Neferine alleviates ovalbumin-induced asthma via MAPK signaling pathways in mice

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Abstract
Purpose: To investigate the role of neferine in ovalbumin (OVA)-induced asthma, and to reveal the possible mechanism.
Methods: In OVA-induced asthmatic mice, enzyme-linked-immunosorbent serologic assay was performed to evaluate the level of interleukin (IL)-4, IL-5, IL-13, immunoglobulin E (IgE) in serum and tumor necrosis factor-α (TNF-α), IL-6, IL-1β, and monocyte chemoattractant protein-1 (MCP-1) in bronchoalveolar lavage fluid (BALF). Eosinophil, neutrophil, and lymphocyte counts in BALF were calculated to assess inflammation. The pulmonary function was measured by airway resistance, peak expiratory flow (PEF) and forced expiratory volume/forced vital capacity (FEV0.4/FVC) ratio, and respiratory rate. Hematoxylin and eosin staining and Masson staining were used to evaluate lung injury. Further, Western blot analysis was conducted to detect phosphorylation of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 of mitogen-activated protein kinase (MAPK) signaling pathways.
Results: Neferine, 20 mg/kg or 40 mg/kg, could significantly decrease the levels of IL-4, IL-5, IL-13, and IgE in OVA-induced serum, and that of TNF-α, IL-6, IL-1β, and MCP-1 in OVA-induced BALF. Moreover, neferine could significantly decline eosinophil, neutrophil, and lymphocyte counts in BALF. Neferine contributed to improve OVA-induced airway resistance, promoted the value of PEF and FEV0.4/FVC ratio, and recovered the respiratory rate. It also reduced mucus secretion, distribution of inflammatory and goblet cells around bronchi, and attenuated collagen deposition in lung tissues. Furthermore, neferine reduced the phosphorylation of p38, JNK, and ERK to inhibit MAPK signaling pathways.
Conclusion: Neferine relieves asthma-induced inflammatory reaction, airway resistance, and lung injury by inhibiting MAPK signaling pathways. This could serve neferine as a novel therapeutic candidate for treating asthma.

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Introduction

Asthma, as one of the common respiratory and inflammatory diseases, is characterized by airway hyperresponsiveness (AHR), inflammation, and allergen-specific immunoglobulin E (IgE) secretion. Its incidence has increased in recent years because of widespread dust, pollen, toxic particles, environmental pollutants, and other allergens. At present, approximately 2,400,000 people are suffering from asthma globally, with 1000 deaths every day. Until now, asthma has not been cured, but only alleviated by use of drugs, resulting in serious physical and mental harm to patients.\(^1\)

Mitogen-activated protein kinase (MAPK) pathway, mainly containing p38 (p38 MAPK), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK1/2), has been reported to be closely associated with multiple aspects of asthma pathology and physiology. For example, airway biopsy samples from asthma patients showed elevated immunostaining signals for phosphorylation of ERK, p38, and JNK, which enhanced clinical severity of asthma.\(^2\) In addition, the cell proliferation and migration are dependent on ERK, p38, and JNK phosphorylation.\(^3\) In primary epithelial cells, ERK and p38 are also engaged in regulating RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted), IL-13, and eotaxin-3, and ERK/p38 are related with differentiation and activation of eosinophils.\(^4\) Thus, these features contribute to take MAPK signaling regulation as a potential target to suppress asthmatic inflammation.

