

Pretreatment of ferulic acid attenuates inflammation and oxidative stress in a rat model of lipopolysaccharide-induced acute respiratory distress syndrome

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Abstract

Acute respiratory distress syndrome (ARDS) is a fatal clinical condition that can be caused by pulmonary and non-pulmonary diseases. Oxidative stress and inflammation play key roles in the development of ARDS. In this study, we investigated whether ferulic acid (FA), an anti-oxidant, was beneficial for prophylaxis of ARDS. We established an ARDS rat model using lipopolysaccharide (LPS) administration. Lung injury was assessed by lung wet/dry ratio and broncho-alveolar lavage fluid (BALF) analysis. Hematoxylin and eosin staining was performed to evaluate the histological changes of the lungs. Enzyme-linked immunosorbent assay (ELISA) and immunoblotting were performed to detect proteins in BALF and lung tissue, respectively. Pulmonary function was determined by testing the oxygen level in BALF. FA pretreatment significantly alleviated LPS-induced pulmonary histological changes. FA reversed LPS-induced changes of lung wet/dry ratio, total protein in BALF, P(A-a)O₂, and PaO₂/FiO₂. In addition, LPS dramatically up-regulated the secretion of interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , and IL-10 in BALF ($P < 0.01$). However, pretreatment of FA significantly improved LPS-induced inflammation. We found that FA indeed reduced oxidative stress in the lungs by testing malondialdehyde level, myeloperoxidase level, and total anti-oxidant capacity. We also proved that FA inactivated multiple mitogen-activated protein kinase signaling pathways in the lungs. In conclusion, FA alleviated LPS-induced ARDS through its anti-inflammatory and anti-oxidant activities.

Keywords

animal model, inflammation, lung injury, reactive oxygen species, respiratory distress syndrome

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Introduction

Acute respiratory distress syndrome (ARDS) is a severe clinical disorder that is caused by increased permeability of alveolar epithelial cells and pulmonary capillary endothelial cells, leading to consequent pulmonary edema and impairment of oxygenation.¹ The physiopathology of ARDS is complicated. Oxidative stress and inflammatory response are two key players in ARDS development.^{2,3} As a common etiology, infection by bacteria or viruses can lead to local immune response

including recruitment and activation of neutrophils and macrophages. Immune reaction is the main cause of lung injury. These immune cells release a

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great amount of cytokines and reactive oxygen species (ROS), further resulting in a cascade of up-regulation of inflammation and oxidative stress.² The crosstalk between pro-inflammatory cytokines and ROS is complicated and interference of such cascade may help block the development of ARDS. Many studies have tried anti-inflammation and anti-oxidant strategies to treat or prevent ARDS. However, as potent anti-inflammatory drugs, the clinical role of corticosteroids is still under consideration.⁴ A number of other anti-inflammatory agents and anti-oxidants also failed to show improved outcomes in ARDS patients.⁵ Therefore, new agents for testing anti-inflammation/anti-oxidant theory in ARDS development and for potential treatment of this intractable disease are urgently needed.

Ferulic acid (FA) is extracted from natural plants and has various biological activities including anti-oxidant and anti-inflammation.⁶ Therefore, FA has been proven to be effective in many disease models such as depression, diabetes, ulcerative colitis, Alzheimer's disease, epilepsy, and hepatotoxicity.⁷⁻¹² FA was previously reported to be able to enhance nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) and up-regulate super oxide dismutase (SOD) expression.^{13,14} Meanwhile, FA could inactivate nicotinamide adenine dinucleotide phosphate, reduced (NADPH) oxidase, nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), and cyclooxygenase-2 (COX-2) and inhibit expression of several pro-inflammatory cytokines including interleukin (IL)-1 β and IL-6.^{9,15} However, whether FA shows benefits in ARDS protection is currently unknown. Here, we established an ARDS rat model using lipopolysaccharides (LPS) administration and tested the effects of pretreatment of FA in histological changes and pulmonary functions. We also evaluated the efficacy of FA in reducing oxidative stress and inflammation in our ARDS model.

