vehicle group, the ALI + melatonin (30 mg/kg; intraperitoneally, i.p.) group and the ALI + melatonin (30 mg/kg; intratracheally, i.t.) group. LPS-induced ALI was performed according to the method previously described with slight modifications [23]. Briefly, mice were anesthetized by isoflurane inhalation, then mice received 25 μg LPS (Escherichia coli O111:B4; Sigma, St. Louis, MO, USA) (i.t.) in 50 μL sterile saline. Control mice received sterile saline. In the ALI + melatonin (i.p.) group, mice were given melatonin (30 mg/kg) i.p. daily for 3 days before ALI induction, on the fourth day mice were given one dose of melatonin 1 hr before ALI induction, and another dose of melatonin was given 24 hr later. In the ALI + melatonin (30 mg/kg, i.t.) group, mice were anesthetized by isoflurane inhalation for i.t. delivery of melatonin (30 mg/kg) at 1 hr after ALI induction, and another dose of melatonin was given 24 hr later. Body weight and temperature were measured every 24 hr. The mice were sacrificed at 48 hr after LPS challenge.

Histology

The lung tissues were harvested 48 hr after LPS treatment. Lung tissues were fixed with 4% paraformaldehyde for 48 hr, then dehydrated, embedded in paraffin, and sliced. Then, the slices were stained with hematoxylin and eosin (H&E) and were viewed under the light microscope. The scores of lung injury were blindly evaluated as previously described [24]. Each histological characteristic was scored from 0 (normal) to 5 (maximal) according to the sum of the score for damage level such as the number of infiltration cells, alveolar wall thickening, patchy hemorrhage and interstitial edema.

Micro-computed tomography experiment

At the time 48 hr post LPS treatment, mice were anesthetized by i.p. administration of sodium pentobarbital and then transferred to an in vivo Micro-computed tomography (Micro-CT) imaging system (Quantum FX, Caliper). CT images were acquired with a resolution of 24 mm FOV, and an exposure time of 4.5 min. Two-dimensional tomographic images were obtained with imaging software (SimpleViewer). Three-dimensional reconstruction was performed by Analyze 11.0 software (AnalyzeDirect. Inc., Overland Park, KS, USA).

BALF

BALF (bronchoalveolar lavage fluid) was harvested via injection and retraction of 1 mL PBS-EDTA three times. The collected BALF was then centrifuged. The cell-free supernatant was aliquoted and stored at −80°C in preparation of histone and cytokine detection. The pelleted cells in the BALF were resuspended in redistilled water for erythrocyte lysis and then 10X PBS was added to recover normal osmotic pressure. Total cell numbers were counted under a hemacytometer, then cells were pelleted and resuspended with FBS for Diff-Quick staining (NOVON, Beijing, China). The percentages of macrophages and neutrophils were determined through counting at least 200 cells for each slide.

Peritoneal macrophages preparation and inflammasome activation assays

Mouse peritoneal macrophages were isolated, as previous described [25]. Mice were primed by i.p. injection of 3 mL of 4% thioglycollate medium (Sigma). Four days later, peritoneal macrophages were harvested through i.p. injection and retraction of 8 mL cold PBS. The peritoneal macrophages were cultured in RPMI 1640 media supplemented with 0.1% BSA at 37°C for 2 hr, then were washed to remove the nonadherent cells. The purity of the macrophages was determined by ink phagocytic assay [26]. The percentage of macrophages was more than 95%. The remaining adherent cells were primed with LPS (1 μg/mL) for 4 hr to activate the NF-κB signaling pathway and induce inflammasome priming. Cells were washed by PBS for three times. The medium was removed and replaced with RPMI 1640 media containing ethanol, melatonin (0.1 mM, 1 mM, 5 mM) for 30 min. Cells were then stimulated with histones (Roche, Germany) for 45 min. Supernatants and cells were collected for ELISA and immunoblot analysis.
ELISA
IL-1β and TNF-α levels were determined by ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. The level of histones was determined by ELISA (Roche, Mannheim, Germany), while purified mixed calf thymus histones were used to generate the standard curves [27].

Mitochondrial reactive oxygen species detection
For histones-induced mitochondrial reactive oxygen species (mt-ROS) generation, peritoneal macrophages were primed with LPS for 4 hr, then incubated with or without 5 mM melatonin for 30 min, stimulated with histones (50 µg/mL) for 30 min, then stained with MitoSox (2.5 µM; Invitrogen, Carlsbad, CA, USA) for 10 min at 37°C. Cells were harvested for FACS analysis and data was analyzed by FlowJo software (Tree Star, Ashland, OR, USA).

