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Urolithin A induces protective autophagy to alleviate inflammation, oxidative stress, and endoplasmic reticulum stress in pediatric pneumonia

Xingli Cao[a], Hong Wan[b], Hao Wan[c]*

[a]*Department of Pediatrics, The Affiliated Huai'an Hospital of Xuzhou Medical University and The Second People's Hospital of Huai'an, Huai'an City, Jiangsu Province, China*
[b]*Department of Digestive System, Jiangxi Provincial Children's Hospital, Nanchang, Jiangxi Province, China*
[c]*Department of Neonatal Surgery, Jiangxi Provincial Children's Hospital, Nanchang, Jiangxi Province, China*

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Abstract
Objective: To investigate the therapeutic effect of urolithin A (UA) on pediatric pneumonia and the underlying mechanisms.
Methods: The pediatric infantile pneumonia model was constructed by intratracheal induction of lipopolysaccharide (LPS) in 1-week-old C57BL/6 mice (male, 4-5 g). UA was also injected intraperitoneally. Lung tissues in each group were examined by histological analysis. Autophagy, inflammation, and oxidative stress were assessed by enzyme-linked-immunosorbent serologic assay and immunoblot analysis. Moreover, pyrophosis and endoplasmic reticulum stress were also evaluated by immunoblot analysis.
Results: UA alleviated lung inflammation in mice, and inhibited cell pyrophosis. In addition, UA A relieved both oxidative and endoplasmic reticulum stress. Furthermore, we found that UA alleviated pneumonia damage by inducing protective autophagy.
Conclusion: UA induced protective autophagy to alleviate inflammation, oxidative stress, and endoplasmic reticulum stress in pediatric pneumonia.

***Corresponding author:** Hao Wan, Department of Neonatal Surgery, Jiangxi Provincial Children's Hospital, No. 1666 Diezihu Avenue, Honggu Beach District, Nanchang, Jiangxi Province, 330038, China. *Email address:* wanhao0908@163.com

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Introduction

Pneumonia is a respiratory disease with increasing frequencies in recent years. Incidence of pneumonia has especially increased in children, with a morbidity of up to 30% in peak season.[1] Pathogenic microorganisms invade the host, and stimulate the immune system to produce inflammatory cytokines and local accumulation of inflammatory cells,[2,3] thus causing bronchitis and pneumonia.[4,5] In serious cases, inflammatory cells may accumulate in the brain, heart, liver, and kidney, followed by complications such as encephalitis, myocarditis, and hepatitis.[6] Pneumonia can lead to lung tissue death in children, neutrophil infiltration, and release of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6.[7] Activated neutrophils promote inflammation and injury by producing reactive oxygen species (ROS) and proteolytic enzymes. ROS induces oxidative stress, which refers to a series of pathophysiological changes caused by oxidative activity *in vivo*.[8] Recent studies have revealed that oxidative stress played an important role in the pathogenesis and progression of pneumonia. Pyrophosis refers to programmed death triggered by various pathological factors and mediated by caspase family proteins, which is closely related to autophagy. Several autophagy-related proteins are involved in the regulation of pyrophosis, which is also closely associated with pneumonia.[9]

Urolithin A (UA) is an end-stage metabolite of ellagic tannin produced by intestinal microbiome.[10] Many studies have shown that UA has anti-inflammatory and antioxidant properties *in vitro* and *in vivo*, and therapeutic effects on myocardial ischemia reperfusion injury, brain injury, neuroinflammation, and other diseases.[11] UA inhibits hyperglucose-induced neuronal amyloid production by regulating transglutaminase 2 (TGM2)-dependent endoplasmic reticulum (ER)–mitochondrial contact and calcium homeostasis.[12] UA improves muscle function by inducing mitochondrial autophagy in muscular dystrophy.[13] In addition, UA inhibits receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast formation and postmenopausal osteoporosis through inhibition of inflammation and downstream nuclear factor kappa B (NF-κB)-activated pyroapoptotic pathway.[14] UA inhibits the growth of prostate and colon cancer cells.[15] UA increases messenger RNA (mRNA) and protein expressions of phospho-p38 mitogen-activated protein kinase (MAPK), and decreases mRNA and protein expression of mitogen-activated protein kinase kinase 1 (MEKK1) and phospho-c-Jun in T24 cells.[16] At present, there is no definite evidence that UA has a therapeutic effect on pediatric pneumonia.