Materials and methods

Establishment of the rat model

Male Wistar rats weighing 200 ± 25 g were purchased from Animal Center in Hebei Medical University. The ARDS rat model was established according to the methods as previously described.¹⁶ Briefly, rats were anesthetized with pentobarbital and were treated with intratracheal instillation of

LPS solution (2 mg/kg diluted in 100 μ L normal saline; Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Control rats were treated with an equal volume of vehicle. In FA-treated groups, FA (50 mg/kg diluted in 100 μ L normal saline; Sigma-Aldrich) was intraperitoneally injected once per day for 30 consecutive days before LPS administration. The protocol of this study was reviewed and approved by the Ethics Committee of Xingtai People's Hospital of Hebei Province. All cultural conditions and procedures of animals complied with the Guidelines for the Care and Use of Laboratory Animals.

Hematoxylin and eosin staining and pathological scoring

Pathology of the lungs was analyzed as previously described.¹⁷ Briefly, the lungs were harvested 24 h after LPS administration and were fixed with formalin solution. The dehydrated samples were embedded in paraffin and were cut into 5- μ m-thick slides. Hematoxylin and eosin (H&E) staining was applied to the slides, which were then reviewed by an experienced pathologist. The pathological scores were evaluated according to the severity of five parameters including inflammation, edema, hemorrhage, atelectasis, and formation of hyaline membrane. The pathological changes of each parameter were scored from 0 (normal) to 4 (severe injury) under a microscope, and the final score of each sample was calculated by adding scores of all the five parameters.

Lung wet/dry ratio assessment

Lung wet weight was acquired by immediate weighing of the lungs after rats were sacrificed. The lungs were then washed with normal saline for three times to thoroughly remove the blood and were baked at 60°C for 72 h. The dry weight of each sample was acquired by weighing the baked lungs. The wet/dry ratio was calculated as the wet weight divided by the dry weight.¹⁶

Broncho-alveolar lavage fluid analysis

The broncho-alveolar lavage fluid (BALF) was harvested from each rat 24 h post LPS treatment. In brief, normal saline was used to wash the left lung for three times through a tracheal cannula. The flushing fluid was collected as much as possible.

Cells and cell debris in the BALF were removed by centrifugation. Concentrations of IL-1 β , IL-6, IL-10, and tumor necrosis factor (TNF)- α were determined using enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Arterial blood gas analysis

The arterial blood gas analysis was performed as previously described.¹⁷ Immediately after the rats' arterial blood was drawn from carotid arteries, the partial pressure of oxygen in arterial blood (PaO₂) and the fraction of inspired oxygen (FiO₂) were determined using an automatic blood gas analyzer (Radiometer, Copenhagen, Denmark). Alveolar-arterial oxygen tension difference [P(A-a)O₂] was calculated using the standard alveolar gas equation, and PaO₂/FiO₂ ratio was calculated as PaO₂ divided by FiO₂.

Oxidative stress evaluation

The oxidative stress of lung tissue was reflected by the malondialdehyde (MDA) level, total anti-oxidant capacity (TAOC), and myeloperoxidase (MPO) level as previously reported.¹⁸ The lungs in each rat were harvested as mentioned earlier, and tissue lysates were obtained. Levels of MDA and MPO were determined using ELISA kits (R&D Systems). TAOC was assessed using a colorimetric assay kit (BioVision Inc., Milpitas, CA, USA).

Immunoblotting

The homogenated tissue samples were lysed using radio immunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with proteinase cocktail (Roche, Basel, Swiss) for total protein extraction.¹⁶ Totally, 40 μ g proteins were loaded for electrophoresis. The proteins were then transferred to a nitrocellulose blotting membrane and were blocked with 5% non-fat milk in room temperature for 1 h. The following indicated primary antibodies were used to incubate the membrane overnight at 4°C: anti-phosphorylated-c-Jun N-terminal kinases (JNK), anti-JNK1/2, anti-phosphorylated-extracellular signal-regulated kinases (ERK), anti-ERK, anti-phosphorylated-p38, anti-p38, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (all from Cell Signaling Technology, Danvers, MA, USA). The membrane was then

incubated with anti-rabbit or anti-mouse secondary antibodies at room temperature for 1 h (Cell Signaling Technology). Enhanced chemiluminescence (Thermo Fisher Scientific) was used to detect the bands. The photodensity of bands was assessed using Image J (NIH, Bethesda, MD, USA), and the levels of phosphorylated protein were normalized to those of corresponding total proteins.

