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Kaempferitrin alleviates LPS-induced septic acute lung injury in mice through downregulating NF- κ B pathway

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Abstract

Background: Acute lung injury (ALI) causes severe and uncontrolled pulmonary inflammation and has high morbidity in dying patients.

Objective: This study aimed to evaluate the potential function of Kaempferitrin (Kae) and uncover its mechanisms in ALI.

Material and Methods: We evaluated the role of Kae in ALI through the lipopolysaccharide (LPS)-induced histopathological changes, lung wet/dry (W/D) ratio, total bronchoalveolar lavage fluid (BALF) cells count, pulmonary inflammation, and the levels of interleukin (IL)-6, tumor necrosis factor- α (TNF- α), and IL-1 β . The effect of Kae on NF- κ B signaling pathway was discovered through the protein expression levels of transcription factors p65, p-p65, I κ B α , and p-I κ B α by Western blot analysis.

Results: The results showed that Kae could improve lung injury by reducing apoptosis, histopathological changes, and lung W/D ratio; more importantly, Kae enhanced the survival of ALI mice. Moreover, Kae relieved inflammation, as it reduced total BALF cells count, and decreased the levels of TNF- α , IL-6, and IL-1 β in serum. In addition, Western blot analysis data suggested that Kae could decrease the protein expression levels of transcription factors p65, p-p65, I κ B- α , and p-I κ B- α , which were promoted by LPS.

Conclusion: The results of this study suggested that Kae could relieve LPS-induced ALI in mice and reduce inflammation and apoptosis through NF- κ B pathway.

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Introduction

Sepsis is a latently life-threatening systemic inflammatory syndrome characterized by inflammatory disorders, coagulation dysfunction, and multiple organ failure.¹ World Health Organization (WHO) has emphasized the importance of sepsis because of its enormous impact on global mortality.² In the context of antibiotic use, the mortality rate of severe sepsis is as high as 20-30%.³ There is an urgent need to identify new targets to improve the survival outcomes of sepsis.⁴ Moreover, acute lung injury (ALI) causes the most usual organ damage in sepsis.⁵ ALI also causes severe inflammation.⁶ Recent epidemiologic studies have suggested that ALI is an important public health issue.⁷ Despite the development of clinical treatment for better outcome, the mortality rate because of ALI remains high.⁸ Thus, it is essential to explore new therapeutic methods for managing ALI.

Lipopolysaccharide (LPS) is an endotoxin and a key constituent of the cell wall of Gram-negative bacteria, and it has a complex function in pneumonia.⁹ Systemic or intranasal administration of LPS has been widely used to induce lung inflammation in animal models of ALI.² Endotoxin can bind to toll-like receptor (TLR)-4, resulting in the production of chemokine and pro-inflammatory cascade reactions, that might be vital for the pathogenesis of ALI.¹⁰ Previous studies suggested that sepsis was constantly adjoined by the up-regulation of nuclear factor κ B (NF- κ B) activation.¹¹ The inhibition of NF- κ B activation could reduce inflammatory reaction, and meliorate improve the survival of septic experimental mice.¹² NF- κ B transcription factor an important regulatory factor of immune response, whereas p65 is a member of NF- κ B family.¹³ In fact, ALI induced by LPS in mice has been a well-established method for investigating inflammatory and histological changes that take place in the lungs.¹⁴

Kaempferitin (Kae) is a potent flavonoid compound extracted from *Cinnamomum osmophloeum*.¹⁵ Kae has an abundant of biological activities, such as anti-inflammatory, antioxidant, anticonvulsant, antidepressant, and anti-tumor activities.¹⁶ It is reported that Kae suppresses NF- κ B and protein kinase B (PKB/Akt)/mammalian rapamycin target protein (mTOR) pathway activation.¹⁵ In addition, treatment with Kae in a mouse model of collagen-induced arthritis decreases interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α) levels in serum.¹⁵ Kae restrains the exudate of white blood cells and bradykinin-induced pleurisy.¹⁷ However, the functioning and mechanism of Kae in sepsis lung injury needs further exploration.

In the present study, the findings suggested that Kae inhibited LPS-induced septic ALI in mice by suppressing NF- κ B pathway.