Here, we selected 1-week-old C57BL/6 mice (male, 4–5 g) for intraperitoneal injection of 2 mg/kg lipopolysaccharide (LPS) to induce pneumonia. It was demonstrated that UA alleviated lung tissue injury and inflammation, inhibited cell pyroapoptosis, oxidative stress and endoplasmic reticulum stress, and alleviated pneumonia injury by inducing protective autophagy in newborn mice.

Materials and Methods

Animal treatment

Male C57BL/6 mice (about 1-week old, 4–5 g, n = 30) were obtained from SLAC (Shanghai, China) and divided into the following five groups: control, LPS, LPS+2.5 mg/kg UA, LPS+ 5 mg/kg UA, and LPS+15 mg/kg 3-methyladenine (3-MA). Ethical approval for the animal study was obtained from the Ethics Committee of The Affiliated Huai'an Hospital of Xuzhou Medical University and the Second People's Hospital of Huai'an. The control group mice were intratracheally instilled with 2 mg/kg LPS (Escherichia coli 0111:B4; Sigma, St. Louis, MO) dissolved in 50-μL PBS and 50-μL phosphate-buffered saline solution (PBS). UA (2.5 and 5 mg/kg, bought from Sigma) and 3-MA (15 mg/kg) were administered intraperitoneally 1 h before LPS treatment. Bronchoalveolar lavage fluid (BALF) was collected for subsequent enzyme-linked-immunosorbent serologic assay (ELISA), and lung tissues were collected for pathological analysis.

Histological analysis

Lung tissues were resected and fixed with 5% paraformaldehyde (PFA), followed by paraffin-embedding. Then the samples were cut into slices and counterstained with hematoxylin and eosin (H&E).

Enzyme-linked-immunosorbent serologic assayELISA

Level of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 in BALF were assessed by ELISA kit (Beyotime, Beijing, China) following the manufacturer's guidelines. Briefly, samples were aspirated into wells. Biotin-conjugated primary antibodies were added followed by avidin-conjugated horseradish peroxidase (HRP). Then enzyme substrate was used for color reaction. The color intensity of each well was measured with a microplate reader.

Detection of antioxidant activity

Levels of superoxide dismutase (SOD), glutathione¤r-glutamyl cysteingl + glycine (GSH), and malonaldehyde (MDA) were measured using detection kits obtained from Nanjing Jiancheng Bio-engineering Institute (Jiangsu, China) and used following the manufacturer's guidelines.

Western blotting analysis

Proteins were extracted from tissues by homogenization with radioimmunoprecipitation assay (RIPA) buffer.

The supernatants were collected through centrifugation and subjected to protein concentration determination with bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China). Proteins were resolved with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked with 5% bovine serum albumin (BSA) followed by incubation with primary antibodies targeting LC3-II/I (1:1000; Thermo Scientific, Waltham, MA, USA), Beclin (1:1000; Abcam, Cambridge, UK), p62 (1:1000; Abcam), gasdermin D (GSDMD, 1:1000; Abcam), GSDMD-N (1:1000; Abcam), NLR family pyrin domain containing 3 (NLRP3, 1:1000; Abcam), pro-caspase-1 (1:1000; Abcam), cleaved-caspase-1 (1:1000; Abcam), pro-IL-1β (1:1000; Abcam), cleaved IL-1β (1:1000; Abcam), activating transcription factor 6 (ATF6, 1:1000; Abcam), C/EBP-homologous protein (CHOP, 1:1000; Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10000; Abcam). Membranes were incubated with HRP-conjugated secondary antibodies at a ratio of 1:1000 for 2 h after rinsing in tris buffered saline with tween (TBST) for 15 min. The signals were detected with enhanced chemiluminescence (ECL) detection kit.