Statistical analysis

Data were presented as the mean \pm standard deviation (SD). All statistical analyses were conducted using two-tailed Student's t-test. *P* values less than 0.05 were considered statistically significant.

Results

FA improves pulmonary pathological changes in LPS-induced ARDS rats

To investigate the preventive role of FA in ARDS, we first pretreated the rats with FA once daily for 30 days. Then, the rats were challenged with LPS and were subjected to analyses after 24 h (Figure 1(a)). According to our previous pilot studies that tried different doses of FA, FA with a dose of 50 mg/kg showed significant effects without noticeable toxicities (data not shown). Here, we again confirmed that 50 mg/kg FA alleviated LPS-induced pathological changes in the lungs. H&E staining showed that LPS led to significant pulmonary inflammation and hyaline membrane formation, both of which were greatly improved in rats with prophylactic use of FA (Figure 1(b)). Histological score reflects the overall lung injury, and as expected, ARDS group showed a dramatic increase in the histological score. However, FA pretreatment significantly reduced the histological score (Figure 1(c)). These findings suggested that FA was able to improve ARDS-associated lung injury in the rat model.

FA rescues lung injury in LPS-treated rats

We further tested whether FA pretreatment improved pulmonary function by detecting four common parameters. Since increased permeability of the pulmonary capillary endothelium and alveolar epithelium results in pulmonary edema that can be evaluated by wet/dry ratio of the lung weights, we tested and found that LPS alone

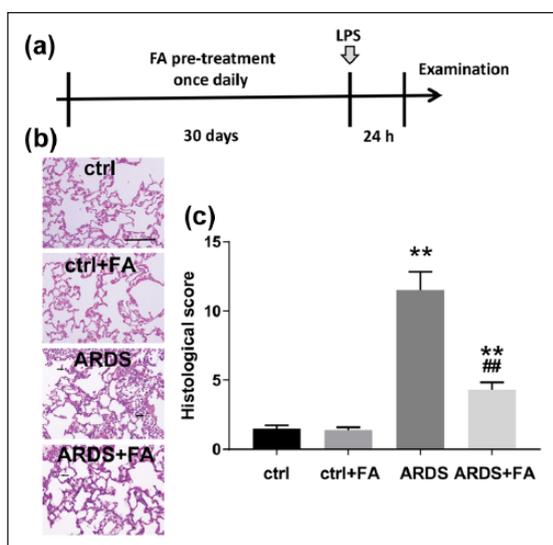


Figure 1. Ferulic acid (FA) alleviates histological changes of the lungs in lipopolysaccharide (LPS)-induced rats. (a) Schematic diagram of the experimental design in this study. Rats were pretreated with FA (50 mg/kg) for consecutive 30 days once per day by intraperitoneal injection and were then subjected to LPS administration (2 mg/kg). After 24 h, the rats were sacrificed for the following examination. FA pretreatment caused significant histological changes in acute respiratory distress syndrome (ARDS) rats, as evidenced by hematoxylin and eosin (H&E) staining in the lung tissues (b) and histological scoring (c). N=8. Data are presented as mean \pm SD. ** P <0.01 compared to the control group (ctrl); ### P <0.01 compared to the ARDS group. Scale bar, 50 μ m. Black arrows indicate samples of abnormality compared to control.

increased lung wet/dry ratio by around 50% (Figure 2(a)), suggesting severe pulmonary edema. FA pretreatment significantly reduced lung wet/dry ratio, though it was still higher compared to that of the control rats. Similar results were observed regarding the total proteins in BALF (Figure 2(b)). Furthermore, we assessed the blood oxygenation in the rats. Obviously, in the ARDS group, $P(A-a)O_2$ increased; while in the presence of FA, $P(A-a)O_2$ decreased to a large extent (Figure 2(c)). Consistently, PaO_2/FiO_2 in rats of ARDS group decreased greatly and was partially rescued by FA (Figure 2(d)). These findings suggested that LPS administration impaired oxygen diffusion, which could be improved by FA pretreatment.