Western blotting
Total protein was extracted using RIPA buffer. The protein content of supernatants was concentrated using Amicon Ultra-4 Centrifugal Filter Devices (Millipore, Billerica, MA, USA). Aliquots of protein extract were electrophoresed in a 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred onto polyvinylidenefluoride membrane (Millipore) for 3 hr with transfer buffer (25 mM Tris, 192 mM glycine, and 20% [v/v] methanol). Membranes were blocked with 2% dried milk in TBS-T (20 mM Tris, 500 mM NaCl, 0.05% Tween 20) and incubated with antibodies against β-actin (sc-47778, 1:4000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-1β (12426S, 1:1000; Cell Signaling Technology, Beverly, MA, USA), or Caspase-1 (AG-20B-0042, 1:1000; Adipogen, San Diego, CA, USA) for 1 hr. The final exposure was completed using enzymatic chemiluminescence (Amersham Pharmacia, London, UK). The film was scanned, and the band density was quantified using Image J software, version 1.34 (NIH).

Statistical analysis
Statistical analysis was performed using the SPSS 17.0.1 Package (SPSS Inc., Michigan, IL, USA). For comparative studies, student’s t-test (unpaired) and one-way ANOVA followed by Dunnett’s post hoc tests were used respectively. P values < 0.05 were considered significant. These data are expressed as the mean ± S.E.M. (standard error of mean). All of the graphs were generated with GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Results
To determine the appropriate administration route of melatonin for ALI, melatonin was administrated locally (i.t.) or systemically (i.p) with the dose of 30 mg/kg. Intra-tracheal instillation of melatonin was more effective for improving acute lung injury pathology (Fig. 1). Thus, intra-tracheal delivery of melatonin was used in all subsequent experiments. As shown in Fig 1A–D, the lung morphology of mice in the ALI group had changed greatly, including alveolar infiltration by leukocytes, alveolar wall thickening, patchy hemorrhage and interstitial edema. However, these histological changes were ameliorated by melatonin, in which i.t. delivery of melatonin was more profound. The results of lung damage scores implied the therapeutic effect was statistical significant (Fig. 1E). The body weight of mice in the ALI group was decreased about 15% at 24 hr after ALI induction, and still decreased until 20% at 48 hr after ALI induction. The body temperature was also decreased greatly during ALI. However, administration of melatonin reduced the loss of body temperature and body weight, while the therapeutic effect of i.t. delivery of melatonin was more significant (Fig. 1F–G).

Micro-CT technology is a powerful tool to study the pathological changes of mouse lungs, with its rapid, non-invasive, serial in vivo imaging capability [28]. The mean lung density and aerated lung volume quantified from repetitive in vivo micro-CT scan are two important biomarkers to evaluate lung injury including fibrotic disease, cancer, inflammation, and infection [29]. The high-resolution tomograms obtained exhibit that the lung density increased during ALI, reflecting that the lungs were filled with foreign substances (Fig. 2A–C). However, with administration of melatonin, the density of the lungs is similar to the control. Three-dimensional remodeling images vividly exhibit the region and degree of lung damage (Fig. 2D–F). The aerated lung volume was calculated. We found a significant loss of the aerated lung volume in the ALI group, while the aerated lung volume in the melatonin-treated group is similar to the control (Fig. 2G).

The cells in the BALF were stained by Diff-Quick and counted. The number of total cells including macrophage, neutrophil was increased greatly during ALI. Melatonin decreased the total cell number while had no effect on cell proportion of macrophages and neutrophils (Fig. 3).