Statistical analysis

Statistical analysis was performed with GraphPad 6.0. All data were expressed as mean ± standard error of mean (SEM). Three replicates were performed for each experiment. $P < 0.05$ was considered as statistically significant.

Results

Urolithin A alleviated lung tissue injury

The molecular formula of UA is shown in Figure 1A. After induction of infantile pneumonia, the histological analysis of lung tissues was performed. LPS induced significant tissue injury, including alveolar shrinkage, severe inflammatory cell infiltration, edema, interstitial hyperemia, and alveolar wall thickening in pneumonia. However, pretreatment with UA significantly decreased lung injury (Figure 1B). These results suggested that UA relieved lung tissue injury.

Urolithin A induced autophagy in LPS-treated mice

Autophagy in LPS-induced infantile pneumonia was analyzed by immunoblot assay. As shown in Figure 2, LPS induced elevated expression ratio of LC3-II to I and Beclin expression, and reduced p62 expression. UA treatment further enhanced the expression levels of LC3-II and I and Beclin but lowered p62 expression in lung tissues (Figure 2). Therefore, UA promoted autophagy in LPS-induced mice.

Urolithin A inhibited oxidative stress and endoplasmic reticulum stress by inducing protective autophagy in newborn mice

The molecular mechanism of UA-mediated lung injury was analyzed. As shown in Figure 5A, LPS stimulation induced elevated MDA and inhibited production of SOD and GSH, which was reversed by UA treatment (Figure 5A). However, 3-MA treatment alleviated the effect of UA on oxidative stress. Moreover, LPS significantly increased the level of ATF6 and CHOP but UA decreased the expressions of ATF6 and CHOP (Figure 5B). 3-MA restored the effect of LPS in lung tissues (Figure 5B). UA inhibited oxidative stress and endoplasmic reticulum stress by inducing protective autophagy in newborn mice.

Urolithin A inhibited cell pyrophosis by inducing autophagy

We detected the role of UA in cell pyrophosis. We observed that LPS stimulated the production of NLRP3,

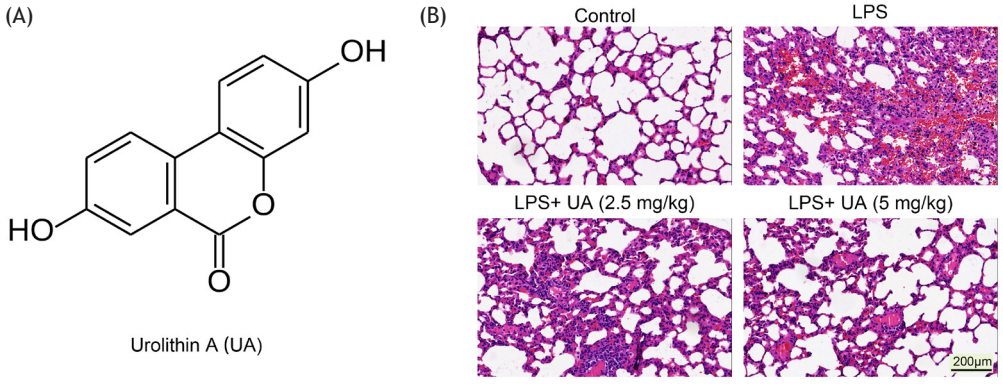

Figure 1 UA alleviated lung tissue injury. (A) The chemical structure of UA. (B) The histological analysis of lung tissues in control, LPS, and UA-treated LPS mice.