Neferine, an alkaloid, is extracted from the green embryos of Nelumbo nucifera Gaerth (Lotus). Its molecular formula is \(C_{28}H_{34}N_{4}O_{6}\). Neferine has been reported to possess biological activities and pharmacological functions, such as anti-inflammatory, antiarrhythmic, antiplatelet aggregation, and antioxidant.\(^5\)\(^6\) As reported, neferine alleviated lipopolysaccharide (LPS)-induced cardiac dysfunction via antagonizing apoptotic and antioxidative effects.\(^5\) Neferine inhibited inflammatory effects on carbon tetra-chloride-induced liver fibrosis by repressing MAPK pathway and nuclear factor kappa B-nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NF-kB/IkBa) pathways.\(^6\) Neferine is an effective natural anti-histaminic and anti-inflammatory product, and can improve dermatitis and mast cell infiltration.\(^7\)

However, the role and involved mechanism of neferine in asthma is still unclear. This study focused on elucidating the effects of neferine on regulating serum-related factors, inflammatory factors, airway resistance, pulmonary function damage, and the lung injury in OVA-induced asthmatic mice. These data demonstrated that neferine relieved asthma-induced inflammatory reaction, airway resistance, and lung injury through inhibiting MAPK signaling pathways.

Materials and Methods

**Ethical statement**

Animal experiments were approved by the Committee of Experimental Animals of Changchun University of Traditional Chinese Medicine. All experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee.

**Mice**

The female BALB/c mice were obtained from SLAC Laboratory Animal Technology (Shanghai, China). The mice were raised in space power facility (SPF) provided with adequate food and drink and 12-h light-dark cycle.

**Establishment of OVA-induced asthmatic mice**

At sensitization stage, specifically on the 1st and 8th day, the mice were injected intraperitoneally 75-μg OVA (IO1280, Solarbio, Beijing, China) and 2-mg Al(OH)\(_3\) dissolved in 200-μL 0.9% NaCl solution. At the maintenance stage (week 3-12), the mice were injected intranasally 20-μL OVA (50 μg/kg) in 0.9% NaCl solution twice a week.\(^8\)

**Treatment**

Neferine (SN8050, Solarbio, Beijing, China) powder was diluted with 0.1% dimethyl sulfoxide (DMSO) and subsequently diluted in 0.9% NaCl solution for oral administration using gavage needles. The total of 25 mice were randomly divided into the following five groups, each group having 5 mice: control group, OVA group, OVA+10-mg/kg neferine group, OVA+20-mg/kg neferine group, and OVA+40-mg/kg neferine group.\(^8\) In control group, no OVA was administered, and only 200-μL 0.9% NaCl solution and Al(OH)\(_3\) were injected at the sensitization stage, and 20-μL 0.9% NaCl solution was injected at the maintenance stage. For OVA group, the administration was the same as described above for establishing OVA-induced asthmatic mice. For the other three OVA+neferine groups, on the basis of the OVA group, the mice were administrated with 20-μL neferine (10, 20, or 40 mg/kg in 0.9% NaCl solution) 2 h before each OVA administration at the maintenance stage.

Airway hyperresponsiveness in mice was examined at the end of OVA administration. Then the mice were anesthetized with 2% sodium pentobarbital in order to collect bronchoalveolar lavage fluid (BALF), blood, lung tissue, and airway tissue samples.

**Assessment of airway hyperresponsiveness**

At 24 h after the last OVA administration, AHR was detected by forced oscillation technique. A catheter was inserted into trachea and connected to a Master Screen (MS)-IOS pulmonary function detector (Jaeger Co, Germany). Airway responsiveness was tested by inhaled methacholine chloride with an increase in dose from 3.125 to 50 mg/mL. The lung function values were reflected by airway resistance (cmH\(_2\)O/mL/s).

**Enzyme-linked-immunosorobent serologic assay (ELISA)**

The serum-related factors and inflammatory factors in OVA-induced BALF were evaluated by using ELISA kits of IL-4 (ab100710), IL-5 (ab204523), IL-13 (ab219634),...
IgE (ab157718), TNF-α (ab208348), IL-1β (ab197742), IL-6 (ab222503), and MCP-1 (ab208979) (Abcam, Cambridge, MA, USA). Briefly, 100-µL sample was incubated for 2 h in ELISA well after washing for five times; 100-µL detection antibody was added and incubated for 1 h. Each well was washed for five times and incubated with 100-µL enzyme working reagent for 30 min. Then each well was washed for five times and incubated with 100-µL tetramethylbenzidine (TMB) reagent for 30 min. Finally, the reaction was completed with 50-µL stop solution. The absorbance value was read at 450 nm.