FA mitigates local inflammation in the lungs

LPS is a potent inflammation inducer and can extensively enhance inflammation, which plays critical roles in ARDS development. We thus evaluated whether FA pretreatment diminished the pro-inflammatory effects of LPS in our rat model. Undoubtedly, the levels of pro-inflammatory cytokines including $TNF-\alpha$, $IL-1\beta$, and $IL-6$ in BALF increased dramatically; however, FA pretreatment significantly limited the secretion of these cytokines (Figure 3(a)–(c)). LPS also slightly

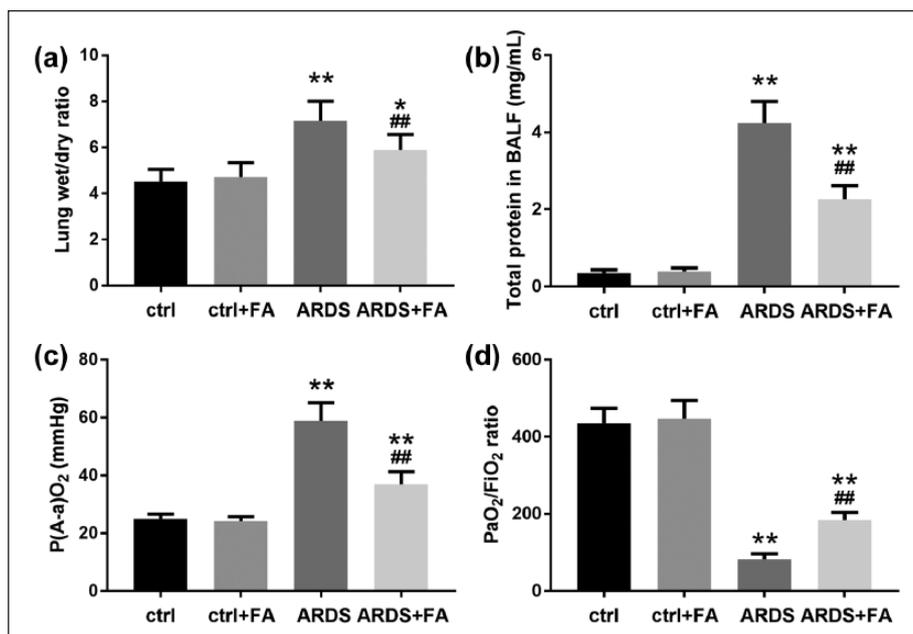


Figure 2. Pretreatment of FA effectively protects against lung injury in LPS-induced ARDS. Lung injury was evaluated by measuring (a) lung wet/dry ratio, (b) total protein level, (c) $P(A-a)O_2$, and (d) PaO_2/FiO_2 ratio in broncho-alveolar lavage fluid, which was collected 24 h following LPS administration. N=8. Data are presented as mean \pm SD. * P <0.05; ** P <0.01 compared to the control group (ctrl); ### P <0.01 compared to the ARDS group.