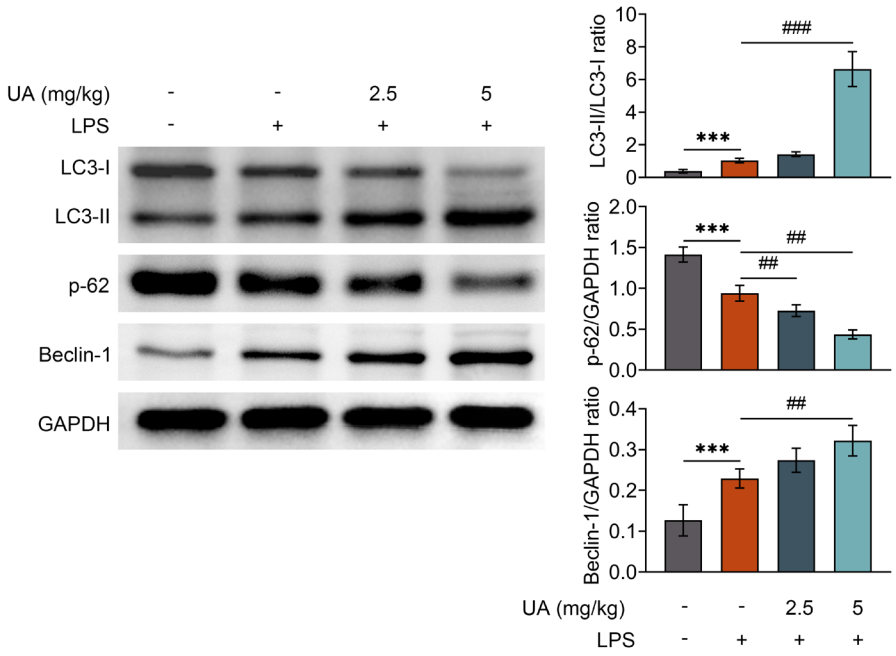

Figure 2 UA induced autophagy in LPS-stimulated mice. (A) Levels of LC3-II/I, p62, and Beclin in each group. ***P < 0.001 vs control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS.

cleaved-caspase-1, pro-IL-1β, cleaved-IL-1β, and GSDMD-N. UA treatment alleviated the production of NLRP3, cleaved-caspase-1, pro-IL-1β, cleaved-IL-1β, and GSDMD-D (Figures 3A and B). Autophagy inhibitor, 3-MA, was used to block cell autophagy. We observed that autophagy inhibition blocked the effect of UA on pyrophosis (Figures 3A and B). FOXP3 reduced the level of p-STAT3 in LPS mice (Figures 3A and B). Therefore, the data confirmed that UA inhibited cell pyrophosis by inducing autophagy.

Urolithin A inhibited lung inflammation in LPS-treated mice by inducing autophagy

Inflammatory cytokines were also monitored in LPS model. The levels of IL-6, IL-1β, and TNF-α were enhanced in LPS-induced mice. However, UA significantly decreased the levels of IL-6, IL-1β, and TNF-α in LPS-treated mice in BALF (Figure 4). UA abolished the effects of UA on inflammation, indicating that the effect of UA on lung inflammation was dependent on autophagy (Figure 4).

Urolithin A inhibited oxidative stress and endoplasmic reticulum stress by inducing protective autophagy in newborn mice

The molecular mechanism of UA-mediated lung injury was analyzed. As shown in Figure 5A, LPS stimulation induced elevated MDA level and decreased levels of SOD and GSH, which was reversed by UA treatment (Figure 5A). However, 3-MA treatment alleviated the effect of UA on oxidative stress. Moreover, LPS significantly induced the expressions of ATF6 and CHOP whereas UA alleviated the induction of ATF6 and CHOP (Figure 5B). 3-MA restored the effect of LPS in lung tissues (Figure 5B). UA inhibited oxidative stress and endoplasmic reticulum stress by inducing protective autophagy in newborn mice.