Methods

Animals

Specific pathogen-free (SPF) C57BL/6 male mice, aged 6-8 weeks, were bought from the Laboratory Animal Center of Zhejiang Chinese Medicine University (Hangzhou, China).

Body weight of the mice was 20-24 g, and lived in cages. The mice were supplemented with free water and food. All experiments involving the animals were approved by the Animal Ethical and Welfare Committee of ZCMU (Approval No. IACUC-20230130-05).

Murine model of LPS-induced ALI

The mice were randomly divided into the following four groups (every group having six animals): control group, LPS group, Kae+LPS group (Kae 10 mg/kg), and Kae+LPS group (Kae 20 mg/kg). Intraperitoneal injection of Kae was given to all animals for 2 days. After 30 min, 30-mg/kg pentobarbital sodium (Beijing Chemical Co., China) was injected to anesthetize the animals. Then, the mice were intraperitoneally injected with LPS (10 μ g/mouse) (L6261; Sigma-Aldrich) to build the model of LPS-induced ALI. Mice were executed after 24 h of LPS stimulation.¹⁸

Collection of bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected through i/v cannulation, and the left lung was rinsed thrice with 2-mL phosphate-buffered saline (PBS).¹⁹ Then BALF was centrifuged at 300 $\times$ g for 10 min. We collected and resuspended the cell pellet of BALF with PBS.

Enzyme-linked immunosorbent serologic assay (ELISA)

The expression levels of TNF- α (PT518; Beyotime Institute of Biotechnology, China), IL-1 β (PI305; Beyotime Institute of Biotechnology), and IL-6 (PI330; Beyotime Institute of Biotechnology) were detected by ELISA kits by following manufacturer's instructions. Briefly, samples or standards of different concentrations were added into the corresponding wells of 96-well plates (100 μ L/well), and incubated at room temperature for 120 min. After the plates were washed for five times, 100- μ L antibody was added into wells of the experiment and incubated for 60 min at room temperature. Then, 100- μ L horseradish peroxidase (HRP)-conjugated streptavidin was added and incubated for 20 min in dark at room temperature. Tetramethylbenzidine (TMB) developing solution, 100 μ L, was added and incubated for 20 min in dark at room temperature. Finally, 50- μ L/well termination solution was added and absorption was measured at 450 nm immediately.

Lung wet/dry (W/D) weight measurement

The W/D ratio was applied for assessing the gravity of pulmonary edema. Lung tissues were collected and metage was made immediately for wet weight (W) after execution of mice. After that, dry lung tissues were obtained from the wet tissues placed in an oven at 60°C for 48 h. Finally, the W/D ratio was obtained by fractioning the weight of dry tissues with the weight of wet tissues.

Hematoxylin and eosin (H&E) staining

Neutral buffered formalin 10% was applied for fixing the right lower lung of each mouse for 24 h. Then, tissues were cut into 3- μ m-thick sections by rotary microtome after they were dehydrated and embedded in paraffin. H&E stain was applied for staining 3- μ m-thick lung tissues to analyze pathological alterations.

TUNEL kit staining was used to detect level of lung tissue apoptosis in each group

The paraffin-fixed sections of mouse lung were dewaxed and stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reagent to detect the apoptosis of cells (green fluorescence) according to the instructions, and 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining (blue fluorescence).

Western blot analysis

Total protein was extracted by radioimmunoprecipitation assay (RIPA) lysis and extraction buffer (89901; Thermo Scientific, Rockford, IL). Protein was quantitated by bicinchoninic acid (BCA) protein assay (23227; Thermo Scientific). Then, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate total proteins. Next, the proteins were transferred on polyvinylidene fluoride (PVDF) membranes. PVDF membranes with protein were blocked in 5% non-fat milk powder dissolved in tris buffered saline with tween (TBST) for 1 h at room temperature. The membranes were probed with primary antibodies at 4°C overnight, including BAX (ab32503, 1:500; Abcam, Cambridge, UK), cleaved caspase-3 (ab32042, 1:500; Abcam), p-65 (ab204604, 1:500; Abcam), p-p65 (ab31624, 1:500; Abcam), I κ B α (ab32518, 1:500; Abcam), p-I κ B α (ab133462, 1:500; Abcam), and β -actin (ab8226, 1:1,000; Abcam). After this, on the second day, anti-rabbit antibody (ab190492, 1:1,000; Abcam) was applied for incubating the membranes for 1 h at room temperature. The immunoreactive bands were quantitated by the Image J software.

