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

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**Research**

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# Abstract

Background: This study aims to investigate the role of Emodin(Emo) on the pulmonary inflammatory response and the neutrophil function in a murine model of lipopolysaccharide (LPS)-induced ALI. The mouse model of lipopolysaccharide (LPS)-induced ALI was produced by acute lung injury of injecting 20 mg/kg LPS via the caudal vein.The right lower lung lobes were harvested, fixed,embedded and stained with hematoxylin and eosin (H&E) for light microscopy analysis to evaluate lung injury. TNF-αand IL-1 ELISA kits were used to detect the levels of TNF-α and IL-1 in the right lung homogenate of rats. The primary rat neutrophils were separated and treated with LPS to mimic a ALI cellular model. The expression of Respiratory burst and Neutrophil NETs Production was examined using spectrophotometer.The Elastase release was detected using Elastase Activity Assay kit.The ROS Production was measured by Luminometer.the phagocytosis of Neutrophils and the rate of apoptotic Neutrophils were measured by flow cytometry.

Results: This study demonstrates that Emo alleviates lung injury and reduces the release of inflammatory cytokines. 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.

Conclusion: Emo has a protection effect on LPS-induced acute lung injury mice. It can alleviate lung injury and reduces the release of inflammatory cytokines, perhaps by affecting the various function of neutrophils in a dose-dependent manner.


# Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are devastating clinical syndromes. They frequently lead to acute respiratory failure characterized by infiltration of a large number of inflammatory cells, alveolar epithelial injury, flooding of protein-rich fluid into the alveolar spaces, and the expression of pro-inflammatory mediators[1-2]. Bacterial or viral pneumonia and sepsis are the most common causes of ALI and ARDS, wherein Gram-negative bacteria are a prominent cause[3]. 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[4-5]. Despite continuous improvements in treating ARDS in recent years, morbidity and mortality rates of ARDS remain as high as 30–40%[6-7]. Alleviating the inflammatory response in a timely and effective manner is critical for minimizing damage to the surrounding tissue and for resolution of inflammation[8].

In infiltrating inflammatory cells, neutrophils are dominant in both the amount and the content of cytotoxic substances in the cytoplasm. They are indispensable in the defense against intruding

microorganisms[9]. Elimination of pathogens by neutrophils involves a series of physiological processes including chemotaxis, phagocytosis, and microbial killing. When pathogenic bacteria invade, neutrophils enhance their phagocytic ability to remove pathogenic microorganisms[10]. Once neutrophils are activated, respiratory burst and degranulation occur in the neutrophils. Respiratory burst is an important process that mediates microbial killing through the formation of reactive oxygen species (ROS)[11]. However, excessive respiratory burst in activated neutrophils produces a large amount of ROS, which is harmful to the tissues surrounding sites of inflammation. As a result, limiting the generation of excessive respiratory burst and ROS is beneficial to the prognosis of inflammation. By contrast, Neutrophil Elastase (NE) is a serine protease present in the azurophilic granules of neutrophils. When exposed to various inflammatory factors, neutrophils release NE via degranulation, which is then involved in the inflammatory response[12]. Through these mechanisms and their other biological functions, neutrophils play an important role in the initiation, development, and transformation of inflammation.

Neutrophils can also play a bactericidal role by phagocytizing pathogenic microorganisms. In response to specific stimuli, neutrophils extrude modified chromatin structures decorated with specific cytoplasmic and granular proteins called neutrophil extracellular traps (NETs)[13]. Early studies described NETs as being beneficial to the host. In more recent years, there are increasing reports that their role in health and disease is more double-edged than initially believed. 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[14-15]. The engulfment of apoptotic cells by phagocytes is essential for maintaining normal tissue homeostasis and a prerequisite for the resolution of inflammation[16]. The removal of apoptotic neutrophils from circulation regulates granulopoiesis, and prevents secondary lysis and spillage of noxious neutrophil substances[17-18] Thus, it can be surmised that efferocytosis of apoptotic neutrophils is a fundamental, innate process required for tissue homeostasis and immunity, and that its dysregulation can lead to unwanted excessive inflammation, autoimmunity, and the exacerbation of infections.

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[19-22]. Modern researches show that Emo extract could significantly inhibit the inflammatory responses in a variety of inflammatory animal models[23]. 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.

# 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 (superoxide dismutase, SOD), Elastase, Hydroxyethylpiperazine Ethylsulfonic Acid (HEPES) and Emodin were obtained from Sigma-Aldrich (St Louis, MO, USA). Tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) ELISA kits were obtained from R&D Systems (Minneapolis, MN). 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

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 the Animal Studies Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

Rats were randomized into five groups (n = 10): control group, LPS group, LPS+Emo 5 mg/kg group, LPS+Emo 10 mg/kg group and LPS+Emo 20 mg/kg group. The LPS-induced ALI model was produced by injecting 20 mg/kg LPS via the caudal vein. In the Emo group, rats received Emo (5 mg/kg, 10 mg/kg and 20 mg/kg) via intraperitoneal injection 30 min before LPS exposure. Emo was dissolved in 100% DMSO at a concentration of 200 mg/mL and diluted in saline to the final concentration of 1 mg/mL. Animals were anesthetized with an intraperitoneal (IP) injection of 5% chloral hydrate (7 ml/kg), after which a tracheostomy tube was placed. Rats were sacrificed after 60 min of mechanical ventilation and lungs were harvested for further analyses.

