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Original Article

Protective activity of asatone against ovalbumin-induced allergic asthma

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Abstract: Allergic asthma is a chronic lung disease characterized by wheezing, coughing, chest tightness and shortness of breath. Clinically, the treatments against asthma focus on controlling the symptoms rather than inhibiting recurrence radically. Additionally, local and systemic side effects caused by current treatments are worthy of attention. Therefore, a novel therapeutic strategy against asthma is needed. Asatone is a pharmacologically active component from Radix et Rhizoma Asari, which has anti-inflammatory effects in lipopolysaccharide-induced lung injury. In the present study, we showed that asatone could protect mice against OVA-induced asthma, as manifested by attenuating inflammation infiltration, mucus production, and airway hyperreactivity and suppressing the elevation of IL-4, IL-5, and IL-13 in broncho-alveolar lavage fluid. Overall, results of the present study support use of asatone as a potent therapeutic strategy for clinical treatment of allergic asthma.

Keywords: Asatone, asthma, Th2 cells, airway hyperreactivity

Introduction

Allergic asthma is a chronic airway inflammatory disease characterized by reversible episodes of airway obstruction, pronounced airway hyperreactivity, peribronchial inflammation, and airway remodelling [1, 2]. The World Health Organization estimated that more than 300 million people worldwide were suffering from this respiratory disease [3]. Although the majority of patients with asthma can achieve a good level of control with existing treatments, asthma still has a chronic disease course, and the effectiveness of current treatments is not satisfactory for numerous patients.

Asatone is a pharmacologically active component in the traditional Chinese medicine Radix et Rhizoma Asari (xixin) [4] and has been reported to prevent acute lung injury by reducing expression of NF-κB, MAPK, and inflammatory cytokines [5]. As allergic asthma is a chronic airway inflammatory disease, we speculate that asatone could help in the treatment of allergic asthma. To address this feasibility, we conducted studies in an Ovalbumin (OVA)-induced asthma model, and then assessed the impact of asatone on the disease development. Administration of asatone attenuated OVA-induced asthma as manifested by blunting lung inflammatory cell infiltration and airway hyper-responsiveness. In addition, the levels of inflammatory cytokines and OVA-specific IgE were markedly reduced after asatone treatment. Together, our data suggested that treatment with asatone may be a novel approach for the therapy of asthma.

Materials and methods

Animals

Female C57BL/6 mice (6-8 weeks old) were obtained from the Animal Experimental Center of Hubei Province (Wuhan, China). All animals were housed in a specific pathogen-free (SPF) animal facility at the Tongji Medical College under a 12 h light/dark photocycle with diet and water available ad libitum. All experimental procedures were approved by the Animal Care and Use Committee of the the Central Hospital of Wuhan.

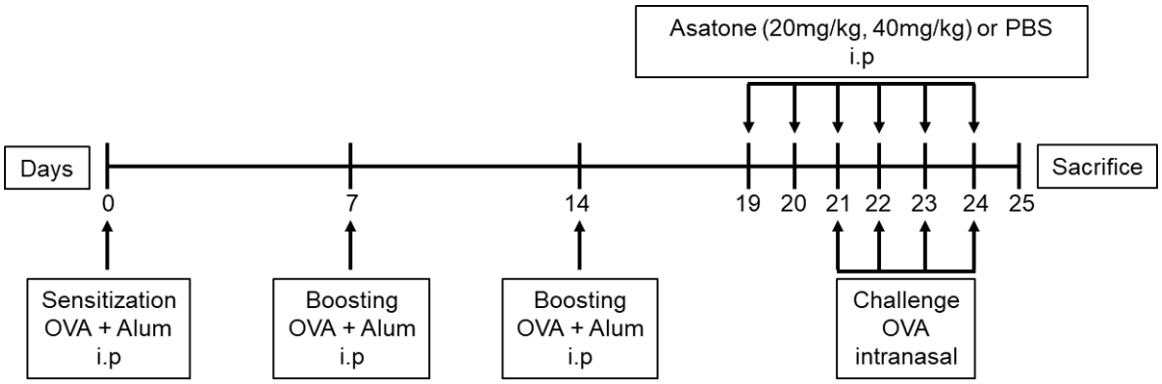

Figure 1. Model of asthma induced by OVA and drug-delivery method of asatone in mice.

