

Emodin alleviates LPS-induced inflammatory response in lung injury rat by affecting the function of neutrophils

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Research

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Abstract

Background: Acute lung injury (ALI) and/or acute respiratory distress syndrome (ARDS) are critical life-threatening syndromes characterized by the infiltration of a large number of neutrophils that lead to an excessive inflammatory response. Emodin (Emo) is a naturally occurring anthraquinone derivative and an active ingredient of Chinese medicine. It is believed to have anti-inflammatory effects. In this study, we examined the impact of Emo on the pulmonary inflammatory response and the neutrophil function in a rat model of lipopolysaccharide (LPS)-induced ALI.

Results: Treatment with Emo protected rat against LPS-induced ALI. Compared to untreated rat, Emo-treated rat exhibited significantly ameliorated lung pathological changes and decreased tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). However, Emo has no protective effect on the rat model of acute lung injury with neutrophil deficiency. In addition, treatment with Emo enhanced the bactericidal capacity of LPS-induced neutrophils via the up-regulation of the ability of neutrophils to phagocytize bacteria and generate neutrophil extracellular traps (NETs). Emo also downregulated the neutrophil respiratory burst and the expression of reactive oxygen species (ROS) in LPS-stimulated neutrophils, alleviating the damage of neutrophils to surrounding tissues. Finally, Emo can accelerate the resolution of inflammation by promoting apoptosis of neutrophils.

Conclusion: Our results provide the evidence that Emo could ameliorates LPS-induced ALI via its anti-inflammatory action by modulating the function of neutrophils. Emo may be a promising preventive and therapeutic agent in the treatment of ALI.

Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are devastating clinical syndromes. Bacterial or viral pneumonia and sepsis are the most common causes of ALI and ARDS, wherein Gram-negative bacteria are a prominent cause ^[1]. Lipopolysaccharide (LPS), an important component of the outer membrane of Gram-negative bacteria, is one of the main pro-inflammatory reaction factors in ALI and leads to neutrophil recruitment and pulmonary edema ^[2-3].

Neutrophils are the most abundant white blood cells. They are essential for killing bacteria and other microorganisms, and they also have a significant role in regulating the inflammatory response. In order to exert its bactericidal function, circulating neutrophils first follow the chemokine gradient to the inflammatory site. They kill the pathogenic microorganisms through mechanisms, such as phagocytosis, secretion of germicidal particles and neutrophil extracellular traps (NETs).

Neutrophil migration through the activated venous wall is a prerequisite for neutrophil entry to interstitial infection injury and emergency sites ^[4]. The local hydrolysis of JAM-C proteins on vascular endothelial cells by neutrophil elastase (NE) is a necessary factor for neutrophils to pass through the vein wall ^[5]. One of the hallmarks associated with the antimicrobial and inflammatory actions of neutrophils is the activation of a powerful respiratory burst, during which neutrophils produce and release large quantities

of antibacterial peptides, proteases, and reactive oxygen species (ROS) to kill pathogens entrapped inside the vacuole. The phagocyte NADPH oxidase activity and ROS production play a key role in host defense against microbial pathogens [6].

In response to specific stimuli, neutrophils extrude modified chromatin structures decorated with specific cytoplasmic and granular proteins called NETs [7]. NETs is a special form of bactericidal method for neutrophils [8]. The engulfment of apoptotic cells by phagocytes is essential for maintaining normal tissue homeostasis and a prerequisite for the resolution of inflammation [9]. The removal of apoptotic neutrophils from circulation regulates granulopoiesis, and prevents secondary lysis and spillage of noxious neutrophil substances [10-11].

Rhubarb is one of the most commonly used herbs in traditional Chinese medicine. Emodin (Emo) is mainly extracted from rhizome and root of rhubarb. Its chemical name is 1,3,8-trihydroxy-6-methyl-anthraquinone, and its molecular weight is 270.23. Its chemical structure belongs to the hydroxyindole family. In recent years, studies have found that Emo has a wide range of pharmacological effects, mainly anti-tumor, anti-microbial, anti-oxidation, and anti-inflammatory [12-15]. Modern researches show that Emo extract could significantly inhibit the inflammatory responses in a variety of inflammatory animal models [16]. This is achieved by directly or indirectly inhibiting the activity of inflammatory cells. Currently, the study of Emo on inflammatory cells is mainly focused on macrophages. Its modulation effects on other immune cells, such as neutrophils which play a very important role in lung inflammation-related disease, are not fully elucidated.

This study was designed to investigate whether Emo could exert protective effects on LPS-induced ALI in vivo and whether Emo plays a protective role by affecting the function of neutrophils. In addition, we investigated the effect of different doses of Emo on the function of neutrophils in vitro.

Results

Effects of Emo on the proliferation of rat neutrophils

As shown in Fig. 1A, the structure of Emo shows that it belongs to the terpenoid family. Pure rat neutrophils can be obtained by gradient centrifugation as mentioned above. Fig.1B shows rat neutrophils after immunofluorescence staining. Red is neutrophil MPO and blue is neutrophil lobulated nucleus. The ratio of rat neutrophils was quantified, and the purity could reach 94.1% of the isolated cells. Subsequently, different concentrations of Emo were administrated to the neutrophils for 4h. As shown in Fig. 1C, Emo at the concentration of 40 μ M and 80 μ M had high cytotoxic effect on neutrophils, while the lower concentration of Emo had little cytotoxic effect on neutrophils. EMO of 5 μ M, 10 μ M and 20 μ M was selected for further study.

Emo protected lung tissues from LPS-induced ALI

First, we evaluated the effect of Emo on LPS-induced ALI. The control group revealed normal pulmonary histology (Fig. 2A). In contrast, lung tissues in the LPS group were significantly damaged, with interstitial edema, hemorrhaging, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitium and alveolar spaces, as evidenced by an increase in lung injury score ($p \leq 0.01$). Compared with the control group, all the morphologic changes observed were less pronounced in the LPS+Emo 5 mg/kg group, LPS+Emo 10 mg/kg group and LPS+Emo 20 mg/kg group. Emo 5 mg/kg attenuated LPS-induced pathologic changes as shown by the decrease in lung injury score ($p \leq 0.05$). As Emo dosage was increased, the attenuation of LPS-induced pathologic also increased. 10 mg/kg and 20 mg/kg Emo could significantly attenuate LPS-induced pathologic changes ($p \leq 0.01$) (Fig. 2B).

As expected, the concentrations of TNF- α and IL-1 β in the lung tissue homogenate were significantly higher in the LPS group than in the control group. By comparison, the level of TNF in LPS+Emo 5 mg/kg group, LPS+Emo 10 mg/kg group and LPS+Emo 20 mg/kg group decreased in varying degrees, and the decrease of TNF level in LPS+Emo 20 mg/kg group was the most significant. (Fig. 2C). After treatment with Emo 10 mg/kg and Emo 20 mg/kg, the level of IL-1 in lung homogenate was also decreased, and Emo 20 mg/kg could significantly reduce the level of IL-1 (Fig. 2D).

Emo impact on respiratory burst and ROS production of neutrophil

As shown in Fig. 3A, the level of O_2^- in which the LPS group has a very significant boosting effect on the production of neutrophil O_2^- following fMLP stimulation was detected ($P < 0.01$). Emo decreased the production of O_2^- in a concentration-dependent manner ($P < 0.01$) (Fig. 3A).

The expression of ROS in neutrophils was measured by Luminometer (Fig. 3B-C). According to the literature, we found that there are four groups of agents that can induce ROS production. The first group of priming agents is composed of physiological inflammatory agents, such as C5a, or formylated peptides/proteins such as fMLP. The second group of priming agents is composed of proinflammatory cytokines and adipokines, such as tumor necrosis factor (TNF- α), IL-8. The third group of priming agents is composed of TLR agonists, such as lipopolysaccharide (LPS or endotoxin). The fourth group of priming agents is Phorbol ester (PMA). Therefore, we chose three agents IL-8, fMLP and PMA to stimulate neutrophils to produce ROS.

It was found that PMA was the best at stimulating neutrophils to produce ROS ($P < 0.01$) (Fig. 3B). Therefore, in the following experiments, PMA was used as an inducer to stimulate neutrophils to produce ROS. Neutrophils were divided into five groups: control group, LPS group (100 ng/ml), LPS+Emo 5 μ M group, LPS+Emo 10 μ M group and LPS+Emo 20 μ M group. Stimulation with Emo was performed for 30 min prior to LPS treatment. PMA (1.5 ng/ml) was used to treat the cells respectively. The ROS production of neutrophil was detected according to the above procedure. In this experiment, the concentration of ROS in the LPS group was significantly increased compared with the control group ($P < 0.01$). Compared with the LPS group, 10 μ M Emo and 20 μ M Emo reduced ROS production, with 20 μ M Emo having an even better effect ($P < 0.01$). At 5 μ M, Emo has no effect ($P \geq 0.05$) (Fig. 3C).