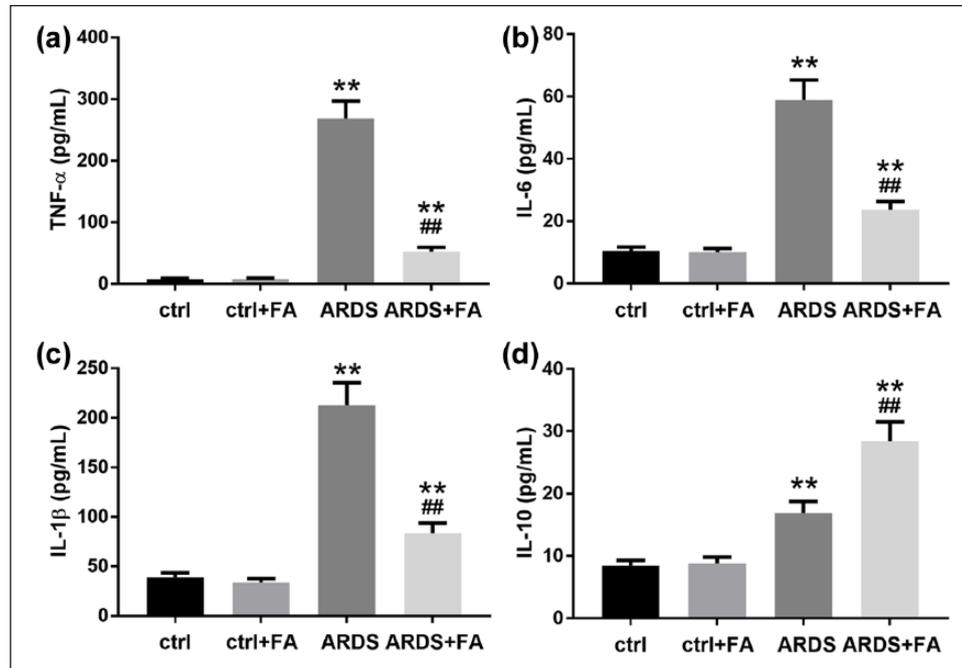


Figure 3. Pretreatment of FA relieves pulmonary inflammatory responses in LPS-induced ARDS. BALF was collected 24 h after LPS administration. Pro-inflammatory cytokines including (a) TNF- α , (b) IL-6, and (c) IL-1 β and anti-inflammatory cytokine (d) IL-10 were determined by ELISA.

N=8. Data are presented as mean \pm SD. ** P <0.01 compared to the control group (ctrl); ## P <0.01 compared to the ARDS group.

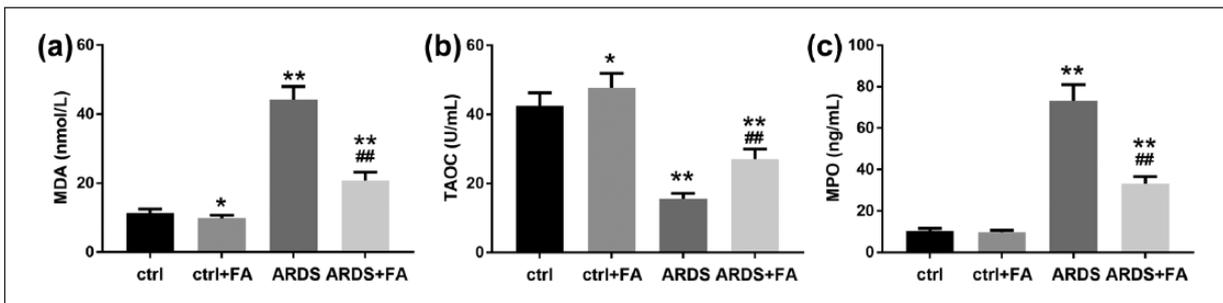


Figure 4. Pretreatment of FA attenuates oxidative stress in LPS-induced ARDS rats. Oxidative stress was characterized by measuring the (a) malondialdehyde (MDA) level, (b) total anti-oxidant capacity (TAOC), and (c) myeloperoxidase (MPO) level in the lungs.

N=8. Data are presented as mean \pm SD. * P <0.05; ** P <0.01 compared to the control group (ctrl); ## P <0.01 compared to the ARDS group.

increased the secretion of IL-10 probably due to a feedback effect induced by an acute inflammation (Figure 3(d)). FA further enhanced IL-10 level in the rats' BALF, displaying an anti-inflammatory effect. These results together showed that long-term pretreatment of FA could limit acute inflammation caused by LPS.