Discussion

Pneumonia is usually accompanied with fever, expectoration, and other typical clinical manifestations. However, pneumonia could be with a few or without clinical manifestations. The most typical clinical manifestations are shortness of breath and dyspnea, or conscious disturbance, lethargy, dehydration, loss of appetite, etc.[17] The fundamental aspect to the treatment of pneumonia is to find out the cause; if it is infectious pneumonia, then anti-infection treatment should be prescribed as a priority.[18] If pneumonia is caused by immune damage, then the immune system should be alleviated. In fact, it is urgent to find effective drugs for pneumonia treatment.[19] Interestingly, this research revealed that UA induced protective autophagy to alleviate inflammation, oxidative stress, and endoplasmic reticulum stress in pediatric pneumonia.

Pneumonia results in destruction of lung tissues, neutrophil infiltration, and release of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Activated neutrophils promote inflammation and injury by producing ROS and proteolytic enzymes. Similarly, the present study also revealed that UA induced protective autophagy to alleviate inflammation, oxidative stress, and endoplasmic reticulum stress in pediatric pneumonia.

H&E staining as well as ELISA revealed that UA alleviated lung inflammation in mice. Immunoblot assay showed that UA inhibited pyrophosis. In addition, UA relieved oxidative stress as well as endoplasmic reticulum stress as revealed by ELISA and Immunoblot assay results. We further confirmed that UA lessened damage caused by pneumonia

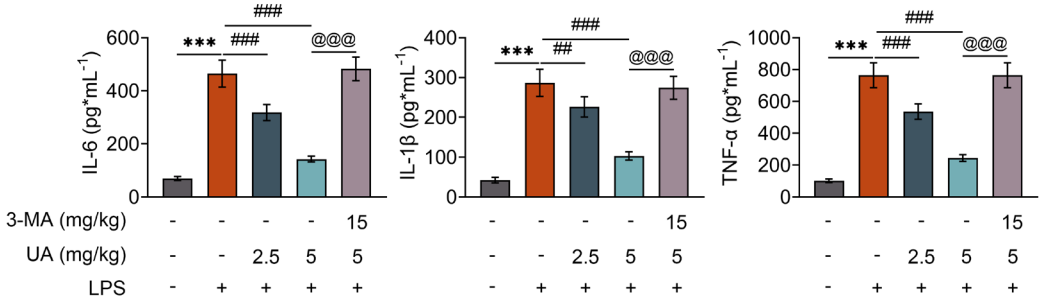

Figure 3 UA inhibited cell pyrophosis by inducing autophagy. (A and B) The protein level of NLRP3, cleaved-caspase-1, pro-IL-1β, cleaved-IL-1β, and N-GSDMD in control, LPS and UA, and 3-MA-treated LPS mice. ***P < 0.001 vs control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS; @@@P < 0.001 vs LPS and UA.