Statistical analyses

The data were analyzed by the SAS 9.0 software. Statistical comparison of two groups was compared by unpaired two-tailed Student's *t*-test. Statistical comparison of more than two groups was compared by one-way ANOVA, followed by Tukey's post hoc test. *P* < 0.05 was considered as statistically significant.

Results

Kaempferitrin improved LPS-induced lung injury in mice

Acute lung injury model was established successfully by the lung pathological alteration assessed via H&E staining. The

pathological changes were presented as destroyed pulmonary architecture, notable inflammatory cell infiltration, thickened alveolar septa, and alveolar hemorrhage in LPS-induced ALI mice. Pulmonary edema of mice was evaluated by lung W/D ratio. As shown in Figure 1A, the W/D ratio in the LPS group was significantly higher than in the control group. However, the W/D ratio was significantly lower in the Kae+LPS group (Kae 10 mg/kg) and the Kae+LPS group (Kae 20 mg/kg) than in the LPS group. As shown in Figure 1B, mice in the LPS group died within 2 days, compared with the control group. One of the mice died on the first day in the Kae+LPS group (Kae 10 mg/kg), another died on the third day, and the third one died on the fourth day. All mice in the Kae+LPS group (Kae 20 mg/kg) were alive, indicating that the survival rate of mice in these Kae+LPS groups was evidently longer than in the LPS group. Compared to the LPS group, pathological analysis indicated that Kae treatment significantly reduced lung weight and improved the condition of scattered lung tissues from acute injury in the Kae+LPS group (Kae 10 mg/kg) and the Kae+LPS group (Kae 20 mg/kg) (Figure 2C). In a word, results suggested that Kae could mitigate pathological process of ALI *in vivo*.

Kaempferitrin lessens LPS-induced pneumonia in mice

Next, the effect of Kae on LPS-induced inflammatory response was explored. First, the total cells count was discovered in BALF. As shown in Figure 2A, the total cells count was significantly increased by LPS in BALF, compared to the control group. However, the total cells count was evidently suppressed by the treatment of Kae in both Kae+LPS (Kae 10 mg/kg) and Kae+LPS (Kae 20 mg/kg) groups, compared with the LPS-treated group. Additionally, TNF- α , IL-1 β , and IL-6 levels in BALF cells count was determined by ELISA. LPS administration led to a significant increase in TNF- α , IL-1 β , and IL-6 levels in BALF (Figure 2B) whereas Kae administration significantly restrained TNF- α , IL-1 β , and IL-6 levels induced by LPS. These results indicated that Kae had a protective effect against pneumonia in ALI by repressing the production of inflammatory factors.

Kaempferitrin lessens LPS-induced apoptosis in mouse lung tissue

Apoptosis is another important pathological feature of ALI.²⁰ We next explored the effect of Kae on apoptosis in LPS-induced ALI mice. The result showed that the number of TUNEL-positive cells was significantly upregulated by LPS, compared to the control group.

Nevertheless, Kae treatment evidently decreased the number of TUNEL-positive cells induced by LPS (Figure 3A). Cleaved capase-3 and Bax are important markers of apoptosis in the development of ALI.²¹ Therefore, the levels of cleaved capase-3 and Bax were observed, and the results showed that the expression levels of Bax and cleaved capase-3 were obviously increased in the LPS group, compared to the control group. Nonetheless, both Kae+LPS (Kae 10 mg/kg) and Kae+LPS (Kae 20 mg/kg) groups showed decreased levels of Bax and cleaved capase-3 induced by