Recent data implied the NLRP3 inflammasome as essential for the development of experimental ALI [7–13]. The products of inflammasome activation (IL-1β and IL-18) were important for exacerbating ALI [30]. In fact, IL-1β is a central proinflammatory cytokine for initiation of inflammation, transient expression of IL-1β alone could induce acute lung injury [31]. To determine whether melatonin could inhibit activation of the NLRP3 inflammasome, we detected the level of caspase-1 and IL-1β in the lung homogenate and BALF at the early phase of ALI (8 hr after LPS instillation). We detected high level of mature IL-1β (IL-1β p17) and caspase-1 p20 (an autoprocessed fragment of caspase-1) in the lung homogenate of ALI mice. However, the levels of IL-1β and caspase-1 p20 were decreased when melatonin was administrated (Fig. 4). The levels of IL-1β and caspase-1 p20 were consistent with the results in BALF (Fig. 5A,B). However, administration of melatonin did not decrease the level of TNF-α in BALF during ALI (Fig. 5C). These results demonstrate that administration of melatonin could effectively attenuate activation of the NLRP3 inflammasome.
We have showed extracellular histones to play a key role to serve as second signal for NLRP3 inflammasome activation during LPS-induced ALI [12]. To determine whether melatonin could inhibit the release of extracellular histones, the histone level in BALF was detected by ELISA. Histones were not detectable in control mice; however, there was a large amount of histones in BALF of ALI mice. Importantly, melatonin significantly decreased the level of histones in BALF (Fig. 5D).

The activation of the NLRP3 inflammasome needs two signals: the LPS-primed NF-κB signal and the inflammasome activation signal [5]. To determine whether melatonin could directly inhibit NLRP3 inflammasome activation, mouse peritoneal macrophages were isolated and primed with LPS; then, cells were washed and incubated with melatonin and lastly stimulated by histones. As Fig. 6A–D shows, histones significantly increased the level of IL-1β in the supernatant, whereas melatonin dose-dependently inhibited the release of IL-1β. The level of caspase-1 p20 was also decreased in the supernatant, suggesting that melatonin inhibited activation of the NLRP3 inflammasome. However, melatonin did not alter the amount of pro-IL-1β (IL-1β p31) and pro-caspase-1 (caspase-1 p45) (Fig. 6E–F). These results imply that melatonin inhibited NLRP3 inflammasome activation irrespective of NLRP3 inflammasome priming.

Although the exact mechanism of NLRP3 inflammasome activation has remained elusive, ROS generation are required for NLRP3 activation [32]. Since melatonin is a well-known scavenger of ROS, we determined the mitochondrial ROS (mt-ROS) levels. Mitochondrial ROS (mt-ROS) levels were increased when LPS-primed macrophages were treated with histones. To our surprise, melatonin did not decrease the level of mt-ROS (Fig. 7).
**Fig. 2.** Two-dimensional and three-dimensional models of lung injury in different experimental groups. Mice were challenged i.t. with LPS. One hour later, melatonin or vehicle was delivered i.t., and another dose of melatonin or vehicle was given 24 hr later. (A–C) In vivo imaging of mouse lung with Micro-CT at 48 hr after LPS challenge. (A) control, (B) acute lung injury (ALI), (C) ALI + melatonin. The density of lung was significantly increased in the ALI group, but the density of the lungs was similar to the control with administration of melatonin. (D–F) Three-dimensional reconstruction images by Analyze11.0. LPS challenge caused the loss of aerated lung volume while melatonin protected it. (D) control, (E) ALI, (F) ALI + melatonin. (G) Quantification of aerated lung volume by Analyze11.0. The results are expressed as the mean ± S.E.M., n = 4. **P < 0.01 versus the control group, and ##P < 0.01 versus the ALI group.

**Fig. 3.** Melatonin decreases the number of leukocytes in the bronchoalveolar lavage fluid (BALF). (A–C) Mice were challenged with LPS. 1 hr later melatonin or vehicle was delivered i.t., and another dose of melatonin or vehicle was given 24 hr later. BALF were harvested 48 hr later. Cells isolated from BALF were counted with a hemocytometer and stained by Diff-Quick. (A) control, (B) acute lung injury (ALI), (C) ALI + melatonin. (D) The number of macrophages/monocytes (black arrowhead) in BALF was counted. (E) The number of neutrophils (gray arrowhead) in the BALF was counted. The results are expressed as the mean ± S.E.M., n = 4. **P < 0.01, ***P < 0.001 versus the control group, and #P < 0.05, ##P < 0.01 versus the ALI group.
The result demonstrates that melatonin did not inhibit the activation of NLRP3 inflammasome through blocking the production of mt-ROS.