## Pathological studies

The right lower lung lobes were harvested, fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for light microscopy analysis. 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[24-25]. The four variables were summed to represent the lung injury score (total score: 0–16). Part of the right lung from individual rats was homogenized and centrifuged, and the levels of TNF-α and IL-1 in the resulting tissue supernatants were determined using TNF-α and IL-1 ELISA kits.

## Separation and of rat neutrophils

20ml of heparinized fresh rat blood was treated with dextran to induce sedimentation of the red blood cells. Lymphocyte separation solution was used to remove lymphocytes and monocytes and distilled water was used to solubilize residual red blood cells. A two-step Histopaque gradient technique was then used to obtain high purity neutrophils. The purity of the rat neutrophils was further evaluated using flowcytometry (Attune, ABI) after staining with RP-1 antibody (BD 550002).

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

## 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. The determination of neutrophil respiratory burst was slightly modified by reference[26]. ① Set blank control group C1, control group C2 and 5 test groups, and the appropriate amount of KRB was added in each group, 100 μl of cytochrome C (1.5 mg/ml) and 100 μl neutrophils ($2\times10^7$/ml) was then added; ②10μl SOD (5000 U/ml) was added, and the corresponding dose was added to the test group, equilibrated in a 5% $CO_2$ incubator at 37 °C for 10min; ③ 10 μl cytochalasin B (1mmol/L) was added to each group and after 3min, 10 μl fMLP (0.1mmol/L) was added for a total of 1 ml and each group was incubated in a 5% $CO_2$ incubator at 37 °C for 30min; ④ Each group was removed and centrifuged at 2000r/min for 10 min;⑤ Supernatant was collected and the OD value was measured with a spectrophotometer. Since the production of $O_2^-$ and the decrease in cytochrome C are in a 1:1 mole stoichiometric relationship, the yield of $O_2^-$ is easily calculated. The millimolar extinction coefficient of the 1 cm optical path is 21.1, and the amount of $O_2^-$ produced by $2 \times 10^6$ / ml of cells in 1 ml of the solution with a diameter of 1 cm can be directly calculated according to the formula:

OD×47.4=nmol $O_2^-$/2×106cells/time unit test group $O_2^-$ inhibition rate = (control $O_2^-$ content-test group $O_2^-$ content)/control group $O_2^-$ content×100%

## Elastase release assay

The detection of neutrophil elastase release was mainly carried out by Neutrophil Elastase Activity Assay kit (ab204730). 5 elastase solution test groups were prepared: No fMLP control group C1, fMLP control group C2; rolipram group(reaction solution concentration: 1.38 μg/ml), Shuanghuanglian powder injection group (reaction solution concentration: 50 mg crude drug/ml), and Emo group (reaction solution concentration: 10, 30, 50 mg crude drug/ml). ▯ The isolated neutrophils were rinsed twice with PBS (pH 7.4), the number of cells was adjusted to $1\times10^7$/ml, 500 μl of solution was added to each group, and the corresponding drugs were added. Finally, each group was supplemented with PBS to 600 μl. The groups were pre-incubated for 30 min at 37 °C in a 5% CO 2 incubator. In addition to the control group C1, 6 μl of cytochalasin B (1 mmol/L) and fMLP (0.1 mmol/L) was added to each group and cultured at 37 °C in a 5% $CO_2$ 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% $CO_2$ 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. Test group inhibition rate of elastase release = (control group C2 elastase content - test group elastase content) / control group C2 elastase content × 100%

## Measuring Neutrophil NETs Production

Clear 96-well flat-bottomed plates were prepared, and 100 μl of cells 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% $CO_2$ 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 1x106/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 IL-8, fMLP, and PMA. The concentration of IL-8, fMLP, and PMA was as shown in the following table. The Luminometer was set up and the ROS level was tested on the instrument.

| | 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 | 1.25 nM |
| fMLP | | 1:500 | 12 µl into 5922 µl | 10 mM | 20 µM | 2.5 µM |
| PMA | A | 1:800 | 1 µl into 799 µl | 1620 µM (1 mg/ml) | 200 nM | 25 nM |
| | B | 1:10 | 55 µl into 495 µl | | | |

## Measuring the phagocytosis of Neutrophils

Neutrophils were isolated and the cell concentration was adjusted to $1\times10^6$/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 ℃ in a 5% $CO_2$ incubator in the dark, and then centrifuged at 250 g and 4℃ for 5 min to remove the supernatant. The cells were resuspended with 100 ul of 2% PBS/BSA, and this was repeated twice before the cell suspension from each well was transferred into flow tubes. 100 µl 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 $1\times10^6$/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.

### Statistical analysis

The data represent the mean ± 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).