FA shows anti-oxidant effects in our ARDS rat model

To test whether FA exerted anti-oxidant activity in preventing ARDS, we detected three common

indicators of oxidative stress. MDA is an indicator of lipid peroxidation,¹⁹ which was found up-regulated in the lungs of our ARDS rats and this up-regulation was limited in the presence of FA (Figure 4(a)). In parallel, the reduced TAOC caused by LPS challenge was largely recovered when FA was pretreated before LPS administration (Figure 4(b)). Noticeably, this anti-oxidant activity of FA could be observed even in rats without LPS challenge, though the effects looked mild (Figure 4(a) and (b)). In addition, we detected the level of MPO, a key regulator of oxidative stress in pro-inflammatory cells,²⁰ and it turned out that ARDS rats

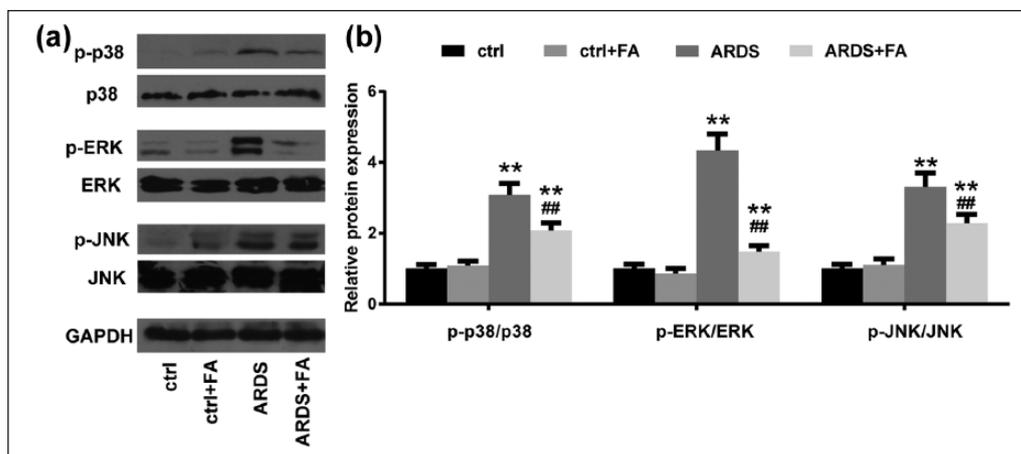


Figure 5. FA down-regulates mitogen-activated protein kinase (MAPK) signaling pathways in rats with LPS administration. (a and b) MAPK signaling pathway-related proteins (phosphorylated and total proteins of p38, ERK and JNK) were analyzed by immunoblotting. GAPDH was employed as the loading control.

N=8. Data are presented as mean \pm SD. * $P < 0.05$; ** $P < 0.01$ compared to the control group (ctrl); ### $P < 0.01$ compared to the ARDS group.

showed an MPO level of approximately six times higher than that of the control rats (Figure 4(c)). Altogether, these results confirmed that oxidative stress increased during ARDS development and could be suppressed by FA.

FA down-regulates mitogen-activated protein kinase signaling in LPS-treated rats

Oxidative stress contributes to lung injury during ARDS development,²¹ and mitogen-activated protein kinase (MAPK) signaling pathways play a key role in mediating the effects of oxidative stress.²² Given that FA is a potent anti-oxidant, we investigated whether FA functioned by blocking MAPK signaling. In healthy conditions, FA had a minimal effect in the phosphorylation of p38, ERK, and JNK (Figure 5(a) and (b)). In ARDS conditions, however, all the three kinases were dramatically up-regulated, indicating enhanced activation of MAPK signaling. When FA was applied to the ARDS rats in advance, the three kinases, especially ERK, were found less phosphorylated, suggesting that pretreatment of FA limited activation of MAPK signaling in the LPS-induced ARDS rat model.

Discussion

In clinic, ARDS is very intractable once developed and only support treatments such as mechanical ventilation and anti-thromboembolism are used.²³ Although people now know that various factors such as inflammation and ROS are involved in ARDS

development, few strategies have been successfully developed to limit its progression. This leads to high mortality and morbidity in patients with primary or secondary ARDS. Many studies have been focused on the enhanced oxidative stress and inflammatory response during ARDS development.²⁴ In the last century, anti-oxidants *N*-acetylcysteine and procysteine were found to shorten the duration of acute lung injury.²⁵ Supplement of vitamin C was also reported helpful in ARDS treatment.²⁶ As a well-established anti-oxidant, FA was proven to be not only beneficial for improvement of pulmonary function but also helpful for recovery of histological changes of the lungs. We thus believe that FA and FA analogues may be promising agents for ARDS management in the future.