**Discussion**

Infectious etiologies, such as sepsis and pneumonia, are leading causes of ALI/ARDS [2]. LPS, a component of the Gram negative bacterial cell membrane, has been widely used to induce pulmonary inflammation in animal models of ALI. Since the mutation of Hiomt gene encoding HIOMT enzyme that catalyzes the synthesis of melatonin, the C57BL/6 strain is a natural ‘melatonin knockout’ mouse which provide convenience for the study to evaluate the therapeutic effect of melatonin in vivo [33]. In the present study, i.t. administration of LPS induces severe lung injury, with characteristic symptoms of ALI, including excessive leukocyte accumulation, pulmonary edema, and intrapulmonary hemorrhage. ALI induced a persistent reduction of body weight and body temperature, which is known to be related to ARDS clinical outcomes [34]. Melatonin sig-

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Fig. 4. Melatonin decreases activation of the NLRP3 inflammasome in lung tissue. Mice were challenged with LPS, 1 hr later melatonin or vehicle was delivered i.t., lungs and bronchoalveolar lavage fluid were harvested 8 hr later. (A) Representative Western blots showing the levels of caspase-1 p20 (an autoprocessed fragment of caspase-1), IL-1β p17 (mature IL-1β) in the lung tissue. (B) The relative band densities of caspase-1 p20. (C) The relative band densities of IL-1β p17. The densities of the bands were analyzed and normalized to β-actin. The data are expressed as a percentage of the controls. The results are expressed as the mean ± S.E.M., n = 4. ***P < 0.001 versus the control group, and #P < 0.05, ###P < 0.001 versus the acute lung injury group.

Fig. 5. Melatonin decreases NLRP3 inflammasome activation in the bronchoalveolar lavage fluid (BALF). (A) Representative Western blots showing the levels of caspase-1 p20 and the relative band densities of caspase-1 p20 in the BALF. The data are expressed as a percentage of the acute lung injury (ALI) groups. (B) IL-1β in the BALF was detected by ELISA. (C) TNF-α in the BALF was detected by ELISA. (D) Histones were measured in the BALF by ELISA. The results are expressed as the mean ± S.E.M., n = 4. ***P < 0.001 versus the control group, and #P < 0.05, ###P < 0.01 versus the ALI group.
significantly ameliorated the above symptoms, and pulmonary-targeted melatonin provided the maximal protective effects against ALI. These results imply that topical application of melatonin is more effective for the therapy of local hyper-inflammatory diseases, which is in accordance with a recent study describing that melatonin protects against oral mucositis: local application into mouth is most effective [21]. Notably, nebulised melatonin could be a more efficient therapeutic clinical strategy for ALI.

**Fig. 6.** Melatonin inhibits NLRP3 inflammasome activation in vitro. Peritoneal macrophages were first primed with LPS (1 µg/mL) for 4 hours, cells were washed with PBS and incubated with ethanol and different concentration of melatonin for 30 min, then stimulated by histones (50 µg/mL) for 45 min. (A) IL-1β levels in the supernatants were determined by ELISA. (B) Representative Western blots showing the level of caspase-1 p20, IL-1β p17 in the supernatant (Sup.) and the level of caspase-1 p45 (pro-caspase-1) and IL-1β p31 (pro-IL-1β) in the cell lysate (Lys.). (C–F) Western blot analysis and densitometric quantification of IL-1β p17 (C), caspase-1 p20 (D), IL-1β p31 (E), caspase-1 p45 (F). The data are expressed as a percentage of the LPS + Histones + Ethanol groups or the controls. The results are expressed as the mean ± S.E.M., n = 3. ***P < 0.001 versus the control group, and #P < 0.05, ##P < 0.01, ###P < 0.001 versus the LPS + Histones + Ethanol groups.
It is well-known that the activation of inflammasome needs the NF-κB signal pathways activation as the first signal, and the second signal induced by DAMPs (damage associated molecular patterns) [5]. Ortiz et al. and also Garcia et al. [21, 22] from the same group demonstrated that melatonin could inhibit the NLRP3 inflammasome pathway in the radiation-induced oral mucositis and cecal ligation-induced-sepsis models, respectively. Beside of the differences in the induced models between our studies, their studies are much focus on the first signal of NLRP3 inflammasome priming. Our experiment proved that melatonin inhibited NLRP3 inflammasome activation by suppressing the second signal (release of extracellular histones), and directly blocking histone-induced NLRP3 inflammasome activation irrespective of NLRP3 inflammasome priming.