## Results

### Effects of emodin 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 cultured and obtained following magnetic bead purification as shown in Fig. 1B, a schematic diagram of the rat neutrophils after Wright's staining, and Fig. 1C, a flow cytogram result. RP-1 positive neutrophil cells were obtained after purification. The ratio of rat neutrophils was quantified and reached 94.1% of the cultured cells. Subsequently, different concentrations of Emo were administrated to the neutrophils for 48 h. As shown in Fig. 1D, a high concentration of 100 μM Emo inhibited the proliferation of rat neutrophils, while a lower concentration of Emo had little effect on the proliferation of neutrophils. 5, 10 and 20 μM of Emo were chosen for further study.

Fig. 1 (A) Schematic diagram of Emo; (B) Wright's staining of rat neutrophils. Scale bar = 20 μm. (C) Flow cytometry results showed that rat neutrophils with higher purity were obtained after 5 days of culture. The left picture shows the flow cell scatter plot, and the right picture shows the further analysis of the circled R1 region cells. Unstained cells were used as isotype controls. (D) MTT assay. The effect of different concentrations of Emo on neutrophil proliferation in rats was examined.

### Emo protected lung tissues from LPS-induced ALI—histopathologic evaluation

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 ($p$ 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 ($p$ 0.05). As Emo dosage was increased, the attenuation of LPS-induced pathologic also increased. 20 mg/kg Emo significantly attenuated LPS-induced pathologic changes($p$ 0.01)(Fig. 2B).

As expected, the concentrations of TNF-α and IL-1 in the lung tissue homogenate were significantly higher in the LPS group than in the control group. By comparison, the concentrations in the Emo treated groups were lower than in the LPS group (Fig. 2. C-D).

Fig. 2. Emo protected lung tissues in LPS-induced ALI. Emo (5 mg/kg,10 mg/kg and 20 mg/kg) was administered to Sprague–Dawley rats 30 min before LPS (20 mg/kg) stimulation through the caudal vein, ventilated for 60 min, and the effect of Emo was assessed (A) by histology in H&E-stained sections (original magnification 3400). Lung injury scores (B) were recorded from 0 (no damage) to 16 (maximum damage) according to the criteria described in Materials and Methods. Part of the right lung was homogenized from individual mice and centrifuged, then the tissue level of TNF-α (C) and IL-1 (D) in the resulting supernatants was determined using ELISA kits. The data are presented as the mean ± SD. n = 6. **P□0.01 versus control group; $^{\&}$P□0.01 versus LPS group; $^{\&\&}$P□0.01 versus LPS group.

## 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, and the inhibition rates of $O_2^-$ production at low, medium and high concentrations were 33.15%, 45.96%, and 62.22%, respectively.

ROS are produced by mitochondria during aerobic respiration as a consequence of electron transport chain activity. The expression of ROS in neutrophils was measured by Luminometer (Fig. 3B-C). IL-8□ fMLP and PMA can all stimulate neutrophils to produce ROS. However, 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. 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 μM Emo and 20 μM Emo reduced ROS production, with 20 μM Emo having an even better effect (P <0.01). At 5μM, Emo has no effect (P□0.05)(Fig. 3C).

Fig 3. Effect of Emo on neutrophil respiratory burst and ROS production of neutrophil. Emo (5 μM,10 μM and 20 μM) was administered to neutrophils. fMLP was then added and incubated for 30 min. Samples were removed and centrifuged at 2000 r/min for 10 min. The supernatant was collected and the OD value was measured with a spectrophotometer. (A) Effect of Emo on neutrophil respiratory burst in rats. Neutrophils were stimulated with IL-8, fMLP, and PMA. (B) The ROS level was tested with Luminometer. Emo (5 μM, 10 μM and 20 μM) was administered to neutrophils. Then the neutrophils were stimulated with PMA. (C) ROS production was tested with Luminometer. 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.

## Emodin 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. Different concentrations of Emo had different effects on the release of elastase. Inhibition of elastase release was observed at low concentrations ($P<0.05$). However, at higher concentrations of Emo, elastase release was increased significantly. At 10 μM Emo, elastase release was significantly higher than the control group ($P < 0.01$), and at 20 μM of Emo elastase release was significantly higher than the control group and the LPS group ($P <0.01$).

NETs are a newly discovered method of killing bacteria used by neutrophils. Necrotic neutrophil DNA forms extracellular fibers combined with proteases that are released outside of cells and can further kill bacteria. IL-8, LPS, fMLP, and PMA can stimulate neutrophils to produce NETs in vitro. 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. 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 μM Emo and 10 μM Emo groups, the generation of NETs did not change significantly compared with the LPS group ($P>0.05$). However, 20μM Emo significantly increased the production of NETs compared with the LPS group ($P <0.05$)(Fig. 4C).