OVA-induced asthma model in mice

The mice were randomly divided into four groups (PBS, OVA, OVA + Asatone 20 mg/kg, and OVA + Asatone 40 mg/kg) with 7 mice in each group. Asthma mouse model was induced as previously described [6]. Briefly, the mice were sensitized using intraperitoneal injection of 100 μg OVA (Sigma, MO, USA) emulsified in 1 mg of aluminum hydroxide gel (Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 200 μl, on Days 0, 7 and 14. Mice injected with an equal volume of saline were served as controls. From Day 21 to Day 24, the sensitized mice were anesthetized with diethyl ether and intranasally challenged with 50 μl OVA (20 μg/μl) or saline for 4 consecutive days. From Day 19 to Day 24, the mice in the Asatone 20 mg/kg group and Asatone 40 mg/kg group received 20 mg/kg or 40 mg/kg of asatone respectively (Melonepharma, Dalian, China) daily through intraperitoneal injection 0.5 h prior to OVA challenged. The mice in the PBS group and OVA group received equal amounts of PBS (**Figure 1**).

Analysis of airway responsiveness

Airway responsiveness to methylcholine was assessed 24 hours after the last OVA challenge by using a computer-controlled small animal ventilator system (flexiVent; SCIREQ Scientific Respiratory Equipment, Inc., Montreal, Canada), as previously described [1]. Briefly, mice were anesthetized by intraperitoneal injection of 10 mg/kg ketamine and 1 mg/kg xylazine, and the values were recorded for airway resistance (R_{aw}; cm/H_2O.sec/ml) in response to incremental doses of methylcholine (0.00, 6.25, 12.5, 25, and 50 mg/mL) administered by means of nebulization through the airway.

Broncho-alveolar lavage preparation

Broncho-alveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lung with 0.6 mL of sterile PBS using previously described techniques [7]. Approximately 0.4 mL of BALF was recovered from each animal. BALF was centrifuged at 300 g for 5 minutes, and the supernatants were stored at -80°C before cytokine measurement. The total cell suspension was resuspended in 1 mL of PBS. Cells were counted with a hemocytometer. Differential cell counts were measured after Diff-Quik staining.

Assessment of liver function and renal function

Liver and renal function was assessed by measuring blood alanine aminotransferase, aspartate aminotransferase, serum urea, and creatinine at the Department of Clinical Laboratories of the Central Hospital of Wuhan.

Histologic analysis

The left lung was inflated with 4% neutral buffered paraformaldehyde and subsequently removed and placed in fresh 4% neutral buffered paraformaldehyde for 24 hours at room temperature. After embedding the tissues in paraffin, they were sliced into 5-μm sections and subjected to hematoxylin and eosin (H&E), and periodic acid-Schiff (PAS) by using the established techniques [8, 9]. The results were assessed by 2 pathologists by using a bright-field or fluorescent microscope in a blinded fashion.