In vivo imaging of mouse lungs with Micro-CT not only enables a more complete analysis without disturbing the tissue but also minimizes the need for data interpolation, therefore yields results that are more accurate [35]. The high-resolution images showed the damage of lungs during ALI and the beneficial therapeutic effect of melatonin. For its noninvasive and superior resolution, Micro-CT technology has opened the new perspectives as a potent scavenger of reactive oxygen species [49], and directly blocking histone-induced NLRP3 inflammasome priming.

In 2002, Tschopp and colleagues firstly described the inflammasome as a large molecular platform that triggers the activation of inflammatory caspases and processing of pro-IL-1β [37]. Five different inflammasomes (NLRP1, NLRP2, NLRP3, AIM2, and IPAF/NLRC4) have been clearly identified to date, while the NLRP3 inflammasome is the most studied inflammasome [38]. Cryopyrin-associated periodic syndromes (CAPS) were the first autoinflammatory disorders found to be caused by gain-of-function mutations in the NLRP3 gene [39]. Subsequently, NLRP3 has also been implicated in the pathogenesis of a number of complex diseases, notably including metabolic disorders such as type 2 diabetes, atherosclerosis, obesity, gout and diseases in the central nervous and lung, as well as in the development of liver disease, kidney disease and aging [40, 41].

As primary organs of respiration, lungs encounter constant exposure to foreign particles and infectious agents [42]. Large number of studies demonstrate the activation of NLRP3 inflammasome plays a crucial role in both the host defense against invading bacteria and pathogens and inflammation-associated tissue injury. IL-1β and IL-18 are the main products of the inflammasome activation. In fact, alveolar macrophages from the patients with ARDS produce excessive amounts of IL-1β [43] and IL-18 [44] which correlated with the increased morbidity and mortality. Additional, applications of the neutralizing antibodies of IL-18 and IL-1β, and IL-1R antagonist as well, reduced the lung injury during experimental ALI in mice and rats [45–47].

Even without the detection of the NLRP3 inflammasome activation, Sun et al. [48] demonstrated that melatonin plus mitochondria could inhibit inflammation in the pure oxygen-induced ALI. Their results encouraged us to study whether and how intratracheal administration of melatonin can inhibit the activation signal of the NLRP3 inflammasome in LPS-induced ALI.

In this study, we demonstrated that melatonin significantly reduces the level of IL-1β in the BALF and lung tissue. The maturation and release of large quantities of IL-1β requires the activated caspase-1. Accordingly, we find that treatment with melatonin greatly reduces caspase-1 p20 in the BALF. However, we find no alteration in the levels of TNF-α suggesting that melatonin specifically affects the activation of NLRP3 inflammasome in vivo.

Extracellular histones are produced during ALI and activate the NLRP3 inflammasome promoting the recruitment of neutrophils (and additional appearance of histones in the extracellular space), suggesting positive feedback and a potential mechanism of inflammatory propagation [12]. Melatonin significantly reduces the level of extracellular histones. Moreover, during in vitro experiments melatonin directly inhibits activation of the NLRP3 inflammasome. Therefore, melatonin inhibits the positive feedback between extracellular histone release and NLRP3 inflammasome activation.

Since melatonin has been recognized by numerous studies as a potent scavenger of reactive oxygen species [49], we also explored whether melatonin could block the production of mt-ROS during the activation of inflammasome. To our surprise, melatonin did not block the histone-induced production of mt-ROS in vitro. We have noticed that a few studies found that melatonin even promoted the generation of ROS through its interaction with calmodulin in several tumor and nontumor cells [50]. These seemingly opposite effects of melatonin may be due to the intrinsic cellular and molecular differences among different cells and the variety of the concentrations of melatonin, obvious making these actions context specific.

The precise mechanism for inhibition of the NLRP3 inflammasome activation by melatonin needs to be further studied.
In conclusion, melatonin can improve the severity of ALI via blocking activation of the NLRP3 inflammasome. Melatonin reduces the release of extracellular histones as well as directly inhibits NLRP3 inflammasome activation.

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Conflicts of interest

None.

Author contributions

Y. Zhang, G-F Gao, and X.-D. Li designed research; Y. Zhang, N. Wang, X.-R. Li, M.-M. Wang, J.-F. Yao, R. Zhong performed research; Y. Zhang, J.-F. Yao, D-X Tan, and X.-D. Li analyzed data; Y. Zhang, J. J. Graier, P. A. Ward, D-X Tan, and X.-D. Li wrote the article.

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413