Fig. 4. Effect of Emo on the release of neutrophil elastase and NETs production. The detection of neutrophil elastase release (A) was mainly carried out by Neutrophil Elastase Activity Assay kit. Neutrophils were stimulated with IL-8, LPS, fMLP, and PMA. They were incubated for 3 h at 37°C in a 5% $CO_2$ incubator before diluted SYTOX green and MNase was added to the neutrophils. They were then incubated at room temp for 10 min. Samples were transferred to micro-centrifuge tubes and they were centrifuged at 5000 rpm for 10 min. (B) The production of NETs in supernatant was tested with the Fluroscan. Emo (5 μM, 10 μM and 20 μM) was administered to the neutrophils. The neutrophils were then stimulated with PMA. (C) NETs production was tested with Fluroscan. 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.

## Emo Promotes Phagocytosis of Neutrophils

Neutrophils were isolated and the cell concentration was adjusted to $1\times10^6$/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 and pHrodo Red E.coli compared with the control group at 45 min and 60 min ($P <0.01$)(Fig. 5A-B). However, there was no significant difference in the phagocytosis of

pHrodo Green S.aureus and pHrodo Red E.coli between the LPS group and the control group at 30 min (P 0.05)(Fig. 5A-B). These results indicate that phagocytosis of neutrophils is enhanced during infection. 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 10μM and 20μM Emo groups (P <0.05)(Fig. 5C-D). The effect of 5μM Emo was not significant (P0.05)(Fig. 5C-D).

Fig. 5. Effect of Emo on Phagocytosis of Neutrophils. Neutrophils were isolated and stimulated with LPS; neutrophils were co-cultured with pHrodo Green S.aureus and pHrodo Red E.coli for 30 min, 45 min, and 60 min. (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. Emo (5 μM, 10 μM, 20 μM) was administered to neutrophils following neutrophil stimulation with LPS. (C) Flow cytometry was used to detect the phagocytosis of pHrodo Green S.aureus by neutrophils in each group. (D) Flow cytometry was used to detect the phagocytosis of pHrodo Red E.coli by neutrophils in each group. The data are presented as the mean ± SD. n = 10. *P0.05versus LPS group in 45 min; **P0.01 versus LPS group in 45 min; $^{\&}$P0.05 versus LPS group in 60 min; $^{\&\&}$P0.01 versus LPS group in 60 min.

## Emo Promotes Apoptosis of Neutrophils

Neutrophils were isolated and the cell concentration was adjusted to $1\times10^6$/ml. Neutrophils were treated with Emo (5 μM, 10 μM and 20 μM) 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 μM group(P0.05)(Fig.6B). However, the apoptosis of cells increased significantly in the LPS+Emo 10 μM group and the LPS+Emo 20 μM group, when compared with the control group (P0.05)(Fig. 6B). We further compared the ratio of living cells, dead cells and necrotic cells between the LPS group and the LPS+Emo 20 μM group, and found that there was no significant difference in the ratio of living cells, dead cells and necrotic cells between the two groups (P0.05)(Fig. 6D). It suggests that Emo 10 μM and Emo 20 μM 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 μM Emo and 20 μM Emo can promote apoptosis of cells following LPS stimulation at 24 h (P 0.05)(Fig. 6C). Moreover, when compared with the LPS group, dead cells in the LPS+Emo 20 μM group were significantly reduced (P0.05)(Fig. 6E). It suggests that 20 μM Emo can not only promote the apoptosis of neutrophils following LPS stimulation but also reduce the ratio of dead neutrophil cells.

Fig. 6. Effect of Emo on apoptosis of Neutrophils. Neutrophils were isolated and treated with LPS and Emo (5 μM, 10 μM and 20 μM) for 4 h and 24 h, before being labeled with Annexin V-FITC and SYTOX. (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 ± SD. n = 10. *P0.05versus 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 20 μM 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 20 μM group at 24 h was detected. The data are presented as the mean ± SD. n = 10. *P□0.05versus LPS group; #P□0.05 versus LPS group.

# Discussion

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[27]. 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 wildly 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[24-25]. 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[28].

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 show that the sterilization ability of neutrophils was enhanced following challenge by LPS and was further upregulated following Emo administration in vitro. Furthermore, the efficacy of Emo increased in a dose-dependent manner. Our data clearly demonstrate that Emo, administered intravenously, reduces lung injury scores of LPS-induced ALI. Our study also shows that following treatment with Emo after ALI, lung injury scores in the LPS+Emo5μM(post) group were lower than the LPS group and that the higher the Emo dose, the lower the lung injury scores. This suggests the potential of Emo as an effective therapeutic agent. Our experimental results also confirm that treatment with Emo inhibits plasma inflammatory cytokines, such as TNF-α and IL-1. Decreased plasma TNF-α and IL-1 levels in Emo-treated mice are consistent with the paradigm that Emo reduces the inflammatory response following ALI, in addition to ameliorating the severity of disease[29].