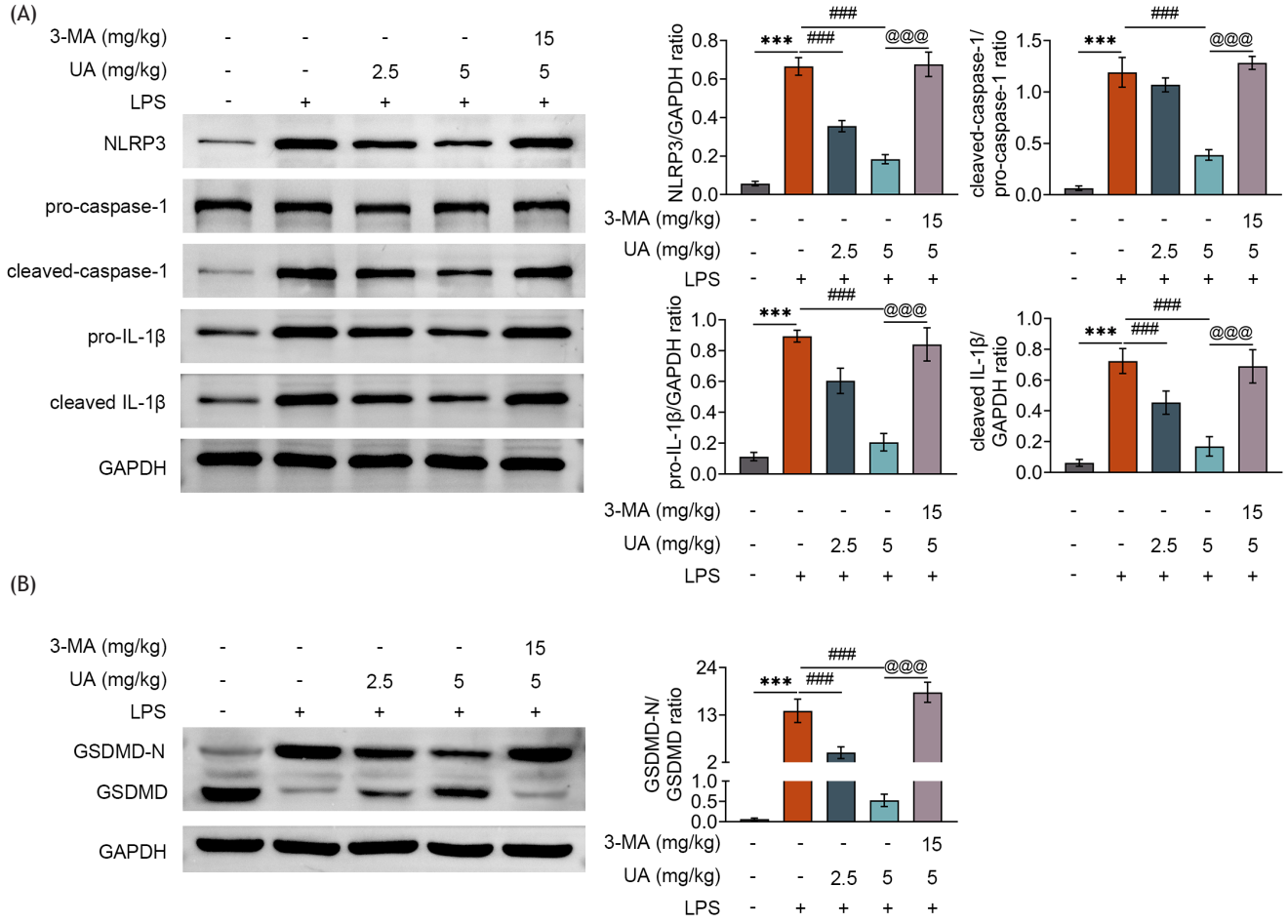

Figure 4 UA inhibited lung inflammation in LPS mice by inducing autophagy. Levels of IL-6, IL-1β, and TNF-α in the lung of control, LPS, and UA and 3-MA-treated LPS mice. ***P < 0.001 vs control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS; @@@P < 0.001 vs LPS and UA.

through protective autophagy. Collectively, these observations confirmed that UA could serve as a promising drug for treating pneumonia. Multiple biological activities of UA have been widely discovered in different diseases.[20,21] UA has anti-inflammatory and antioxidant properties *in vitro* and *in vivo*, and has therapeutic effects in myocardial ischemia-reperfusion injury, brain injury, neuroinflammation, and other diseases.[22] UA inhibits production of hyperglucose-induced neuronal amyloid by regulating TGM2-dependent endoplasmic reticulum-mitochondrial contact and calcium homeostasis.[23] UA inhibits replication of enterovirus 71 (EV71) and contributes to autophagy as well as apoptosis of infected cells *in vitro*.[24] UA improves muscle function by inducing mitochondrial autophagy in muscular dystrophy. In addition, UA inhibits RANKL-induced osteoclast formation and postmenopausal osteoporosis through inhibition of inflammation and downstream NF-κB-activated pyroapoptotic pathway.[25]