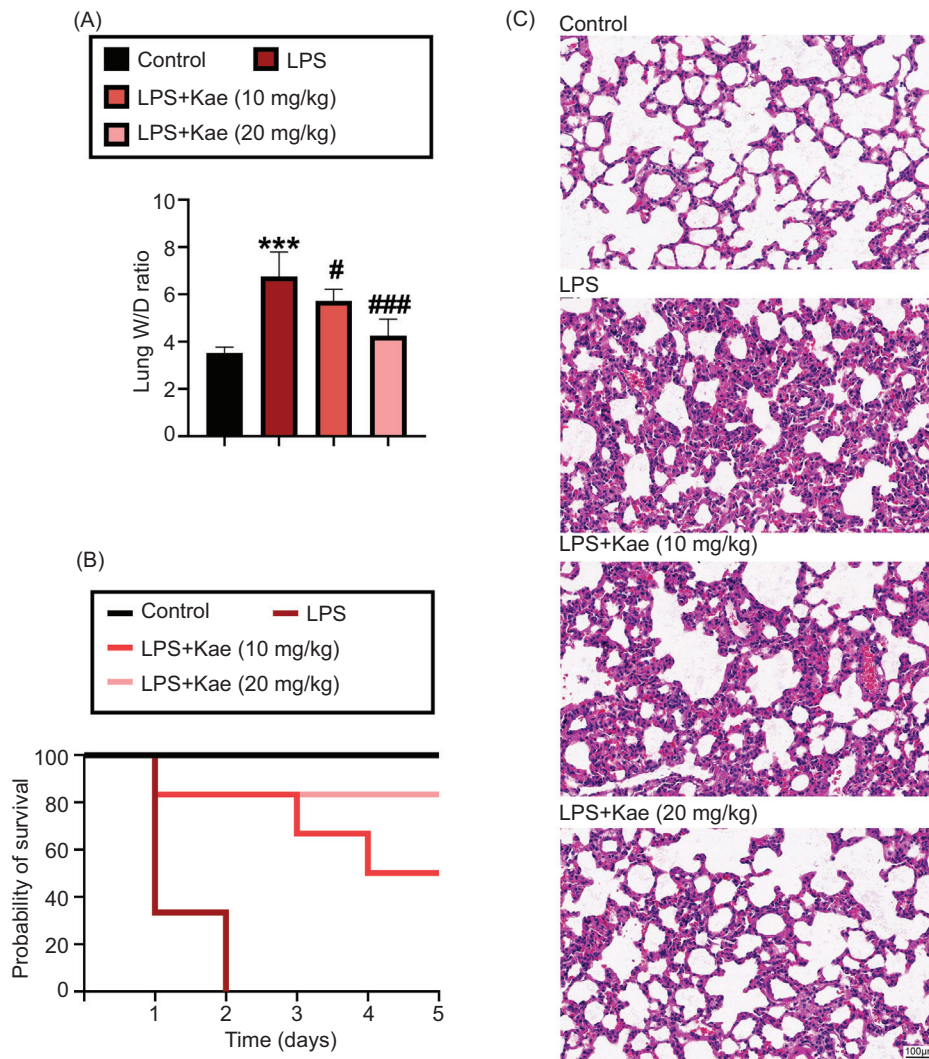


Figure 1 Successful establishment of LPS-induced ALI *in vivo*. (A) The lung wet/dry (W/D) weight ratio. (B) Probability of survival. (C) Histological changes in the lungs are assessed by H&E staining. * $P < 0.005$, ** $P < 0.001$, *** $P < 0.0001$ ($n = 3$), # $P < 0.005$, ## $P < 0.001$, ### $P < 0.0001$ ($n = 3$). *LPS group, compared with the control group; #Kae+LPS group, compared with the LPS group.

LPS (Figure 3B). Hence, the results established that Kae attenuated LPS-induced apoptosis in ALI mice.

Kaempferitrin inhibits activation of NF- κ B pathway

Finally, the mechanism of Kae on the NF- κ B pathway was explored in lung tissue. Here, the proteins related to the NF- κ B pathway were observed, including transcription nuclear factors p-p65, p65, I κ B α , and p-I κ B α . The proteins of lung tissues were extracted and subjected to Western blot analysis for testing p-p65, p65, I κ B α , and p-I κ B α . The protein levels of p-p65/p65 and p-I κ B α /I κ B α were evidently upregulated in the LPS group, compared to the control group (Figure 4). On the contrary, Kae treatment decreased the levels of p-p65/p65 and p-I κ B α /I κ B α in both Kae+LPS (Kae 10 mg/kg) and Kae+LPS (Kae 20 mg/kg) groups. These results emphasized that the LPS-induced inflammatory response was

suppressed by Kae by diminishing the level of pro-inflammatory cytokines by inhibiting the activation of NF- κ B pathway.