Emo effect on the release of neutrophil elastase and NETs production of neutrophils

As shown in Fig. 4A, the amount of elastase released was significantly higher in the LPS group following stimulation with fMLP than in the control group ($P < 0.01$). This indicates that neutrophils increase the release of elastase in order to fight infection. elastase release in LPS+Emo 10 μ M and LPS+Emo 20 μ M groups were significantly higher than control group and LPS group ($P < 0.01$).

The literature also confirmed that four group of reagents that induce ROS production can also stimulate the production of NETs, so we also choose four agents LPS, IL-8, fMLP and PMA to induce the production of NETs. Our study found that PMA stimulates neutrophils to produce the most NETs compared with IL-8, LPS and fMLP ($P < 0.01$) (Fig. 4B). As a result, PMA was used as the inducer.

Therefore, in the following experiments, PMA was used as an inducer to stimulate neutrophils to produce NETs. Neutrophils were divided into five groups: control group, LPS group(100ng/ml), LPS+Emo 5 μ M group, LPS+Emo 10 μ M group and LPS+Emo 20 μ M group. Stimulation with Emo was performed for 30min prior to LPS treatment. PMA (1.5 ng/ml) was used to treat the cells respectively. The NETs production of neutrophil was detected according to the above procedure. This study found that after PMA stimulation, the generation of NETs in the LPS group increased significantly compared with the control group ($P < 0.05$). In 5 μ M Emo and 10 μ M Emo groups, the generation of NETs did not increase compared with the LPS group. However, 20 μ M Emo significantly increased the production of NETs compared with the LPS group ($P < 0.05$) (Fig. 4C).

Emo promotes phagocytosis of neutrophils

Neutrophils were isolated and adjusted concentration to 1×10^6 /ml. After administration of LPS and Emo, neutrophils were co-cultured with pHrodo red E.coli and pHrodo green S.aureus for 30 min, 45 min, and 60 min. We found that the average fluorescence intensity of neutrophils phagocytosis of pHrodo green S.aureus increased from 2749.44 ± 469.95 at 30 min to 12305.01 ± 1425.02 at 60 min ($P < 0.01$)(Fig. 5A). In addition, the average fluorescence intensity of neutrophils phagocytosis of pHrodo red E.coli increased from 4159.30 ± 357.72 at 30 min to 7340.26 ± 597.80 at 60 min ($P < 0.01$)(Fig. 5B). This result suggests that neutrophils phagocytosis of pHrodo green S.aureus and pHrodo red E.coli increases with time, reaching a peak at 60 min. We also found that LPS-stimulated neutrophils showed increased phagocytosis of pHrodo green S.aureus compared with the control group at 45 min and 60 min ($P < 0.01$) (Fig. 5A). While the phagocytosis of neutrophils to pHrodo red E.coli at 30 min, 45 min and 60 min was higher than control group, especially at 60 min ($P < 0.05$ & $P < 0.01$)(Fig. 5B). In the groups treated with Emo, the experimental results showed that the neutrophil phagocytosis of pHrodo green S.aureus and pHrodo red E.coli at 45 min and 60 min was significantly enhanced for the 10 μ M and 20 μ M Emo groups ($P < 0.05$)(Fig. 5C-D).

Emo promotes apoptosis of neutrophils

Neutrophils were isolated and adjusted concentration to 1×10^6 /ml. Neutrophils were treated with Emo (5 μ M, 10 μ M and 20 μ M) for 4h and 24h. After labeling with Annexin V-FITC and SYTOX, the apoptosis of neutrophils was measured by flow cytometry (Fig. 6A). Our 4 h apoptosis experiment showed that apoptosis of cells did not increase significantly in the LPS group and the LPS+Emo 5 μ M group ($P \geq 0.05$) (Fig. 6B). However, the apoptosis of cells increased significantly in the LPS+Emo 10 μ M group and the LPS+Emo 20 μ M group, when compared with the control group ($P \leq 0.05$) (Fig. 6B). We further compared the ratio of living cells, dead cells and necrotic cells between the LPS group and the LPS+Emo 20 μ M group, and found that there was no significant difference in the ratio of living cells, dead cells and necrotic cells between the two groups ($P \geq 0.05$) (Fig. 6D). It suggests that Emo 10 μ M and Emo 20 μ M can promote the apoptosis of neutrophils following LPS stimulation.

The results of the 24 h apoptosis experiment are consistent with those of the 4 h experiment, confirming that 10 μ M Emo and 20 μ M Emo can promote apoptosis of cells following LPS stimulation at 24 h ($P \leq 0.05$) (Fig. 6C). Moreover, when compared with the LPS group, dead cells in the LPS+Emo 20 μ M group were significantly reduced ($P \leq 0.05$) (Fig. 6E). It suggests that 20 μ M Emo can not only promote the apoptosis of neutrophils following LPS stimulation but also reduce the ratio of dead neutrophil cells.

Emo has no protective effect on the rat model of acute lung injury with neutrophil deficiency.

On the second day after receiving cyclophosphamide immunosuppression, the rats showed poor mental state, reduced diet and activity, gray and yellow hair, and the above performance was aggravated after injection of LPS.

From the H&E staining samples of rat lung tissue, we can see that the lung histology of the control group is normal (Fig. 7A). In LPS group, the lung tissue injury was markedly damaged, with interstitial edema, hemorrhaging, thickening of the alveolar wall, and infiltration of inflammatory cells into the interstitium and alveolar spaces, as evidenced by an increase in lung injury score ($p \leq 0.01$). In LPS+Emo 20 μ M group, the histomorphology of lung tissue was similar to that of LPS, and the injury was severe (Fig. 7B). Emo could not reduce the concentration of TNF- α and IL-1 β in lung tissue homogenate of LPS group. (Fig. 7C-D).

Discussion

Acute lung injury is a rapid non-cardiogenic bilateral lung infiltration syndrome characterized by alveolar vascular injury, neutrophil infiltration and accompanied by the release of pro-inflammatory factors^[17]. Neutrophils and macrophages play an important role in the process of lung injury, in which neutrophils are the first line of defense against the invasion of pathogens. Macrophages play a key role in the subsequent clearance of apoptotic neutrophils and promoting the regression of inflammation. This study focuses on the effect of Emo on the function of neutrophils in ALI, so as to explore the anti-inflammatory mechanism of Emo.

Neutrophils are the first line of defense against bacterial invasion and make up the largest proportion of leukocytes, which play a vital role in nonspecific immunity [18]. When the body is invaded by foreign microorganisms, neutrophils first gather at the inflammatory lesion site to play a defensive role. Emo (1,3,8-trihydroxy-6-methylanthraquinone) is a naturally occurring anthraquinone derivative and an active ingredient of some Chinese herbs. These herbs have been widely used as traditional medicines in many countries, especially in eastern Asia. Currently, a number of researchers are focusing on the pharmacological effects of this compound. In the last 5 years, there have been many reports on the anti-tumor and anti-inflammatory effects of Emo [19-20]. These pharmacological properties suggest that Emo might be a valuable therapeutic option for the prophylaxis and treatment of various diseases, including asthma, atopic dermatitis, osteoarthritis, diabetes and diabetic complications, atherosclerosis, Alzheimer's disease (AD), hepatic disease and several types of cancers such as pancreatic cancer, breast cancer, hepatocellular carcinoma and lung carcinoma.

In this study, we have identified a unique role for Emo related to the function of neutrophils in LPS-induced inflammatory lung injury. Our data clearly demonstrate that exogenous administration of Emo can reduce the lung injury score of ALI rats induced by LPS. Our experimental results also confirm that treatment with Emo inhibits plasma inflammatory cytokines, such as TNF- α and IL-1 β . Decreased plasma TNF- α and IL-1 β levels in Emo-treated rats are consistent with the paradigm that Emo reduces the inflammatory response following ALI, in addition to ameliorating the severity of disease [21]. This suggests the potential of Emo as an effective lung protective therapeutic agent.