Neutrophils are over-activated during ALI, which releases neutrophil respiratory burst and ROS to damage the surrounding tissues[30]. Our experimental results supported this conclusion. 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. Elastase is the main enzyme involved in neutrophil degranulation release. It plays an important role in the various pathological processes such as the various inflammatory reactions, adult respiratory distress syndrome, and acute lung injury, and promotes the invasion of viruses and bacteria. the results showed that Emo exhibited a dual regulation

effect on the release of neutrophil elastase caused by fMLP: inhibition at low concentrations and promotion at high concentrations.

cAMP is involved in the inhibition of neutrophil degranulation, and fMLP causes neutrophil degranulation through the cAMP-PKA pathway. Therefore, the inhibition of elastase release by Emo at low concentrations may be related to its inhibition of cAMP-PDE activity; the promotion of elastase release at high concentrations may be due to its active ingredient initiating neutrophils at high concentrations. In view of the dual role of elastase in the inflammatory response, the pharmacological effects and regulatory significance of Emo on neutrophil elastase release need further study. This will help to further understand the anti-inflammatory mechanism of Emo and optimize the quality of its preparation.

During an inflammatory injury, neutrophils gather at the injury site to exert phagocytosis, an important aspect of neutrophils to kill pathogenic microorganisms[30]. 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 μM did not promote the phagocytosis of neutrophils, 10 μM Emo and 20 μM Emo significantly promoted neutrophil phagocytosis of S.aureus and E.coli, indicating that Emo needs to reach a certain dose to promote neutrophil phagocytosis .

Recent studies have shown that neutrophils also have another bactericidal mechanism, NETs, which is a reticular extracellular structure formed when neutrophils repel genomic DNA containing antimicrobial proteins, including histones, MPO, neutrophil elastin and cathepsin G[32]. NETs are a double-edged sword in the inflammation process. Some studies have shown that NETs can combine and kill a large number of microorganisms such as S. aureus[32], E.coli, Candida albicans[34], etc. However, other studies have also shown that a large number of extensive NETs formations can damage epithelial cells and endothelial cells[35]. 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 are helpful in enabling neutrophils to play a bactericidal function in the acute stages of inflammation.

When 5 μM Emo was added to neutrophils in the LPS group, the generation of NETs did not increase further but decreased. However, when 10 μM Emo and 20 μM Emo were added, the generation of NETs was significantly higher than the LPS group. This data showed that small doses of Emo could not further promote the bactericidal function of neutrophils through NETs, but medium and large doses of Emo could significantly promote the bactericidal ability of neutrophils through NETs.

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[36]. 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, 20μM Emo can not only promote the apoptosis of neutrophils but also reduce the number of dead neutrophil cells.

In summary, this study demonstrates that Emo alleviates lung injury and reduces the release of inflammatory cytokines. 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. Only, medium and large doses of Emo can significantly affect the various function of neutrophils 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 mice. It can alleviate lung injury and reduces the release of inflammatory cytokines, perhaps by affecting the various function of neutrophils in a dose-dependent manner.

# Declarations

### Ethics approval and consent to participate

The use of animals in this study was approved by the Animal Studies Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

### Consent for publication

Not applicable

### Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests

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### Authors' contributions

MHX performed the experiments and TY collected the raw data. ZTH performed the statistical analysis and QF 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.All authors read and approved the final manuscript.