**Hematoxylin and eosin staining**

Hematoxylin and eosin (H&E) staining was performed using H&E stain kit (G1120; Solarbio Life Sciences, Beijing, China) according to the manufacturer’s protocol. Briefly, lung or airway tissues were fixed, embedded in paraffin, sectioned, and dewaxed. The tissue slices were stained with hematoxylin for 10 min and treated with differentiation solution for 30 s. After washing for twice, the tissues were stained with eosin for 1 min, and dehydrated in gradient ethanol. Finally, the sample slices were soaked in xylene and sealed with neutral gum. A DMLA automatic microscope (Leica, Solms, Germany) was used to capture images.

**Masson staining**

Masson staining was performed using Masson stain kit (G1340; Solarbio Life Sciences) according to the manufacturer’s protocol. Briefly, lung or airway tissues were fixed, embedded into paraffin, sectioned, and dewaxed into double distilled water. The tissue slices were stained with Weigert’s iron hematoxylin solution for 5 min, differentiated with acid alcohol differentiation solution for 10 s, and washed twice. After being treated with Bluing solution for 5 min, the slices were stained with Ponceau acid fuchsin solution for 5 min, and differentiated in phosphomolybic acid. Then the slices were directly moved into acetic acid working solution for staining, and dehydrated in gradient ethanol. Finally, the sample slices were soaked in xylene and sealed with neutral gum.

**Western blot analysis**

The cellular protein was extracted by radioimmunoprecipitation (RIPA) lysis buffer (89901; Thermo Scientific, Carlsbad, CA). The lysates were processed for immunoblot with the primary antibodies listed in Table 1. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were used as secondary antibodies (B900210; ProteinTech Group; 1:5000). Finally, the target bands were visualized with ECL Western blotting detection kit (Solarbio Life Sciences). For quantification of Western blot signal, the relative intensity of each band was measured by the ImageJ software, and the relative expression levels were normalized to relative β-actin levels.

**Quantification and statistical analysis**

Statistical analysis was done by GraphPad Prism 8. Data were presented as mean ± standard error (SE) from three biological replicates. Differences between any two groups were compared by unpaired t-tests. Multiple group comparisons were analyzed with ANOVA. P < 0.05, <0.01, and <0.001 were considered statistically significant.

**Results**

**Neferine declined serum-related factors in OVA-induced asthmatic mice**

The structure of neferine is shown in Figure 1A. To explore the effect of neferine in asthma, an OVA-induced asthma model was established with mice (Figure 1B). To study the effect of neferine on serum-related factors, four typical factors, including IL-4, IL-5, IL-13, and IgE, in serum samples were measured using ELISA. First, the expression of all four factors was elevated drastically in the OVA group, compared to their control group, indicating that serum-related factors were induced to a higher level in asthmatic mice. Although no significant decline was observed in the low-dose group of neferine (10 mg/kg), the levels of IL-4, IL-5, IL-13, and IgE were significantly decreased in the mid-dose group (20-mg/kg neferine) and high-dose group (40-mg/kg neferine) (Figures 1B–E). Therefore, neferine declined the concentration of serum-related factors in OVA-induced asthmatic mice.