Discussion

In spite of the advantages of understanding the pathogenesis of sepsis, clinical treatments still have limitations. ALI was the most common organ injury in sepsis, which can lead to severe diseases of the lung and lead to uncontrolled and self-amplified pulmonary inflammation.²² The treatments available for ALI in clinical practice are limited to supportive treatment. Therefore, it is of great value and significance to discover a new drug for treating ALI.⁸ By establishing ALI model using LPS, the action of Kae on ALI was explored by spotting specific characteristic proteins and pathways.

Recently, a number of components extracted from herbs have been demonstrated to attenuate ALI by regulating different pathways. For instance, glycyrrhizic acid

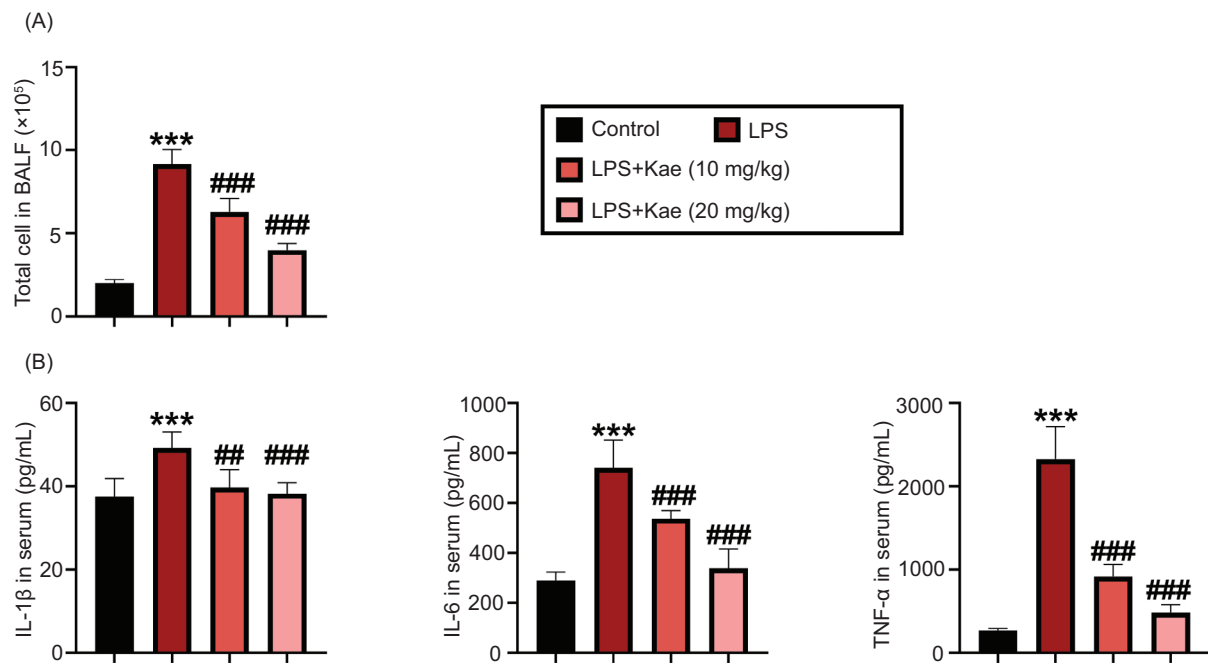


Figure 2 Kaempferitrin improves LPS-induced mouse pneumonia factor. (A) Total cells count in BALF in different groups. (B) Pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6, levels in serum determined by ELISA. * $P < 0.05$ vs control group. ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$ ($n = 3$), # $P < 0.005$, ## $P < 0.001$, ### $P < 0.0001$ ($n = 3$). *LPS group, compared with the control group; #Kae+LPS group, compared with the LPS group.