Neutrophils are over-activated during ALI, which releases neutrophil respiratory burst and ROS to damage the surrounding tissues. Priming of the neutrophil ROS production is believed to be involved in many inflammatory diseases, such as acute respiratory distress syndrome (ARDS), rheumatoid arthritis (RA), atherosclerosis, ischemia-induced tissue injury, hypertension, diabetes, kidney disease, and sepsis [22]. Our experimental results supported this conclusion. In this study, we evaluated the initiation of respiratory burst of suspended neutrophils in vitro using a SOD-inhibited cytochrome c reduction test, and luminol-amplified chemiluminescence to measure ROS [23]. SOD-inhibitable cytochrome c reduction assay is a standard technique for measuring superoxide dismutase production and has the advantages of being a quantitative method that is specific for extracellular superoxide anions [24]. Luminol-amplified chemiluminescence method has the advantages of being very sensitive and able to detect both extracellular and intracellular ROS. We found that Emo reduces neutrophil respiratory burst and ROS production in a dose-dependent manner, thus alleviating the damage from over-active neutrophils to surrounding tissues.

NE is a serine protease secreted by polymorphonuclear neutrophils, which plays an important role in many physiological and pathological processes such as the various inflammatory reactions, adult respiratory distress syndrome, and acute lung injury [25]. When exposed to various inflammatory factors, neutrophils release NE via degranulation, which is then involved in the inflammatory response. Some studies believe that NE is a destructive elastase that attacks the extracellular matrix and modulates

inflammation and tissue remodeling. Its involvement may be direct (tissue damage) or indirect (proinflammatory or proapoptotic) [26-28]. However, it is undeniable that NE is a necessary condition for neutrophil migration to inflammatory sites, and the proteolytic activity of NE contributes to the body's defence against infectious agents by promoting the destruction of pathogenic bacteria [29-31]. Our results show that EMO at the concentration of 10 μ M and 20 μ M can promote neutrophil elastase release induced by fMLP. To analyze the reasons, we consider that the time point selected in this study is 4 hours after the successful establishment of acute lung injury model induced by LPS. This is the peak of inflammation and it is the time when a large number of neutrophils migrate to the inflammatory site. At this time, Emo promotes the release of neutrophil elastase is conducive to neutrophil migration to the site of inflammation to play a bactericidal function.

NETs are a kind of outer network structure, composed of complex three- reticular structure of DNA, and contains the main protein, elastase, myeloperoxidase (MPO), cathepsin G and other antibacterial proteases. It is a special bactericidal mechanism for neutrophils [8]. NETs are a double-edged sword in the inflammation process. Some studies have shown that a large number of extensive NETs formations can damage epithelial cells and endothelial cells, resulting in the spread of inflammation [32]. However, there are still a large number of studies that show that in the acute stages of inflammation, NETs not only capture microorganisms but also limit the diffusion of cytotoxic antimicrobial proteins and reduce host tissue damage [33-34]. Our research results show that the production of NETs is higher than that of the control group following LPS stimulation of neutrophils, which indicates that the production of NETs is helpful in enabling neutrophils to play a bactericidal function in the acute stages of inflammation. Compared with the LPS group, 5 μ M Emo did not increase the generation of NETs, but 10 μ M Emo and 20 μ M Emo could significantly promote the generation of NETs. This data showed that Emo at concentrations of 10 μ M and 20 μ M could enhance the bactericidal ability of neutrophils by promoting the production of NETs.

During an inflammatory injury, neutrophils gather at the injury site to exert phagocytosis, an important neutrophil mechanism to kill pathogenic microorganisms. Our study found that Emo promotes phagocytosis of neutrophils: the phagocytosis of *S.aureus* and *E.coli* by neutrophils increases with time, reaching a peak at 60 min. At 30 min, 45 min and 60 min, the phagocytosis of *S.aureus* and *E.coli* by LPS-stimulated neutrophils was significantly stronger than the control group. We also found that while Emo 5 μ M did not promote the phagocytosis of neutrophils, 10 μ M Emo and 20 μ M Emo significantly promoted neutrophil phagocytosis of *S.aureus* and *E.coli*, indicating that Emo needs to reach a certain dose to promote neutrophil phagocytosis.

It is well known that neutrophils have a short life span, mainly due to apoptosis of neutrophils in the circulatory system over time. The resolution of an acute inflammatory response requires subsequent phagocytosis of apoptotic neutrophils by macrophages. Delayed apoptosis of activated neutrophils can lead to persistent acute lung inflammation and can eventually develop into one of the mechanisms of ARDS [35]. In this experiment, we mainly compared the effects of different doses of Emo on LPS-

stimulated neutrophil apoptosis. We found that 5 μM Emo had no effect on neutrophil apoptosis after 4 h and 24 h of treatment in the LPS group. However, 10 μM Emo and 20 μM Emo significantly promote the apoptosis of neutrophils in the LPS group after treating the neutrophils for 4 h and 24 h in a dose-dependent manner. This indicates that medium and large doses of Emo can promote the apoptosis of neutrophils and that the larger the dose, the better the effect. We have further compared the effects of 20 μM Emo on dead neutrophil cells and necrotic neutrophil cells in the LPS group. The results show that 20 μM Emo can only promote the apoptosis of neutrophils at 4 h, but has no effect on dead cells and necrotic cells. However, at 24 h, 20 μM Emo can not only promote the apoptosis of neutrophils but also reduce the number of dead neutrophil cells.

Finally, we used cyclophosphamide to inhibit circulating neutrophils in rats, and made a rat model of acute lung injury with neutrophil deficiency. The results showed that Emo could not reduce the pulmonary inflammatory infiltration and lung injury score in the rat model of acute lung injury with neutrophil deficiency, nor could it reduce the levels of inflammatory factors TNF- α and IL-1 β in lung homogenate. It is further confirmed that Emo had a protective effect on ALI rats by affecting the function of neutropenia.

In summary, this study demonstrates that Emo alleviates lung injury and reduces the release of inflammatory cytokines in rats with acute lung injury induced by LPS, but has no protective effect on acute lung injury in rats with neutrophil deficiency. Moreover, Emo also downregulated neutrophil respiratory burst and the production of ROS in the LPS-stimulated neutrophils, thus reducing the damage of neutrophils to the surrounding tissues. Emo can also up-regulate the ability of neutrophils to phagocytize bacteria and generate NETs, thereby enhancing the bactericidal ability of neutrophils. In addition, Emo can promote the apoptosis of neutrophils and accelerate the resolution of inflammation. Interestingly, our research has confirmed that small doses of Emo have no obvious effect on neutrophil function. High-dose Emo also had obvious toxic effect on neutrophils. Only Emo (animal: 10 -20 mg/kg; cells: 10-20 μM) had an obvious effect, and in this range in a dose-dependent manner. Our findings reveal a novel mechanism for Emo to attenuate the inflammatory reaction and shows that Emo could be exploited therapeutically for acute lung injury.

Conclusions

Emo has a protection effect on LPS-induced acute lung injury rats. It can alleviate lung injury and reduces the release of inflammatory cytokines, by affecting the various function of neutrophils. The appropriate dose (animal: 10 -20 mg/kg; cells: 10-20 μM) has the best effect.

Materials And Methods

Reagents

LPS (Escherichia coli serotype 055:B5), formyl methionyl leucyl phenylalanine (fMLP), interleukin-8 (IL-8), phorbol ester (PMA), cell chromatography C (Cytochrome C), superoxide dismutase (SOD), Elastase, Hydroxyethylpiperazine Ethylsulfonic Acid (HEPES) and Emo were obtained from Sigma-Aldrich (St Louis,

MO, USA). Tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) ELISA kits were obtained from R&D Systems (Minneapolis, MN). Anti-Myeloperoxidase (MPO) antibody (abcam, ab65871), MNase, RP-1 antibody (BD 550002), SYTOX Green, and Annexin V-FITC were obtained from eBioscience (San Diego, CA). pHrodo red E. coli (Cat.No.4615), pHrodo green S.aureus (Cat. No. 4620) were obtained from Sartorius (Göttingen, Germany). RPMI 1640, fetal bovine serum (FBS), trypsin, and enzyme-free cell dissociation buffer were purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin in saline citrate buffer were from Invitrogen (Carlsbad, CA, USA). Other chemical reagents are of analytical grade.

Animals, experimental procedure, and treatments

Experiments were performed on adult male Sprague Dawley rats (250–300 g; Shanghai Experimental Animal Center of China). Rats were provided with water and food ad libitum. The use of animals in this study was approved by Animal Studies Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

Rats were randomized into five groups (n = 6): control group, LPS group, LPS + Emo 5mg/kg group, LPS+Emo10 mg/kg group and LPS + Emo 20mg/kg group. In LPS group, rats received LPS (20mg/kg) through the tail vein. LPS was dissolved in 0.9% normal saline. In the Emo group, rats received Emo (5mg/kg, 10mg/kg and 20mg/kg) via intraperitoneal injection 30 min before LPS exposure. Emo was dissolved in 100% DMSO at a concentration of 200mg/mL and diluted in saline to the final concentration of 1 mg/ml. The action time of LPS-induced acute lung injury in rats was 4 hours.