**Neferine inhibited the expression of inflammatory factors in OVA-induced BALF**

The effect of neferine was investigated on inflammation. ELISA was performed to measure the expression level of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Antibodies information.</th>
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<tbody>
<tr>
<td><strong>Proteins</strong></td>
<td><strong>Cat. No.</strong></td>
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<tr>
<td>Phosphorylated (p)-p38</td>
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</tr>
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<td>p38</td>
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<td>p-JNK</td>
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<td>ERK</td>
<td>ab179461</td>
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<tr>
<td>GAPDH</td>
<td>10494-1-AP</td>
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Neferine improved OVA-induced airway resistance and pulmonary function damage

Given that neferine ameliorated OVA-induced asthma, the airway resistance reflected by RI (cmH₂O/mL/s) was also measured. In brief, OVA induced RI to a considerable higher level; for example, RI elevated drastically in the OVA group than in the control group at 50 mg/mL methacholine. While there was no obvious change in RI in the 10-mg/kg Neferine group, 20- or 40-mg/kg neferine significantly ameliorated the airway resistance (Figure 3A). Thus, concentration of neferine played a significant role in improving OVA-induced airway resistance.

Furthermore, pulmonary function was evaluated by two parameters, including peak expiratory flow (PEF) and forced expiratory volume/forced vital capacity (FEV₁/FVC) ratio. For OVA induction, the values of both PEF and FEV₁/FVC ratio declined significantly. However, 20- or 40-mg/kg neferine in OVA-induced asthmatic mice promoted PEF and FEV₁/FVC ratio, but no significant difference was observed.
Neferine alleviates asthma via MAPK signaling pathways

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![Graph](image)

**Figure 3** Neferine improved OVA-induced airway resistance and pulmonary function damage. (A) The airway resistance reflected by RI (cmH₂O/mL/s) was measured to demonstrate that Neferine alleviated the airway resistance from OVA-induced asthma. (B and C) PEF and FEV₀.₄/FVC were measured to assess the effect of neferine on attenuating pulmonary function damage. (D) Neferine recovered the respiratory rate of OVA-induced asthmatic mice.

![Images](image)

**Figure 4** Neferine suppressed OVA-induced lung injury. (A) H&E staining was performed to detect pathological changes in the lung tissue of mice. Neferine reduced mucus secretion, and the number of goblet and inflammatory cells around the bronchi. (B) Masson staining was used to detect collagen deposition in the lung tissue of mice. Neferine decreased collagen deposition in the lung tissue of OVA-induced mice. Scale bar: 200 μm.

in 10-mg/kg neferine group, compared to the OVA group (Figures 3B and C). In addition, OVA induction accelerated the respiratory rate, but 20- or 40-mg/kg neferine could help mice to recover respiratory rate nearly to a normal level (Figure 3D). The above data demonstrated that neferine improved OVA-induced pulmonary function damage.

Neferine suppressed OVA-induced lung injury

Since neferine improved pulmonary function, it is to be investigated whether neferine could attenuate lung tissue injury. First, H&E staining was performed to detect pathological injury in lung tissues. In the control group, the lung tissue structure was well organized, the bronchi were intact, and neither inflammatory cell infiltration around the bronchi nor goblet cell hyperplasia was observed. However, the lung tissue structure in the OVA group was disordered, as the alveolar wall was significantly thickened, and the infiltration of inflammatory cells around the bronchi, the secretion of mucus, and the proliferation of goblet cells were significantly increased. By contrast, structure of the lung in mice of the high-dose (40 mg/kg) neferine group improved significantly. Mucus secretion, number of goblet cells, and inflammatory cells around the bronchi were reduced significantly (Figure 4A).

![Images](image)
Neferine inhibited MAPK signaling pathways

It is known that neferine inhibited MAPK signaling pathways in carbon tetrachloride-induced liver fibrosis; however, it is to be investigated whether neferine could regulate MAPK pathway in OVA-induced asthma. Western blot analysis was conducted to test the phosphorylation and expression levels of three kinases—p38, JNK, and ERK. OVA induction or neferine treatment did not affect expression levels but instead regulated the phosphorylation of p38, JNK, and ERK. Specifically, their phosphorylation was increased drastically in the OVA group than in the control; however, 20- or 40-mg/kg neferine could significantly decline the phosphorylation of p38, JNK, and ERK, although the difference was not significant in the group treated with 10-mg/kg neferine (Figure 5). These data indicated that neferine inhibited OVA-induced MAPK pathway.