can regulate PI3K/AKT/mTOR pathway of LPS-induced inflammation in ALI.²³ The factors of inducible nitric oxide synthase (iNOS), NF- κ B, and mitogen-activated protein kinase (MAPK) signaling were regulated by *Picrasma quassioides* in LPS-induced ALI.²⁴ Emodin restrained pulmonary inflammation induced by LPS through regulation of NF- κ B pathway.²⁵ Our study showed that Kae could suppress NF- κ B and Akt/mTOR pathway activation.²⁶ Meanwhile, inhibition of TLR4/NF- κ B pathway attenuated the injury and inflammation of lung tissues in ALI.²⁷ Thus, TLR4/NF- κ B pathway plays an important role in LPS infections. In this study, we confirmed that Kae could inhibit the protein expression levels of transcription factors p65, p-p65, I κ B α , and p-I κ B α , thus attenuating the activating of NF- κ B pathway induced by LPS. These findings suggested that Kae attenuated ALI by blocking NF- κ B-hedgehog pathway.

Conclusion

The results of this study revealed the multiple functions of Kae in constricting LPS-induced ALI in mice through its anti-inflammatory and anti-apoptosis effects. Mechanistic studies showed that Kae repressed inflammatory response by inhibiting NF- κ B pathway activation, indicating that Kae might be a potential therapeutic component to treat ALI.

Funding

No funding was used in this study.

Conflict of Interest

The authors stated that there was no conflict of interest to disclose.

Data Availability

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

Author Contributions

Xiaoli Zhu, Yongyue Pan, Xin Xu, and Jing Xu designed and carried out the study. All the authors supervised data collection, and analyzed and interpreted the collected data. They prepared the manuscript for publication and reviewed the draft. All the authors read and approved the final manuscript.