Pathological studies

Rats were anesthetized with chloral hydrate (7 ml/kg, intraperitoneally), intubated and connected to the animal ventilator (respiratory parameters are tidal volume: 10ml / kg and respiratory rate: 40-60bpm) 4 hours after injection of LPS. After anesthesia and mechanical ventilation with pure oxygen for 1 hour, rats were killed by cutting off the abdominal aorta and bloodletting. Rats were subjected to thoracotomy and PBS (25ml/min) was injected into the right ventricle to flush the pulmonary vessels. Finally, the right lower lung lobe of rats was cut and fixed in 4% paraformaldehyde for 24 hours at room temperature, and 4 μ m sections were embedded in paraffin and stained with hematoxylin and eosin (H&E) for light microscopy analysis. The rest of the lung tissue was frozen in liquid nitrogen for 48 hours and stored in refrigerator at-80 °C.

A semi-quantitative scoring system was adopted to evaluate lung injury, which included alveolar congestion, alveolar hemorrhaging, neutrophil infiltration or aggregation in the airspace or vessel wall, and alveolar wall/hyaline membrane thickness and inflammatory cell infiltration. The grading scale for the light microscopy pathologic findings was as follows: 0 = no injury; 1 = slight injury (25%); 2 = moderate injury (50%); 3 = severe injury (75%); and 4 = very severe injury (almost 100%). The results were graded from 0 to 4 for each item, as described previously. The four variables were summed to represent the lung injury score (total score: 0–16).

Determination of inflammatory cytokines in lung homogenate by enzyme-linked immunosorbent assay (ELISA)

Part of the right lung from individual rats was homogenized and centrifuged, and the levels of TNF- α and IL-1 β in the resulting tissue supernatants were determined using TNF- α and IL-1 β ELISA kits.

After ultrasonic lysis of lung homogenate, the supernatant was obtained by centrifugation at 4 °C for 5000 r/min for 15 minutes. Follow the reagent instructions. 100 μ l of standard or sample to be tested was added to each hole, and the reaction plate was fully mixed and placed at 37 °C for 30 minutes. Wash the reaction plate fully with washing solution for 4 times and print it on the filter paper for 6 times. 100 μ l of enzyme-labeled antibody working solution was added to each well. Put the reaction plate at 37 °C for 30 minutes. The washing board is the same as before. 100 μ l of substrate working solution was added to each hole and reacted in the dark at 37 °C for 15 minutes. Add 100 μ l terminating liquid to each hole and mix well. The absorbance value of 450nm was measured by enzyme labeling instrument within 30 minutes.

Separation and of rat neutrophil

20ml of heparinized fresh rat blood was treated with dextran to induce sedimentation of the red blood cells. Prepare a Percol gradient in a 15ml Falcon Tube by first pipetting 5ml 56% Percol, then put the sucker to the bottom of the tube, and slowly pipetting 2.5ml 80% Percol to the bottom. Then carefully draw up the plasma and white blood cell suspension from the blood sample with a pipette and slowly layer them on top of the Percol gradient. 4 °C, 220g, centrifuge for 20 minutes, accelerate to 1, decelerate to 0, remove the top layer of serum, suck out the neutrophil layer, add PBS to wash twice, the cells were resuscitated with RPMI-1640 medium containing 5% FBS and then counted so that the cell concentration was 1×10^6 /ml.

Neutrophils were divided into five groups: control group, LPS group(100ng/ml), LPS+ Emo 5 μ M group, LPS+ Emo 10 μ M group and LPS+ Emo 20 μ M group. Stimulation with Emo was performed for 30min prior to LPS treatment.

Immunofluorescence staining

Neutrophils were isolated and adjusted concentration to 5×10^5 /ml. They were centrifuged for 5 minutes in order to fix the cells on glass slides and dried. Neutrophils were fixed with 4% paraformaldehyde at room temperature for 15 minutes. Then they were permeabilize with 0.5% TritonX-100PBS for 20 minutes and blocked with blocking solution buffer for 30 minutes. Neutrophils were incubated overnight at 4 °C with primary antibody (MPO antibody 1: 500), and then incubated the secondary antibody (1:200) onto glass slides for 1 h at room temperature in moist environmental box. DAPI was diluted into PBS in accordance with the instructions and added onto glass slides for 5 minutes at room temperature. 20 μ L of mounting medium was promptly added onto the slide, then put a cover slip on it, followed by mounting the coverslip with nail enamel. Finally, the slides were observed under a fluorescence microscope.

Cell counting kit-8 (CCK8 assay)

Neutrophils were isolated and adjusted concentration to 1×10^6 /ml. Neutrophils were added to 96-well plate (100 μ l per hole). Three multiple holes and blank control holes were set up at the same time (no cells). Emo was added into test wells as inhibitor, which was divided into five concentration gradients of 5 μ M, 10 μ M, 20 μ M, 40 μ M and 80 μ M. PBS was used as negative control. After 4 hours of Emo intervention, 10 μ l of CCK-8 reagent was added into each well. The plate was cultured in 5%CO₂ incubator at 37 °C for 3 hours, the OD value of each well of wavelength 450nm was detected by enzyme labeling instrument. Cell inhibition rate (IC) can be calculated according to the formula:

Cell inhibition rate (IC) = [(control group OD value - experimental group OD value) / (control group OD value - zeroing group OD value) \times] 100%

Respiratory burst detection

The reactive oxygen species released by the activated inflammatory cells can reduce the membrane non-penetrating cytochrome C. The reduced cytochrome C has an absorption peak at 550 nm. Therefore, the amount of reduced cytochrome C is measured using a spectrophotometer. The amount of active oxygen produced can be inferred from this data. \square Set control group, LPS group and 3 Emo groups, and the appropriate amount of Emo was added in each group, 100 μ l of cytochrome C (1.5 mg/ml) and 100 μ l neutrophils (2×10^7 /ml) was then added; \square 10 μ l SOD (5000 U/ml) was added, and the corresponding dose was added to the test group, equilibrated in a 5% CO₂ incubator at 37 °C for 10min; \square 10 μ l cytochalasin B (1mmol/L) was added to each group and after 3min, 10 μ l fMLP (0.1mmol/L) was added for a total of 1 ml and each group was incubated in a 5% CO₂ incubator at 37 °C for 30min; \square Each group was removed and centrifuged at 2000r/min for 10 min; \square Supernatant was collected and the OD value was measured with a spectrophotometer. Since the production of O₂⁻ and the decrease in cytochrome C is in a 1:1 mole stoichiometric relationship, the yield of O₂⁻ is easily calculated. The millimolar extinction coefficient of the 1 cm optical path is 21.1, and the amount of O₂⁻ produced by 2×10^6 / ml of cells in 1 ml of the solution with a diameter of 1 cm can be directly calculated according to the formula:

$OD \times 47.4 = \text{nmol O}_2^- / 2 \times 10^6 \text{ cells} / \text{time unit}$
test group O₂⁻ inhibition rate = (control O₂⁻ content - test group O₂⁻ content) / control group O₂⁻ content \times 100%.

Elastase release assay

The detection of neutrophil elastase release was mainly carried out by Neutrophil Elastase Activity Assay kit (ab204730). Five elastase solution test groups were prepared: Control group, LPS group, and Emo group (reaction solution concentration: 5 μ M, 10 μ M, 20 μ M). \square The isolated neutrophils were rinsed twice with PBS (pH 7.4), the number of cells was adjusted to 1×10^7 /ml, 500 μ l of solution was added to each group, and the corresponding drugs were added. Finally, each group was supplemented with PBS to 600 μ l. The groups were pre-incubated for 30 min at 37 °C in a 5% CO₂ incubator. In addition to Control

group, 6µl of cytochalasin B (1 mmol/L) and LPS (100ng/ml) was added to each group and cultured at 37 °C in a 5% CO₂ incubator for 20 min. The tubes were then placed in an ice-water bath to terminate the reaction. They were centrifuged at 1500 r/min for 5 min, and the supernatant was dispensed and stored at -80 °C until use. **Elastase determination.** Elastase standard was diluted with PBS: 50, 37.5, 25, 18.75, 12.5, 9.38, 6.25, 4.69, 3.125 series concentrations (µg/ml) and a PBS blank were used to generate a standard curve. Using a 96-well microtiter plate containing a standard or a 50 µl sample to be tested, 100 µl buffer was added (containing elastase substrate 1 mmol/L, HEPES 0.1 mol/L, NaCl 0.5 mol/L, pH 7.5). The OD value was read at 405 nm by a microplate reader (the emodin absorption is at 405 nm), and then cultured at 37 °C in a 5% CO₂ incubator for 60 min. The OD value at 405 nm was then read again, and the difference between the two OD values was recorded. The OD value of the substrate decomposes, and the elastase content is calculated according to the standard curve.