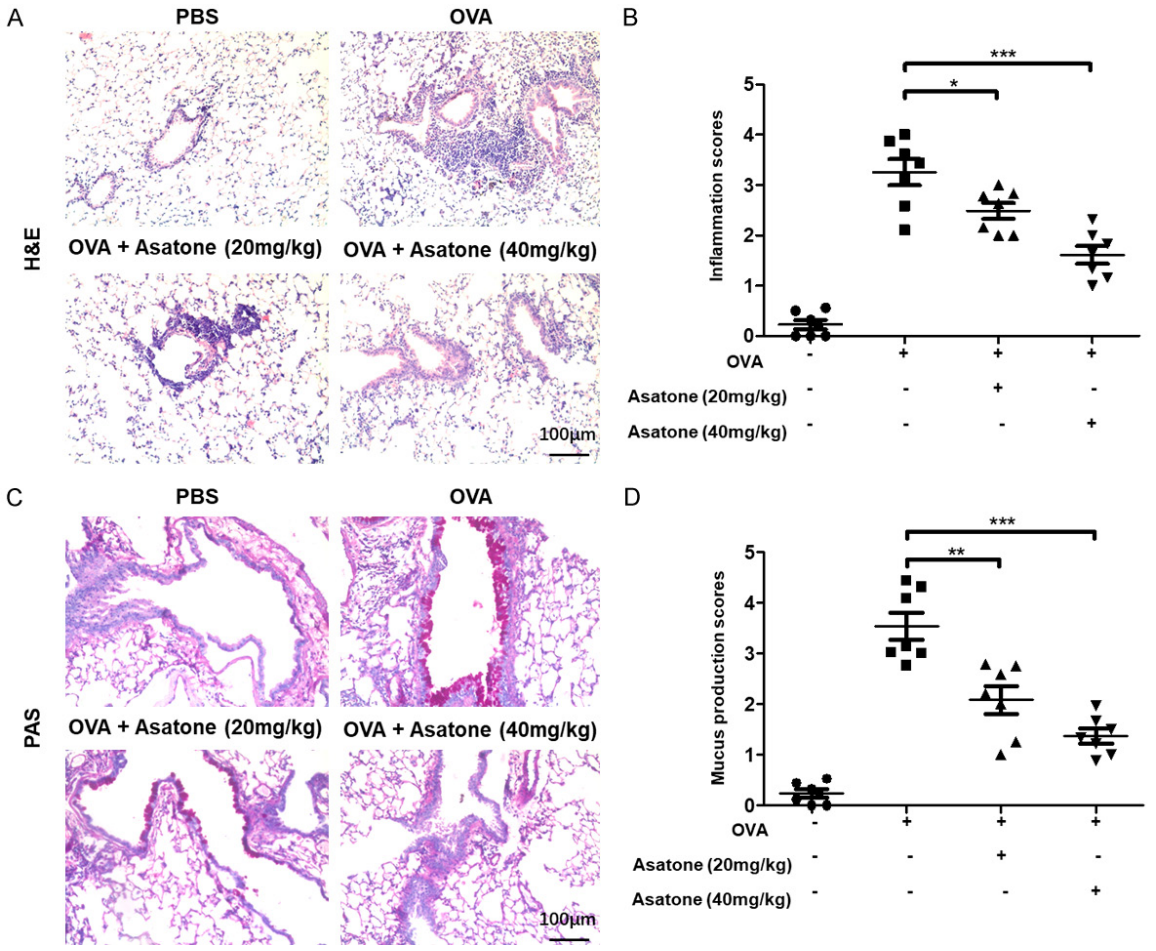

Figure 2. Comparison of the severity of lung inflammation after asatone treatment. A. H&E staining for analysis of lung inflammation. B. Bar graphs represent the quantitative mean score of the severity of inflammation. C. PAS staining for analysis of mucus hypersecretion. D. Bar graphs represent the quantitative mean score of mucus production. Images were captured at a magnification of 200×. Seven mice were included in each study group. Experimental results are expressed as mean ± standard deviation. *P < 0.05; **P < 0.01.

Enzyme-linked immunosorbent assay of inflammatory cytokines and OVA specific IgE

Levels of the inflammatory cytokines IL-4, IL-5, IL-13 in BALF samples and OVA Specific IgE in peripheral blood were measured by using commercial ELISA kits (Neobioscience, Beijing, China), as previously described [10].

Statistical analysis

All data are presented as mean ± SEM. Statistical analyses of the data were conducted using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) and two-way analysis of variance followed by Bonferroni's post hoc test was used for the comparison of multiple groups. P<0.05 was considered a significant difference.