Discussion

Asthma, being a common respiratory and inflammatory disease, affects a large number of populations, with 1000 deaths reported annually. As it leads to serious physical and mental harm to patients, it is emergent to develop more effective drugs to control this disease. The typical characteristics of asthma are AHR, airway inflammation, and allergen-specific IgE secretion. In this study, IL-4, IL-5, IL-13, and IgE were first evaluated in serum using ELISA. The results demonstrated that these factors were induced to a higher level, which was consistent with the results of the previous studies. Meanwhile, this study confirmed that neferine declined the concentration of serum-related factors in OVA-induced asthmatic mice, revealing the potential of neferine to relieve asthma manifestations.

Neferine has been reported to exert anti-inflammatory effects in carbon tetrachloride-induced liver fibrosis. The effect of neferine in asthma remains to be studied. Hence, it is necessary to study the function of neferine in airway inflammation. This study revealed that neferine declined the protein level of inflammatory factors, including TNF-α, IL-1β, IL-6, and MCP-1, and the counts of eosinophils, neutrophils, and lymphocytes in BALF.

For OVA-induced asthma, it is necessary to evaluate airway resistance and pulmonary function. For example, previous studies have demonstrated that celastrol, extracted from the roots of Tripterygium wilfordii, alleviates AHR in obese asthmatic mice, because airway resistance is closely associated with the production of cytokines in the lungs. Glabridin, a component of licorice, weakened AHR and inflammation in a mice model of OVA-induced asthma. Pulmonary function measurements are important for studying respiratory disease models. Here, two parameters, PEF and FEV₁/FVC ratio, were evaluated, and OVA induction significantly reduced the value of both parameters. The same method was used in other studies, and these parameters were evaluated in OVA-induced mice to assess the function of glabridin in weakening airway resistance and improve pulmonary function. In addition, respiratory rate was the other indicator for assessing pulmonary function. Furthermore, H&E and Masson staining were used to observe the effect of neferine in relieving lung injury in OVA-induced asthma. Thus, it could be concluded from the results of the present study that neferine lessens both airway resistance and pulmonary function damage.

The activity of MAPK and signal transducer and activator of transcription 1 (STAT1) has been reported to increase in OVA-treated mice, compared to the control, activation of MAPK engaged in inflammation, and airway remodeling in asthma. Further, neferine inactivated the MAPK and NF-κB/IKKα signaling pathway and suppressed the nuclear translocation of phosphorylated (p)MAPK. In this study, regulation of MAPK by neferine was elucidated. Three MAPKs (p38, JNK, and ERK) were evaluated, including the protein level and phosphorylation. As a result, neferine was discovered to inhibit MAPK pathway by reducing the phosphorylation of p38, JNK, and ERK, rather than affecting their expression levels.

Conclusion

This study demonstrated that neferine declined serum-related factors and the expression of inflammatory factors, improved airway resistance and pulmonary function damage, and alleviated lung injury in OVA-induced asthmatic mice. Neferine reduced the phosphorylation of p38, JNK and ERK to inhibit MAPK signaling pathway. Therefore, neferine could be considered as a novel therapeutic candidate for treating asthma.

Availability of Data and Material

All data generated or analyzed in this study are included in this published article. The datasets used and/or analyzed are available from the corresponding author on reasonable request.

Competing Interests

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

Author Contributions

Tonggang Zhu designed the study, completed the experiment, and supervised data collection. Xue Xiao analyzed and interpreted the data. Yufu Dong and Chengbo Yuan prepared the manuscript for publication and reviewed draft of the manuscript. All authors read and approved the final manuscript.
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Figure 5 Neferine inhibited the MAPK pathway. Western blot analysis was conducted to test the phosphorylation and expression of three molecules, including p38, JNK, and ERK. Neferine did not affect the expression level but declined the phosphorylation of p38, JNK, and ERK.

References