Measuring neutrophil NETs production

Clear 96-well flat-bottomed plates were prepared, and 100 µl of neutrophils were added to the relevant wells. Lipopolysaccharide (LPS, 100 ng/ml), interleukin-8 (IL-8, 100 ng/ml), phorbol ester (PMA, 1.5 ng/ml) and N-formylthionyl-leucyl-phenylalanine (fMLP, 1000 ng/ml) were used to treat the cells respectively. The control group was treated with an equal volume of medium. They were incubated for 3 h at 37 °C in a 5% CO₂ incubator. SYTOX Green was diluted 1:500 (5mM Stock; 1ul SYTOX Green into 499ul PBS), and then stored in the dark. 20 µl of diluted SYTOX green was added to each well using a fresh tip for each well. 1 µl of MNase was added to each well using a fresh tip for each well. They were then incubated at room temp for 10 min in the dark. Samples were transferred to 0.5 ml micro-centrifuge tubes without any pipetting of the liquid up and down. They were immediately centrifuged at 5000 rpm for 10 min in the micro-centrifuge before 160 µl of the supernatant was removed and transferred to a black 96-well flat-bottomed plate. Fluorescence was measured immediately (programme: Gen5; excitation 485nm, emission 528nm with optics position in top 50% of well with a 10-second 'medium' shake immediately prior to read).

Measuring ROS production by isolated neutrophils

Following isolation, cells were resuspended at 1×10⁶/ml in HBSS (with Ca²⁺ and Mg²⁺) (4.5 ml total) in 15 ml Falcon. 100 µl of neutrophils were added to each well of a 96-well plate. Cells were stimulated with Luminol(0.5mM),IL-8(1.25nM), fMLP(2.5µM), and PMA(25nM) for 1 hour. The luminometer was set up and the ROS level was tested on the instrument.

Measuring the phagocytosis of neutrophils

Neutrophils were isolated and adjusted concentration to 1×10⁶/ml. Following LPS and Emo treatment, neutrophils were inoculated into 96-well plates at 100 µl/well. pHrodo red E. coli and pHrodo green S. aureus were added to neutrophils respectively to stimulate neutrophils for 30 min, 45 min, and 60 min. Neutrophils were incubated at 37°C in a 5% CO₂ incubator in the dark, and then centrifuged at 250g and

4°C for 5 min to remove the supernatant. The cells were resuspended with 100ul of 2% PBS/BSA, and this was repeated twice before the cell suspension from each well was transferred into flow tubes. 100µl of 2% PBS/BSA was added to each tube, gently mixed and placed on ice. Finally, the phagocytosis of neutrophils was measured using flow cytometry.

Measuring the rate of apoptotic neutrophils

Neutrophils were isolated and inoculated into six-well plates at an adjusted concentration of 1×10^6 /ml. The groups were divided into groups and treated for 4 h and 24 h. Cells were harvested as normal and cells were transferred to the appropriate FACS tubes. They were centrifuged at 600 g for 4 min before the supernatant was poured off. Cells were resuspended in 200 µl Annexin V buffer to wash the cells and then pelleted again. The cells were incubated in 100 µl Annexin V-FITC diluted 1:100 in Annexin V buffer for 15-20 min on ice and protected from the light. 200 µl Annexin V buffer was added to each tube. SYTOX was removed from the freezer and defrosted while being protected from the light. A SYTOX stock diluted 1:500 in Annexin V buffer was prepared. Immediately prior to running the sample on the CyAN, 30 µl of the SYTOX solution was added to each tube and they were vortexed well to mix. The FITC and Violet 1 channels on the FACS machine were used to measure.

To establish a rat model of acute lung injury with neutrophil deficiency

Cyclophosphamide (CTX) is a kind of non-specific chemotherapeutic drug in cell cycle, which is widely used in clinic. It can kill the cells in each phase of the proliferation cycle and inhibit the number of leukocytes in bone marrow.

In this experiment, rats were injected intraperitoneally with cyclophosphamide (75 mg /kg)4 days before and 1 day before the acute lung injury induced by LPS. And one day before the acute lung injury model was prepared and one day after the model was prepared, the number of neutrophils in rat tail vein blood was less than 2×10^5 /ml by using the blood cell count version technology, Therefore, the rat model of acute lung injury with neutrophil deficiency was successfully prepared.

Statistical analysis

The data represent the mean \pm SD. There were no missing, lost, or excluded data. Based on previous experience, no prior power analysis was conducted; all data were analyzed by one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. All tests were two-sided, and significance was determined at the $p < 0.05$ level. Statistical analyses were performed using Prism 6.0 software (GraphPad Software, San Diego, CA).

Abbreviations

ALI: Acute lung injury; ARDS: acute respiratory distress syndrome; Emo: Emodin; LPS: lipopolysaccharide; TNF- α : tumor necrosis factor- α ; IL-1 β : Interleukin-1 β ; NETs: neutrophil extracellular traps; ROS: reactive

oxygen species; NE: neutrophil elastase; fMLP: formyl methionyl leucyl phenylalanine; IL-8: interleukin-8; PMA: phorbol ester; Cytochrome C: cell chromatography C; SOD: superoxide dismutase; HEPES: Hydroxyethylpiperazine Ethylsulfonic Acid; FBS: fetal bovine serum; ELISA: enzyme-linked immunosorbent assay; CCK8: Cell Counting Kit-8; CTX: Cyclophosphamide; MPO: myeloperoxidase; RA: rheumatoid arthritis.

Declarations

Ethical Approval and Consent to participate

All animals received care in compliance with the Principles of Laboratory Animal Care and National standards.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Hongxia Mei performed the experiments and Ying Tao collected the raw data. Tianhao Zhang performed the statistical analysis and Feng Qi drafted the manuscript. All authors contributed towards data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

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Table

	Dilution		Concentrations		
	Factor	Volumes	Stock	Working	Final (in cells)
Luminol	1:10	1 ml into 9 ml; pH to 7.3	30 mM	3 mM	0.5 mM
IL8	1:625	1 µl into 624 µl	6.25 µM	10 nM	1.25nM
fMLP	1:500	12 µl into 5922 µl	10 mM	20 µM	2.5µM
PMA	A 1:800	1 µl into 799 µl	1620µM (1 mg/ml)	200nM	25nM
	B 1:10	55 µl into 495 µl			