Results

Administration of asatone attenuates airway inflammation and mucus production in OVA-induced asthmatic mice

To address the therapeutic effects of asatone on OVA-induced asthma, we first examined pathologic alteration in the lungs from mice after onset of asthma. As expected, severe inflammatory responses were observed in mice following 4 days of repeated OVA challenges, as demonstrated by the infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues. However, the inflammatory responses were attenuated after treatment with low-dose of asatone (20 mg/kg), and a more prominent alleviating effect was observed in the mice treated with high-dose of asatone (40 mg/kg), indicating that asatone may reduce

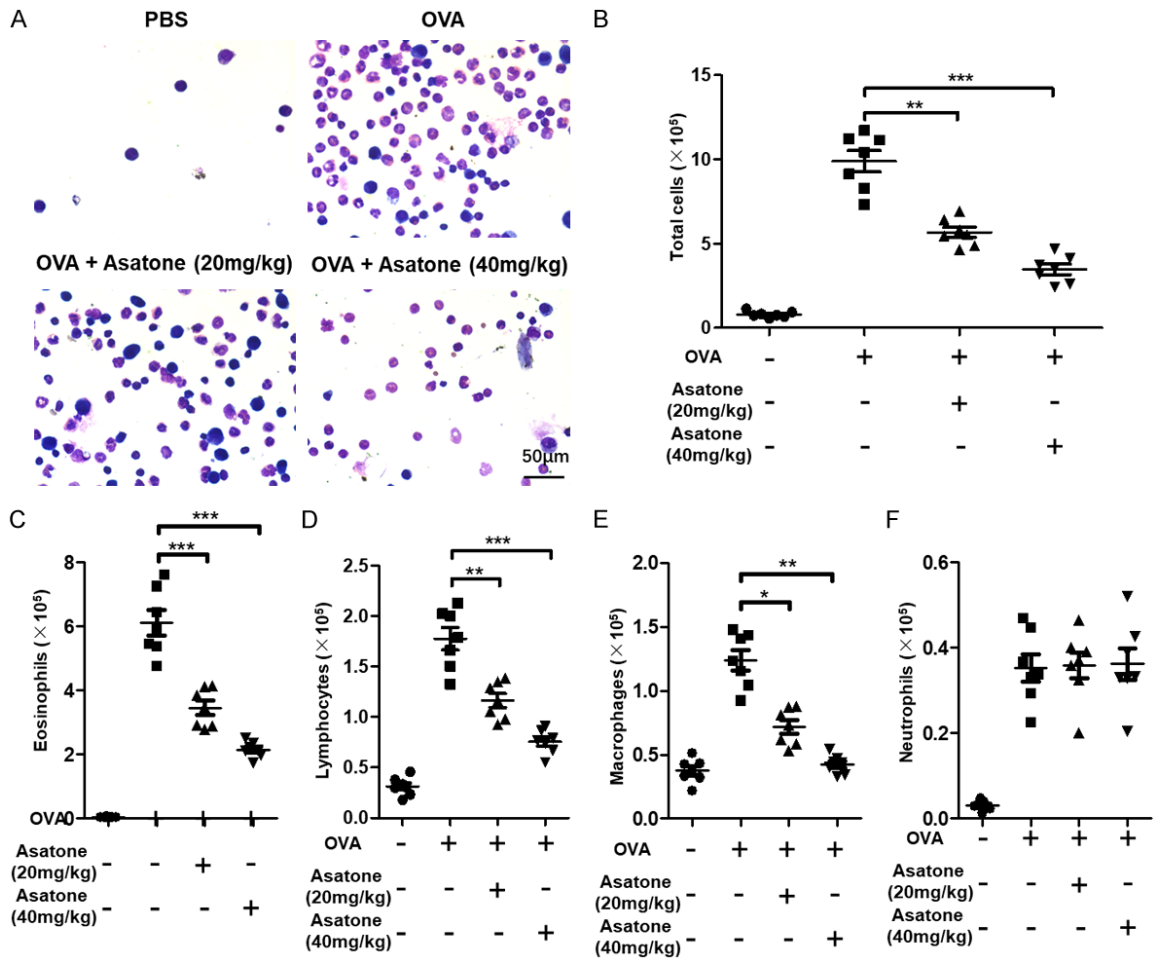

Figure 3. Comparison of the number of inflammatory cells in BALF samples after asatone treatment. (A) A representative Diff-Quik staining result. (B) Analysis of the total number of inflammatory cells in BALF samples from OVA-challenged mice. (C-F) Analysis of eosinophils (C), lymphocytes (D), macrophages (E), and neutrophils (F) in BALF samples. Images were captured using a microscope at a magnification of 400×. Seven mice were included in each study group. Experimental results are expressed as mean ± standard deviation. *P < 0.05; **P < 0.01; ***P < 0.001.