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Not applicable

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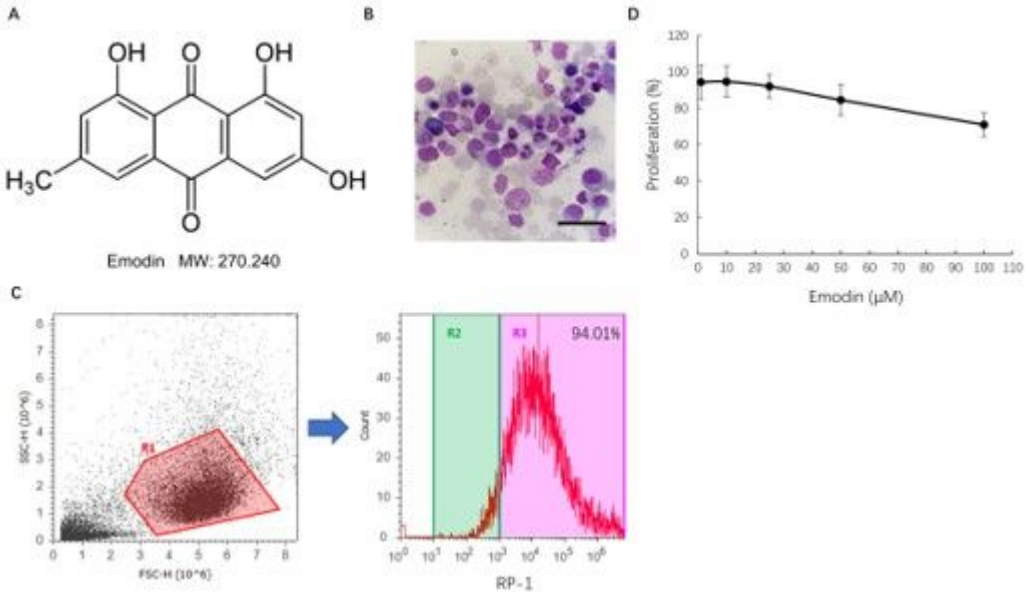
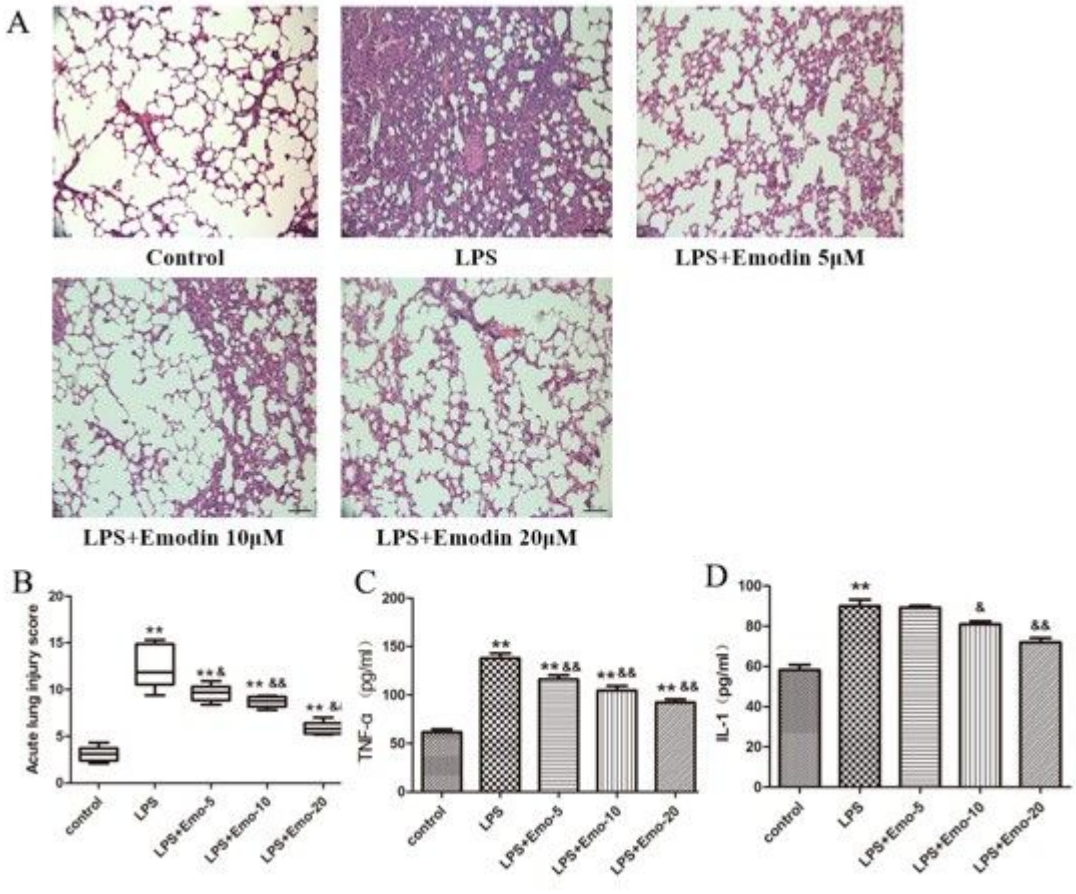
# Figures



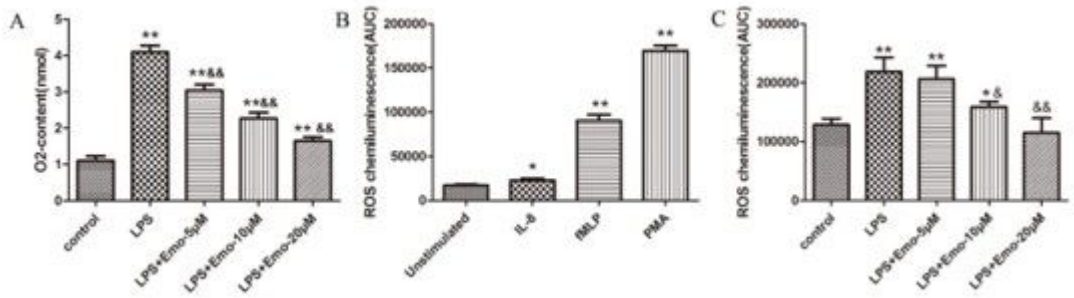

## Figure 1

(A) Schematic diagram of Emo; (B) Wright's staining of rat neutrophils. Scale bar = 20 μm. (C) Flow cytometry results showed that rat neutrophils with higher purity were obtained after 5 days of culture. The left picture shows the flow cell scatter plot, and the right picture shows the further analysis of the circled R1 region cells. Unstained cells were used as isotype controls. (D) MTT assay. The effect of different concentrations of Emo on neutrophil proliferation in rats was examined.