Figures

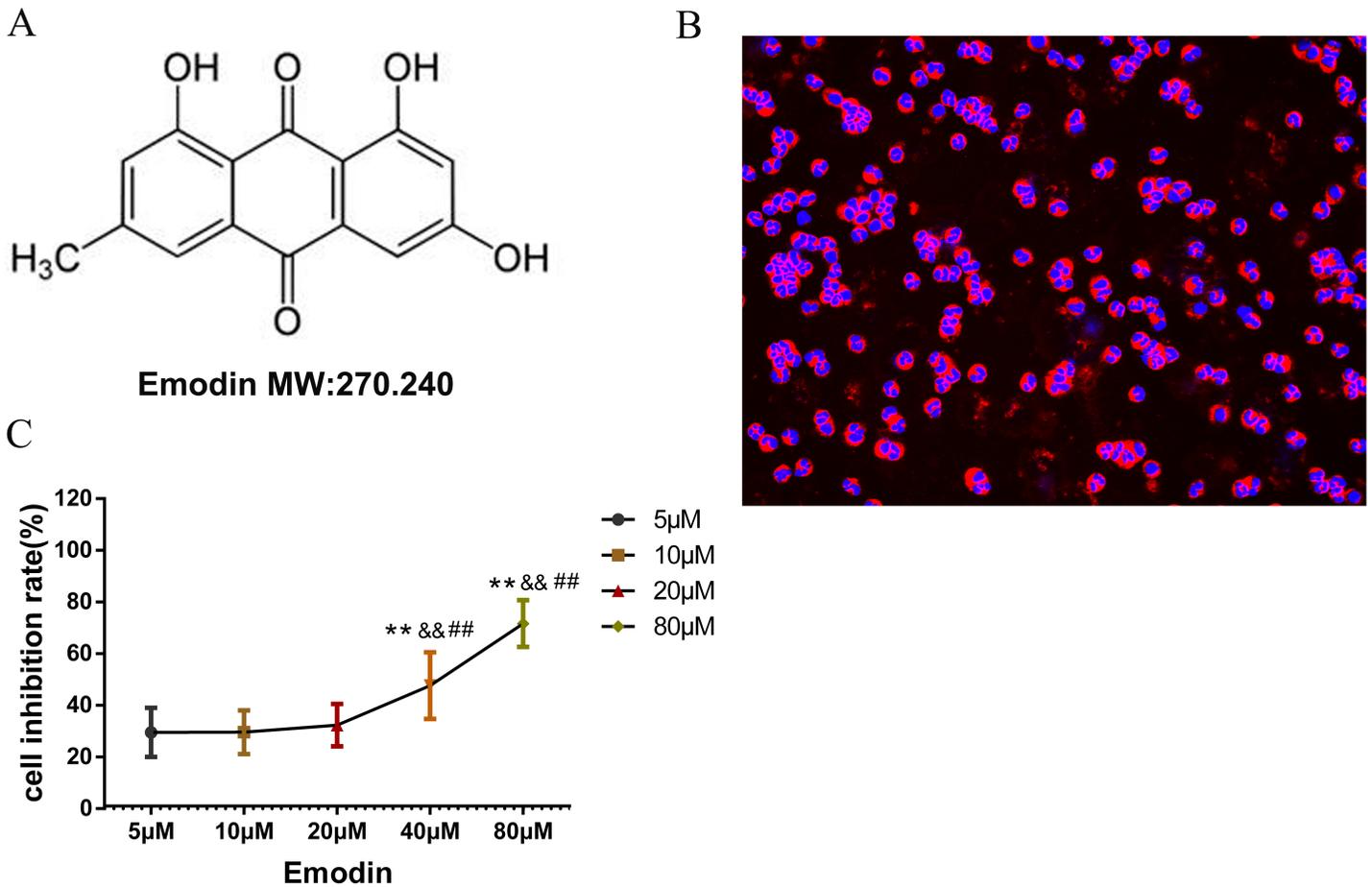


Figure 1

(A) Schematic diagram of Emo; (B) Immunofluorescence staining of rat neutrophils. (C) CCK8 assay. The effect of different concentrations of Emo on neutrophil Cytotoxicity in rats was examined. The data are presented as the mean \pm SD. n = 6. **P \leq 0.01 versus Emo 5 μ M group; &&P \leq 0.01 versus Emo 10 μ M group; ##P \leq 0.01 versus Emo 20 μ M group.

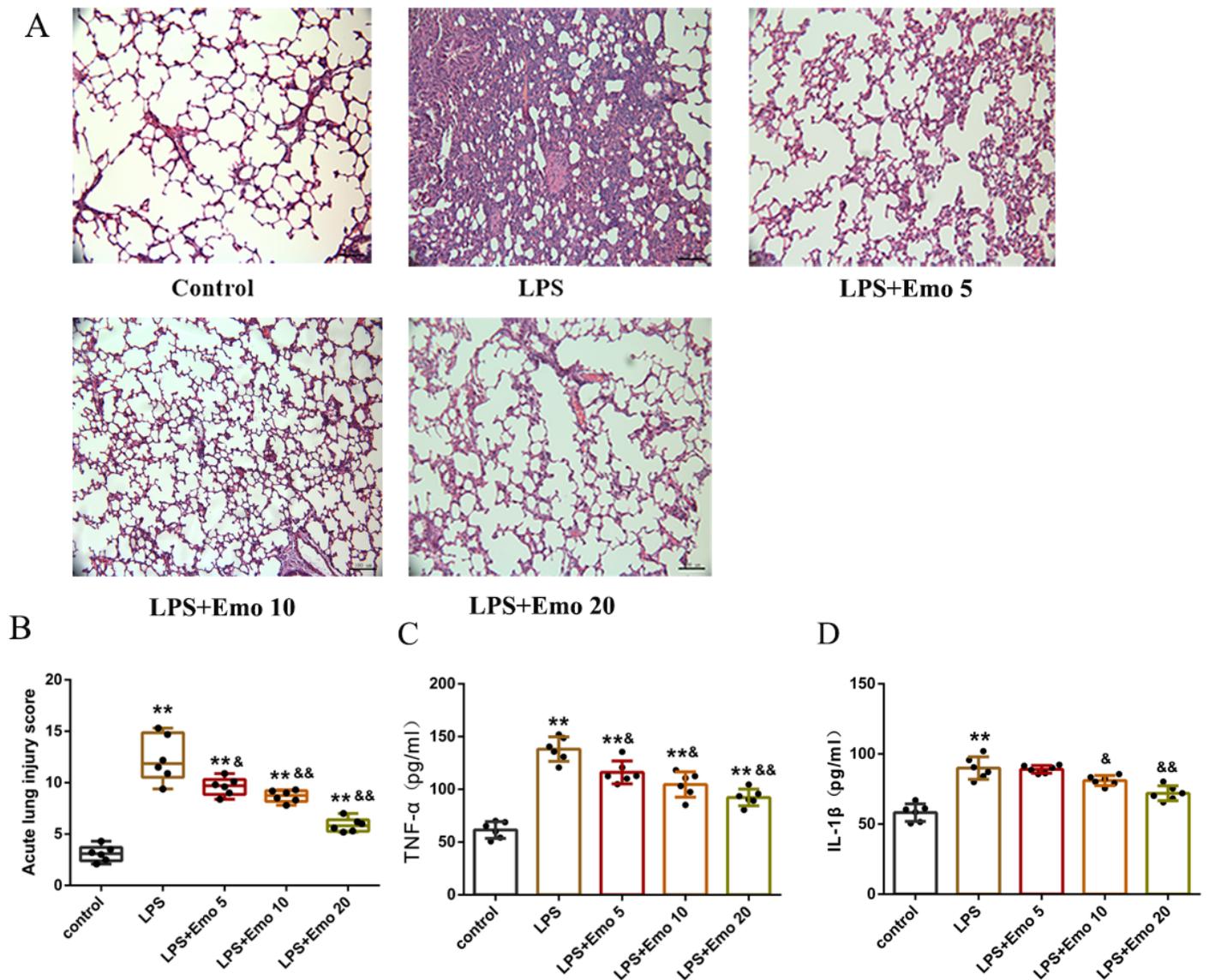


Figure 2

Emo protected lung tissues in LPS-induced ALI. (A) The lung tissues were obtained immediately after exsanguination (4h after LPS), and the effect of Emo was assessed histologically in H&E-stained sections (original magnification $\times 200$). (B) Lung injury scores were recorded from 0 to 16 according to the criteria described in Materials and Methods. (C) The lung tissue homogenate TNF- α protein expression and (D) the lung tissue homogenate IL-1 β protein expression. The data are presented as the mean \pm SD. $n = 6$. ** $P < 0.01$ versus control group; & $P < 0.01$ versus LPS group; && $P < 0.01$ versus LPS group.

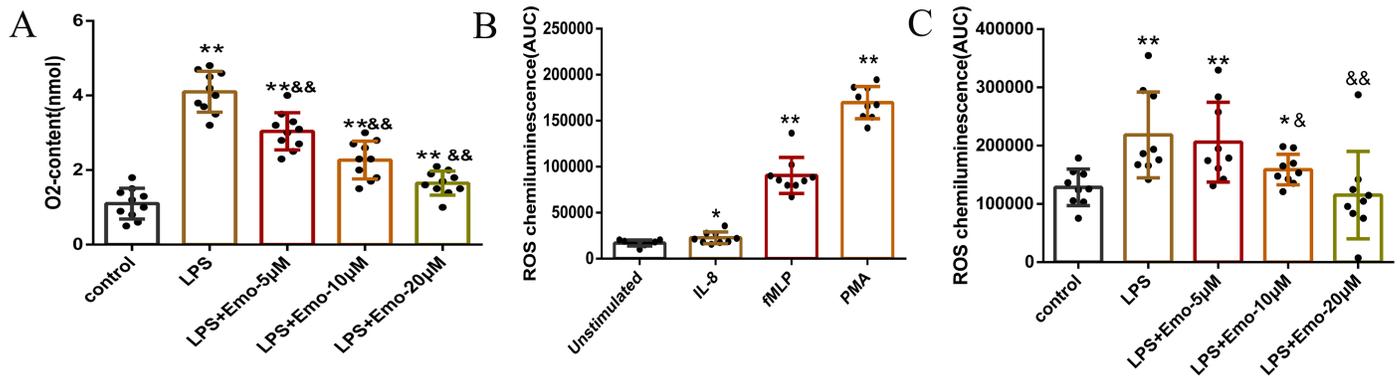


Figure 3

Effect of Emo on neutrophil respiratory burst and ROS production of neutrophil. (A) Effect of Emo on neutrophil respiratory burst in rats. (B) Comparison of the ROS level produced by neutrophils after stimulation with IL-8, fMLP, and PMA. (C) Comparison of the ROS level production by neutrophils stimulated by Emo at different concentrations (5µM, 10µM and 20µM). The data are presented as the mean ± SD. n = 10. *P<0.05 versus control group; **P<0.01 versus control group; &P<0.05 versus LPS group; &&P<0.01 versus LPS group.