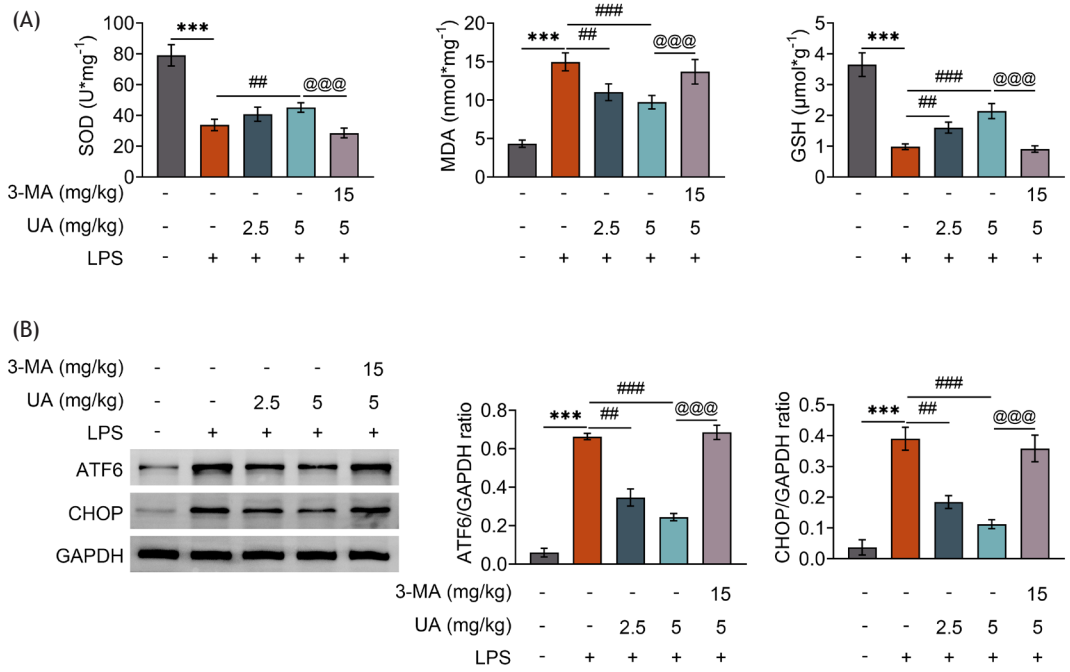

Figure 5 UA inhibited oxidative stress and endoplasmic reticulum stress by inducing protective autophagy in newborn mice. (A) Levels of SOD, MDA, and GSH in control, LPS and UA, and 3-MA-treated LPS mice. (B) The protein level of ATF6 and CHOP in control, LPS and UA, and 3-MA-treated LPS mice. ***P < 0.001 vs control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs LPS; @@@P < 0.001 vs LPS and UA.

The present also revealed that UA relieved pneumonia injury by stimulating protective autophagy. The protective autophagy plays a crucial role in the pathology of pneumonia. When cells are damaged due to physiological, pathological, and chemical factors, the fusion of autophagosome and lysosome produces a large number of autophagosomes, which induces the initiation of autophagy, thus helps in protecting cells from injury.[24] Recent studies have established that autophagy-related signaling pathways are closely associated with different types of pneumonia.[26] Therefore, targeting autophagy could serve as a promising method for the treatment of pneumonia. However, whether UA could serve as a drug for treating pneumonia needs further study, as the present is an experimental study on mice and its clinical efficacy and safety have not been evaluated.

Conclusion

In this study, UA relieved lung tissue injury and inflammation, inhibited cell pyroapoptosis, oxidative stress, and endoplasmic reticulum stress, and eased pneumonia injury by inducing protective autophagy in newborn mice.

Competing Interests

The authors stated that there were no conflicts of interest to disclose.

Data Availability

The authors state that all data supporting the findings of this study are available in the paper, and any raw data can be obtained from the corresponding author upon request.

Xingli Cao[a], Hong Wan[b], Hao Wan[c]

Author Contributions

Xingli Cao and Hao Wan designed and carried out the study. Xingli Cao, Hao Wan, and Hong Wan supervised data collection, analyzed and interpreted the collected data, and prepared and reviewed the draft manuscript. All authors read and approved the final manuscript.

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