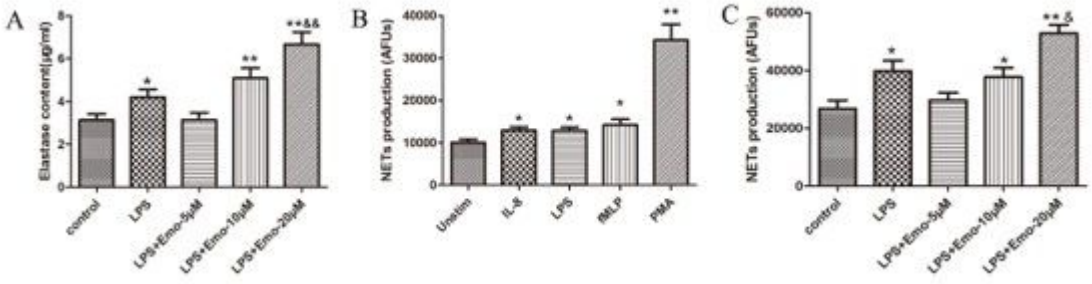
## Figure 2

Emo protected lung tissues in LPS-induced ALI. Emo (5 mg/kg,10 mg/kg and 20 mg/kg) was administered to Sprague–Dawley rats 30 min before LPS (20 mg/kg) stimulation through the caudal vein, ventilated for 60 min, and the effect of Emo was assessed (A) by histology in H&E-stained sections (original magnification 3400). Lung injury scores (B) were recorded from 0 (no damage) to 16 (maximum damage) according to the criteria described in Materials and Methods. Part of the right lung was homogenized from individual mice and centrifuged, then the tissue level of TNF-α (C) and IL-1 (D) in the resulting supernatants was determined using ELISA kits. The data are presented as the mean ± SD. n = 6. **P<0.01 versus control group; &P<0.01 versus LPS group; &&P<0.01 versus LPS group.




## Figure 3

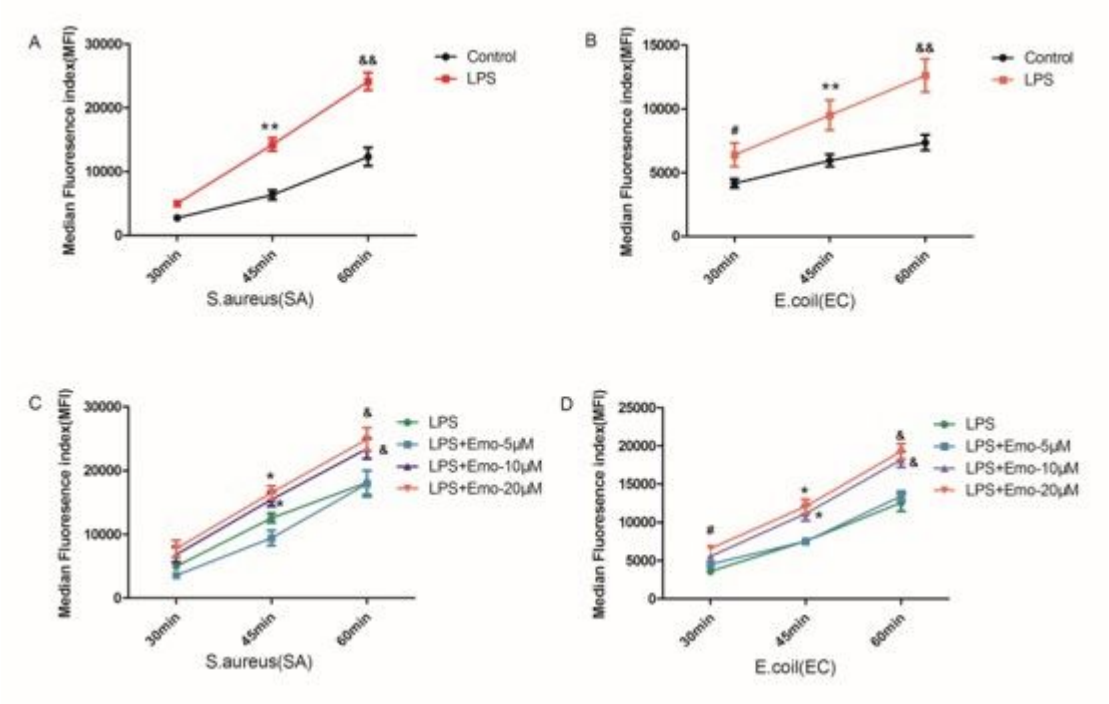
Effect of Emo on neutrophil respiratory burst and ROS production of neutrophil. Emo (5 μM,10 μM and 20 μM) was administered to neutrophils. fMLP was then added and incubated for 30 min. Samples were removed and centrifuged at 2000 r/min for 10 min. The supernatant was collected and the OD value was measured with a spectrophotometer. (A) Effect of Emo on neutrophil respiratory burst in rats. Neutrophils were stimulated with IL-8, fMLP, and PMA. (B) The ROS level was tested with Luminometer. Emo (5 μM, 10 μM and 20 μM) was administered to neutrophils. Then the neutrophils were stimulated with PMA. (C) ROS production was tested with Luminometer. 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