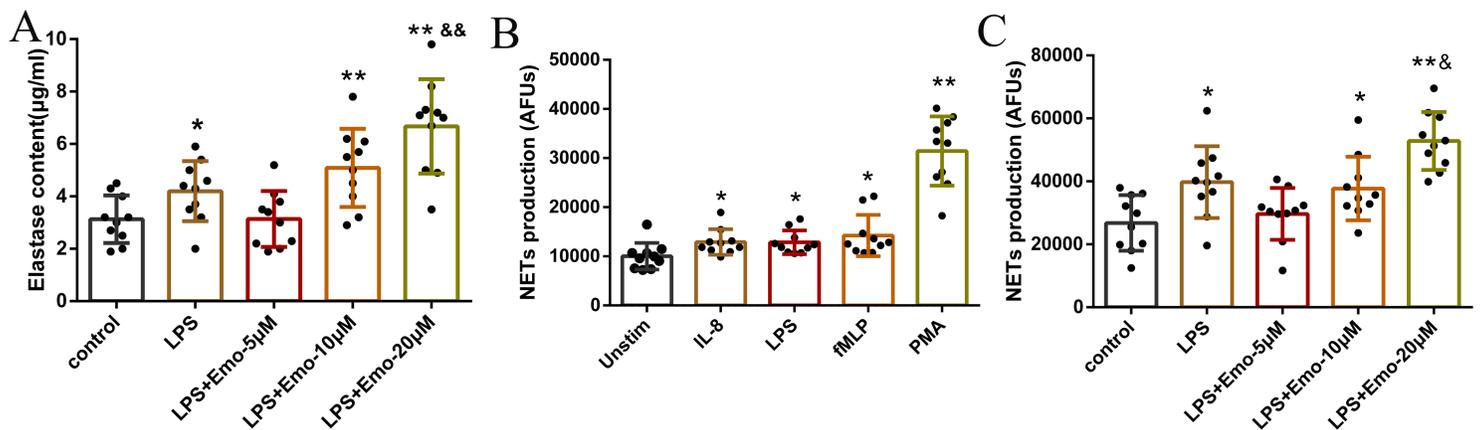


Figure 4

Effect of Emo on the release of neutrophil elastase and NETs production. (A) The detection of neutrophil elastase release was mainly carried out by Neutrophil Elastase Activity Assay kit. (B) Comparison of the NETs produced by neutrophils after stimulation with IL-8, LPS, fMLP, and PMA. (C) Comparison of the NETs production by neutrophils stimulated by Emo at different concentrations (5µM, 10µM and 20µM). The data are presented as the mean ± SD. n = 10. *P<0.05 versus control group; **P<0.01 versus control group; &P<0.05 versus LPS group; &&P<0.01 versus LPS group.

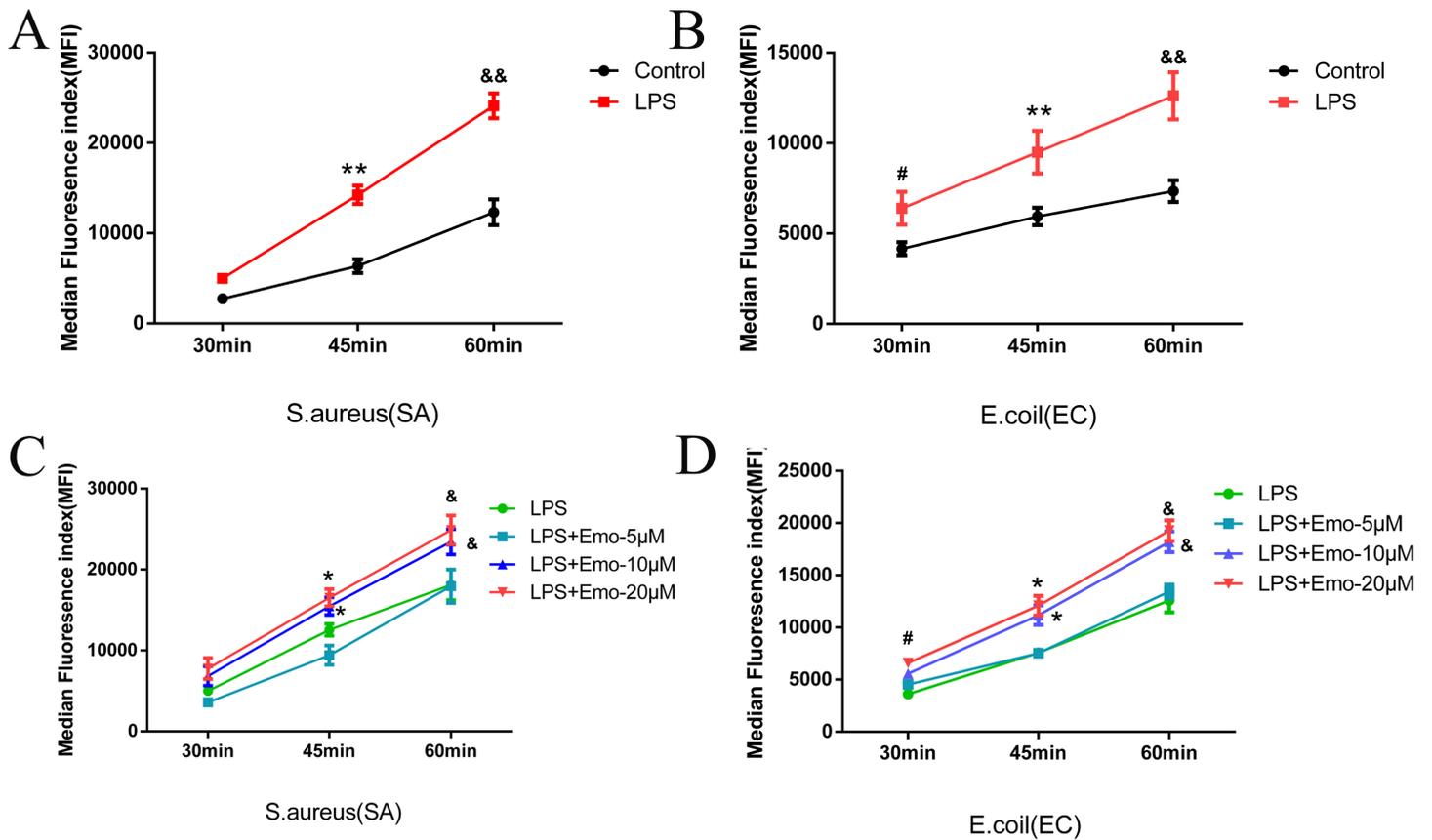


Figure 5

Effect of Emo on Phagocytosis of Neutrophils. (A) The neutrophil phagocytosis of pHrodo Green *S.aureus* was tested with flow cytometry. (B) The neutrophil phagocytosis of pHrodo Red *E.coli* was tested with flow cytometry. The data are presented as the mean \pm SD. $n = 10$. # $P \leq 0.05$ versus LPS group in 30min; ** $P \leq 0.01$ versus LPS group in 45 min; && $P \leq 0.01$ versus LPS group in 60 min. (C) Comparison of the phagocytosis of pHrodo Green *S.aureus* by neutrophils stimulated by Emo at different concentrations (5 μ M, 10 μ M and 20 μ M). (D) Comparison of the phagocytosis of pHrodo Red *E.coli* by neutrophils stimulated by Emo at different concentrations (5 μ M, 10 μ M and 20 μ M). The data are presented as the mean \pm SD. $n = 10$. # $P \leq 0.05$ versus LPS group in 30min; * $P \leq 0.05$ versus LPS group in 45 min; & $P \leq 0.05$ versus LPS group in 60 min.