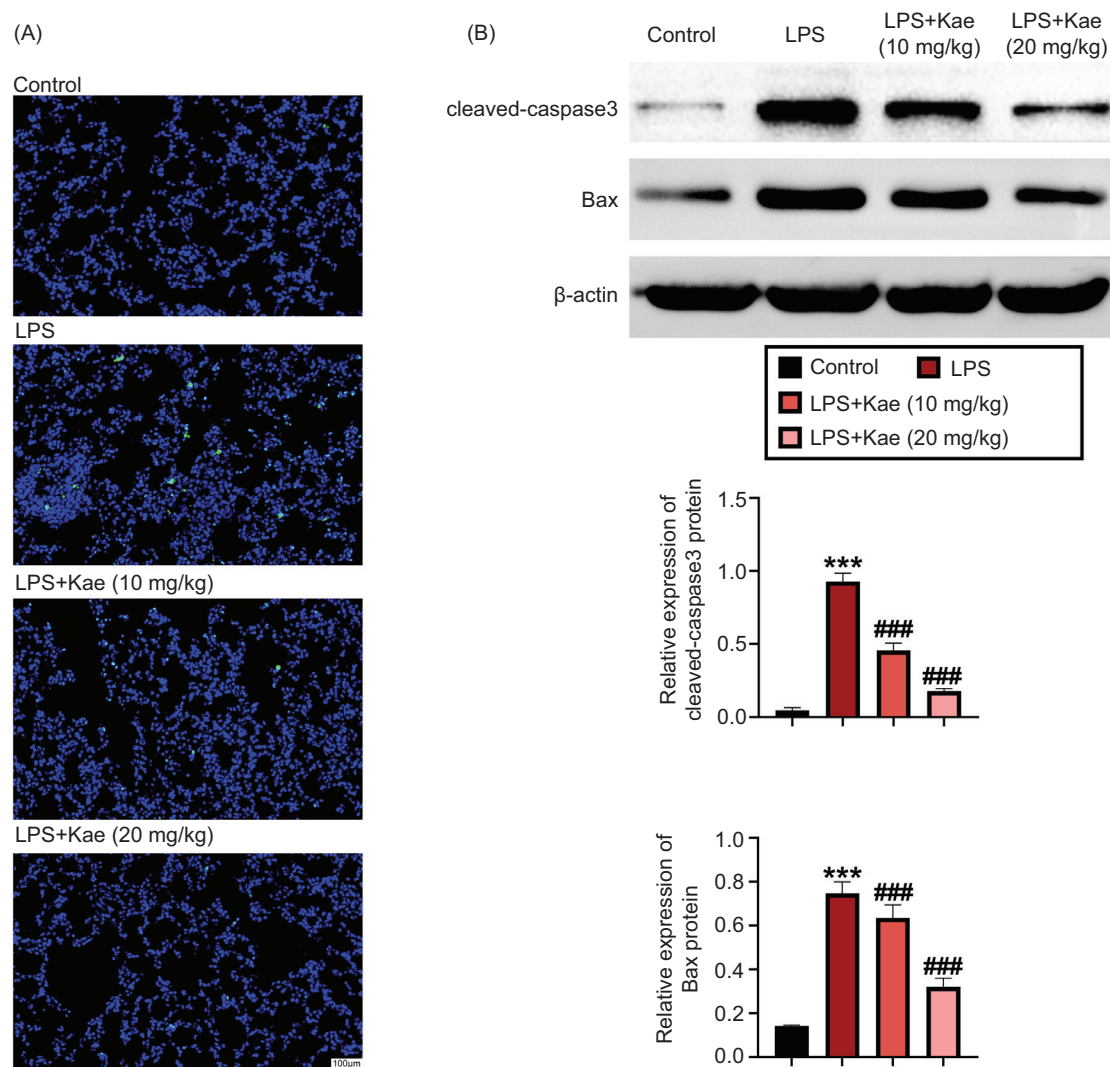


Figure 3 Kaempferitrin improves LPS-induced apoptosis in mouse lung tissue. (A) Apoptotic cells in lung tissues were observed by TUNEL. (B) The protein expression levels of Bax and cleaved caspase-3 were discovered by Western blot analysis. * $P < 0.05$ vs control group. ** $P < 0.005$, *** $P < 0.0001$ ($n = 3$). # $P < 0.005$, ## $P < 0.001$, and ### $P < 0.0001$ ($n = 3$). *LPS group, compared with the control group; #Kae+LPS group, compared with the LPS group.

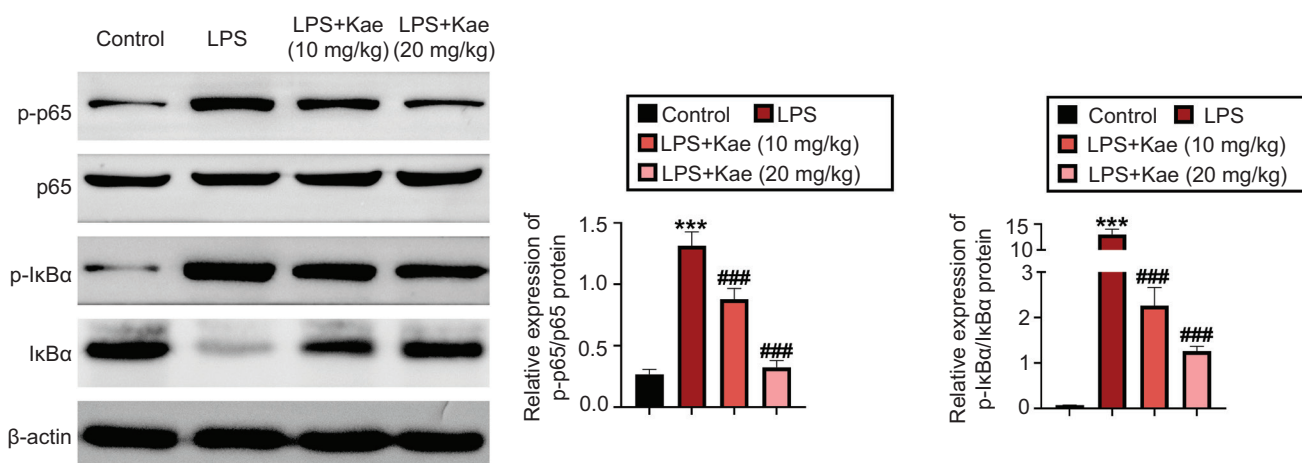


Figure 4 The mechanism study of Kae on NF-κB pathway. The protein expression levels of p-P56, p-56, p-IκBα, and IκBα were discovered by Western blot analysis. * $P < 0.05$ vs control group. * $P < 0.005$, ** $P < 0.001$, and *** $P < 0.0001$ ($n = 3$). # $P < 0.005$, ## $P < 0.001$, and ### $P < 0.0001$ ($n = 3$). *LPS group, compared with the control group; #Kae+LPS group, compared with the LPS group.

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