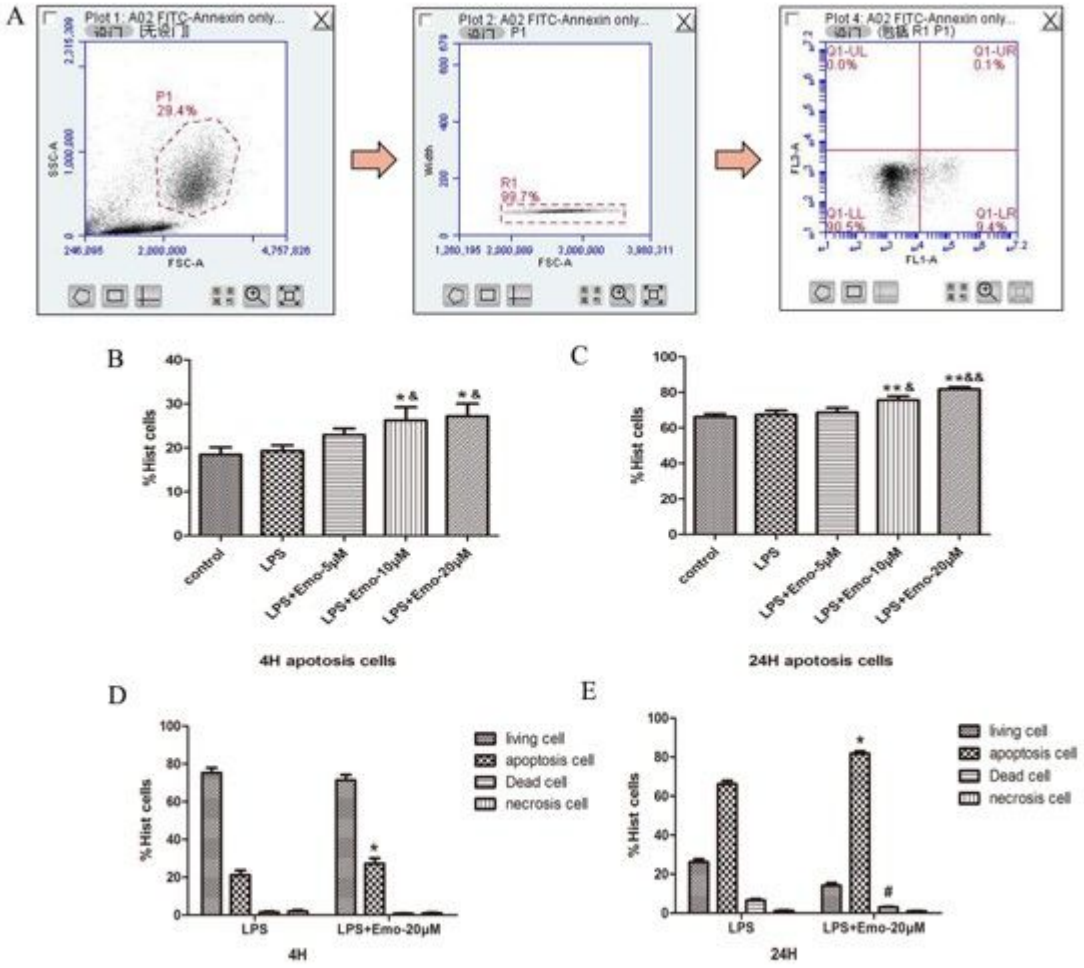
Effect of Emo on the release of neutrophil elastase and NETs production. The detection of neutrophil elastase release (A) was mainly carried out by Neutrophil Elastase Activity Assay kit. Neutrophils were

stimulated with IL-8, LPS, fMLP, and PMA. They were incubated for 3 h at 37°C in a 5% CO2 incubator before diluted SYTOX green and MNase was added to the neutrophils. They were then incubated at room temp for 10 min. Samples were transferred to micro-centrifuge tubes and they were centrifuged at 5000 rpm for 10 min. (B) The production of NETs in supernatant was tested with the Fluroscan. Emo (5 μM, 10 μM and 20 μM) was administered to the neutrophils. The neutrophils were then stimulated with PMA. (C) NETs production was tested with Fluroscan. The data are presented as the mean ± SD. n = 10. *P 0.05versus 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. Neutrophils were isolated and stimulated with LPS; neutrophils were co-cultured with pHrodo Green S.aureus and pHrodo Red E.coli for 30 min, 45 min, and 60 min. (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. Emo (5 μM, 10 μM, 20 μM) was administered to neutrophils following neutrophil stimulation with LPS. (C) Flow cytometry was used to detect the phagocytosis of pHrodo Green S.aureus by neutrophils in each group. (D) Flow cytometry was used to detect the phagocytosis of pHrodo Red E.coli by neutrophils in each group. The data are presented as the mean ± SD. n = 10. *P 0.05versus LPS group in 45 min; **P 0.01 versus LPS group in 45 min; &P 0.05 versus LPS group in 60 min; &&P 0.01 versus LPS group in 60 min.




## Figure 6

Effect of Emo on apoptosis of Neutrophils. Neutrophils were isolated and treated with LPS and Emo (5 μM, 10 μM and 20 μM) for 4 h and 24 h, before being labeled with Annexin V-FITC and SYTOX. (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 ± SD. n = 10. *P＜0.05versus 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 20 μM 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 20 μM group at 24 h was detected. The data are presented as the mean ± SD. n = 10. *P＜0.05versus LPS group; #P＜0.05 versus LPS group.