Up to date, FA has been shown effective in a variety of disease models with up-regulated oxidative stress because of distinct causes.^{9,12-15} However, no study had used FA for the purpose of ARDS prevention or treatment. LPS is commonly used to induce ARDS in animal models and is capable of inducing oxidative stress,²⁷ and the etiology and physiopathology of LPS-induced ARDS are very similar to those in human patients. We thus hypothesized that FA may be useful in ARDS management. Since most studies in this field tried preventive effects of certain materials, we also tested the prophylactic role of FA in this study. As expected, FA pretreatment reserved the anti-oxidant activity in the injured lungs. Although subtle changes in MDA and TAOC levels were also observed in the absence of LPS, the potential of FA

was significantly evoked when LPS existed. From this view, FA may be safely used in patients with a high risk of ARDS to provide anti-oxidant reserve. Thus, the prophylactic use of FA looks reasonable in such clinical scenarios.

Besides the anti-oxidative activity of FA, we also detected a weakened inflammatory response in the lungs. The decreased levels of TNF- α , IL-1 β , and IL-6, together with the increased level of IL-10, suggested an immunomodulating effect of FA. Since the expression and secretion of these four cytokines are differently regulated (e.g. inflammasome is required for IL-1 β but not other cytokines), it is not likely that FA directly modulates the expression of them at the same time. Therefore, we hypothesized that FA influences an upstream target that functions as an immunomodulator and finally inhibits inflammation. Studies performed in other disease models suggested that NF- κ B and nucleotide binding and oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) were regulated by FA and showed very similar alterations of TNF- α , IL-1 β , and IL-6.²⁸ It is thus possible that FA also regulates NF- κ B and NLRP3 in conditions of ARDS; however, this needs further investigations.

We further found that FA could be a potent MAPK inhibitor, showing significant inactivation of three main kinases in the signaling pathways. Intriguingly, it was previously reported that FA could activate p38 MAPK signaling in an ischemia-reperfusion rat model,²⁹ while other studies supported that FA inhibited p38 MAPK signaling in neurons and microglia.^{30,31} Opposite effects of FA in ERK were also reported in lymphocytes in the presence or absence of radiation activation.^{32,33} These discrepancies indicate that the role of FA is highly dependent on disease models and suggest complicated effects of FA. Given the complicated influences caused by FA in our rat model, it can be speculated that FA may be a multi-target chemical. Therefore, the application of FA needs caution.

This study has some limitations. First, we only tested the efficacy of FA as a pretreatment. Whether FA has treatment effect when ARDS is already developed is unknown. Unfortunately, the initiation of ARDS is obscure and difficult to observe clinically.³⁴ When signs and symptoms can be detected, it is usually hard to stop the development of ARDS. It can be foreseen that the treatment effect of FA may be not as good as the prophylactic use of FA.

Second, we pretreated the rats with FA for 30 days, which is relatively a long term. The minimal duration that guarantees the efficacy of FA is currently unknown. Third, we used LPS, which is from gram-negative bacteria, to establish an ARDS rat model. We thus are unaware of whether FA is also effective in virus-induced ARDS, which is also a common clinical scenario. In addition, we only showed the possible efficacy of FA without investigating its targets and the detailed mechanisms by which FA functioned as an MAPK inhibitor. However, given the efficacy of FA in our model, further study is valuable to understand its molecular mechanisms in preventing ARDS.

We here demonstrate that FA can ameliorate ARDS-related lung injury through its anti-oxidant effect by interfering MAPK signaling pathways in a LPS-induced ARDS rat model. Mechanically, FA pretreatment can reduce inflammation and ROS level in the lungs. As far as we know, this is the first study showing that pretreatment of FA is able to restrain ARDS development and shed light on its clinical use. Other ARDS animal models are required to verify our conclusions, and the efficacy of FA in existing ARDS needs to be studied.

Declaration of conflicting interests

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