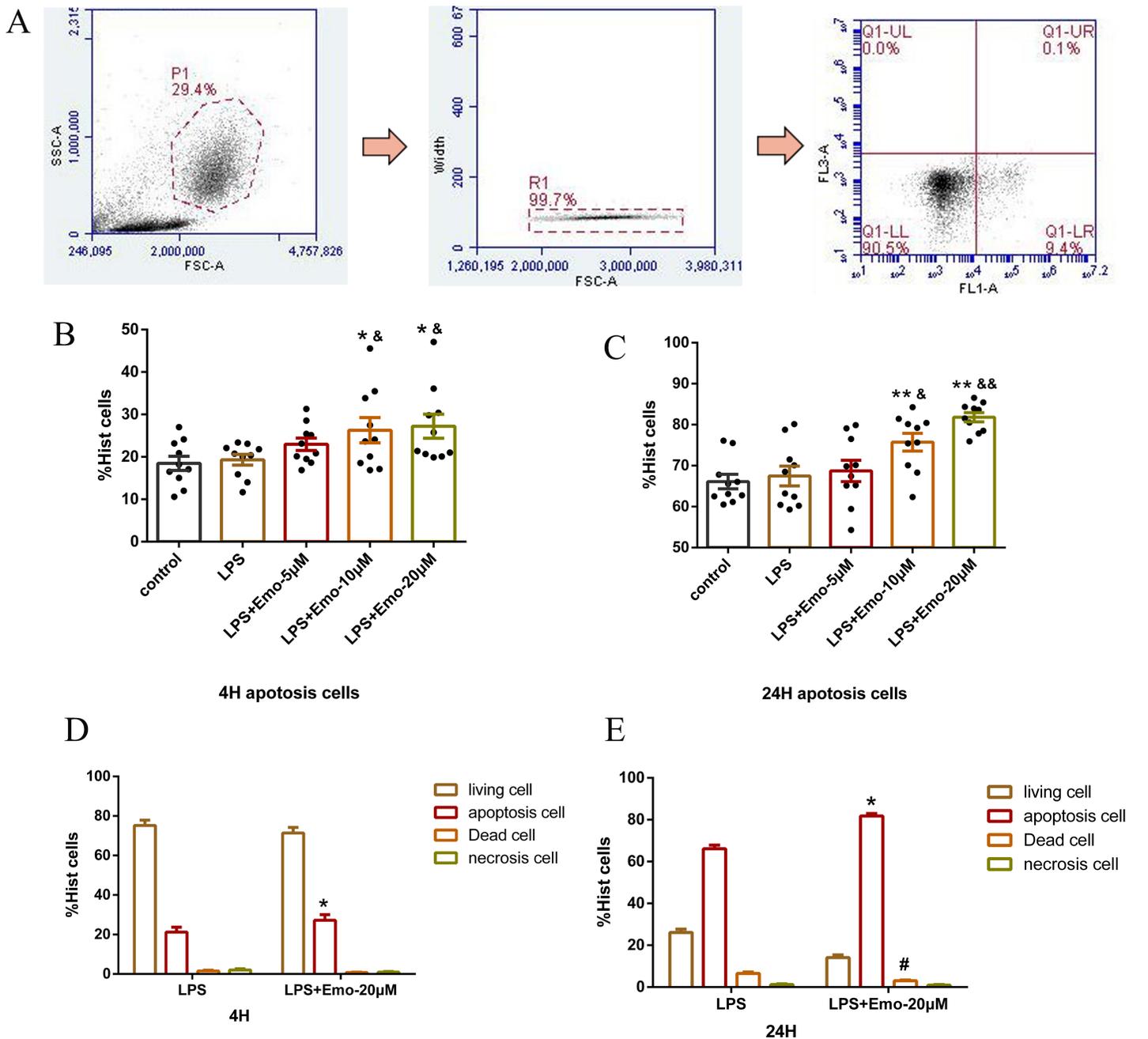


Figure 6

Effect of Emo on apoptosis of Neutrophils. (A) The apoptosis of neutrophils was measured by flow cytometry. (B) The proportion of apoptosis of neutrophils in each group at 4 h was measured. (C) The proportion of apoptosis of neutrophils in each group at 24 h was measured. The data are presented as the mean \pm SD. $n = 10$. * $P < 0.05$ versus control group; ** $P < 0.01$ versus LPS group; & $P < 0.05$ versus LPS group; && $P < 0.01$ versus LPS group. (D) The ratio of living cells, apoptosis cells, dead cells and necrotic cells between the LPS group and the LPS+Emo 20µM group at 4 h was detected. (E) The ratio of living cells, apoptotic cells, dead cells and necrotic cells between the LPS group and the LPS+Emo 20µM group

at 24 h was detected. The data are presented as the mean \pm SD. $n = 10$. * $P < 0.05$ versus apoptosis cell in LPS group; # $P < 0.05$ versus dead cell in LPS group.

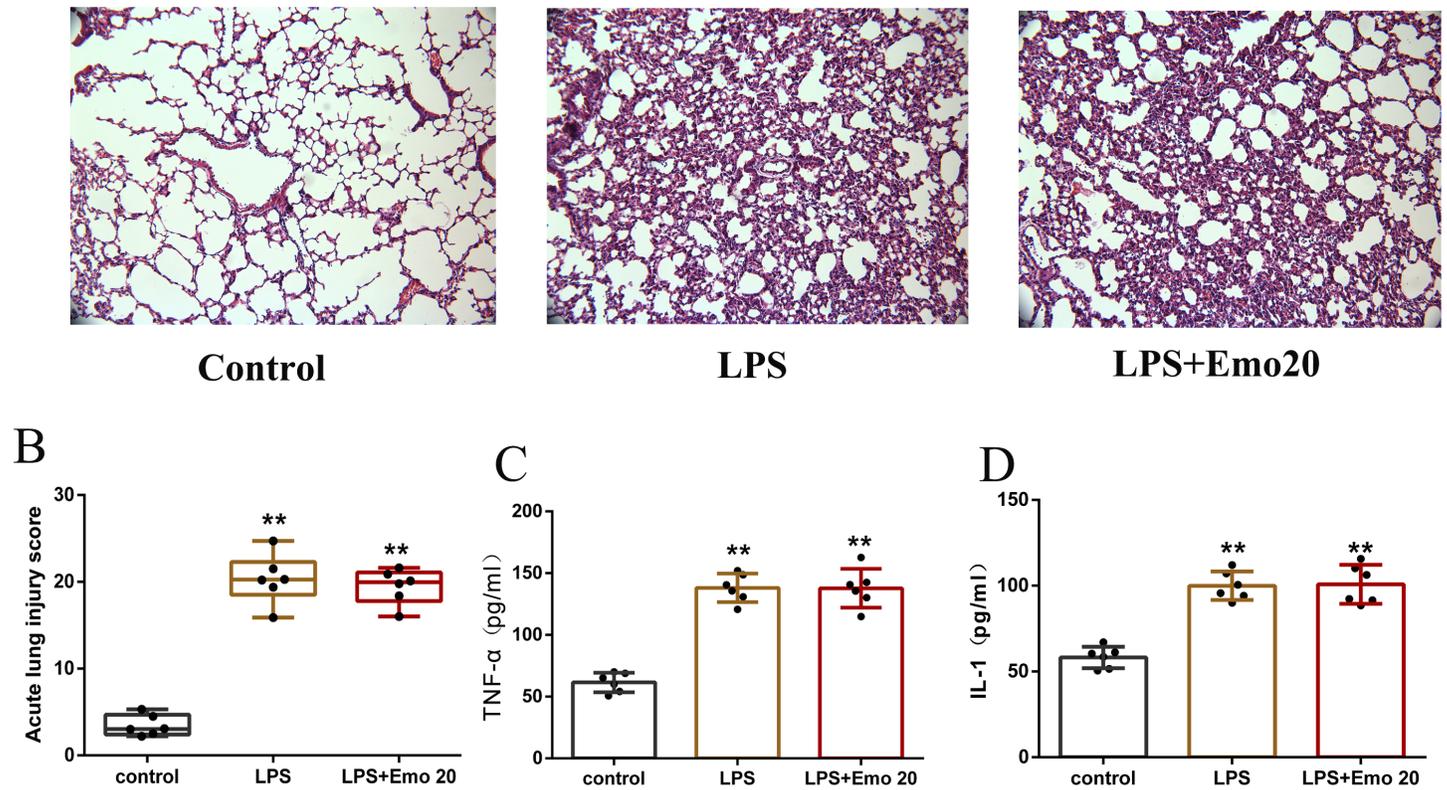


Figure 7

Emo has no protective effect on the rat model of acute lung injury with neutrophil deficiency. (A) The lung tissues were obtained immediately after exsanguination (4h after LPS), and the effect of Emo 20mg/kg was assessed histologically in H&E-stained sections (original magnification $\times 200$). (B) Lung injury scores. (C) The lung tissue homogenate TNF- α protein expression and (D) the lung tissue homogenate IL-1 β protein expression. The data are presented as the mean \pm SD. $n = 6$. ** $P < 0.01$ versus control group.