the airway inflammation in a dose-dependent manner (**Figure 2A** and **2B**).

Since goblet cell hyperplasia and mucus hypersecretion is a feature of airway remodelling, we thus next assessed mucus production by PAS staining. Indeed, repeated exposure to OVA for 4 consecutive days increased mucus production in OVA group compared with the PBS group. Administration of asatone attenuated OVA-induced mucus production in a concentration-dependent manner (**Figure 2C** and **2D**).

Asatone decreases inflammatory cells in BALF from OVA-induced asthmatic mice

To further confirm the above observation, we compared the number and subtype of inflammatory cells in the BALF. As expected, OVA-induced asthmatic mice manifested significantly higher total inflammatory cell numbers in the BALF than control group (**Figure 3A** and **3B**). By contrast, the number of total inflammatory cell in the BALF was decreased significantly after low-dose asatone treatment. Similarly, administration of high-dose asatone caused a significant decrease in the number of total inflammatory cells. Consistent with above data, BALF derived from asatone-treated mice contained significantly fewer eosinophils (**Figure 3C**), lymphocytes, (**Figure 3D**) and macrophages (**Figure 3E**) compared with OVA-challenged mice. However, the total number of neutrophils was not significantly different after asatone treatment (**Figure 3F**).

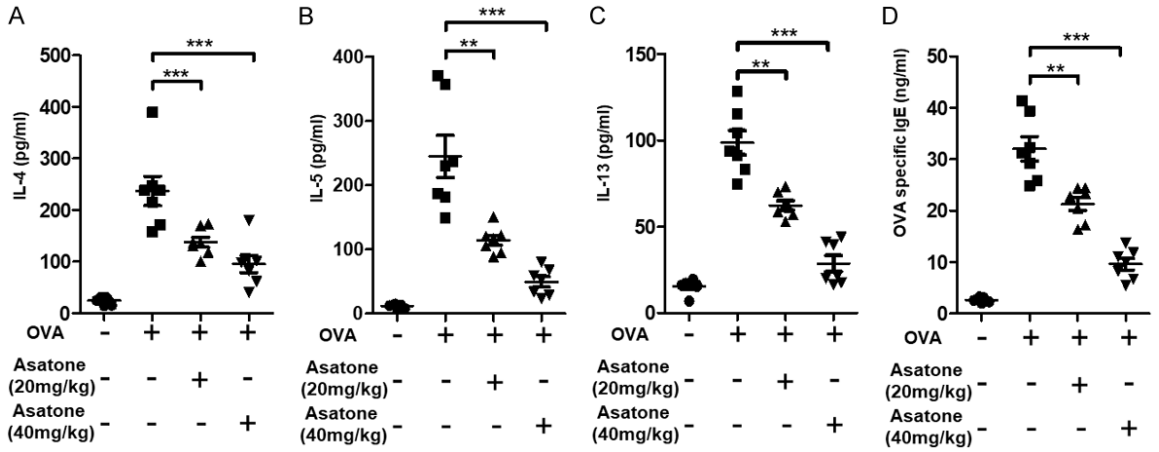

Figure 4. Asatone attenuated the levels of Th2 cytokines and OVA-specific IgE in an OVA-induced asthma model. (A-C) The levels of IL-4 (A), IL-5 (B), IL-13 (C) in BALF. (D) The levels of OVA-specific IgE. Seven mice were included in each study group. Experimental results are expressed as mean ± standard deviation. *P < 0.05; **P < 0.01; ***P < 0.001.

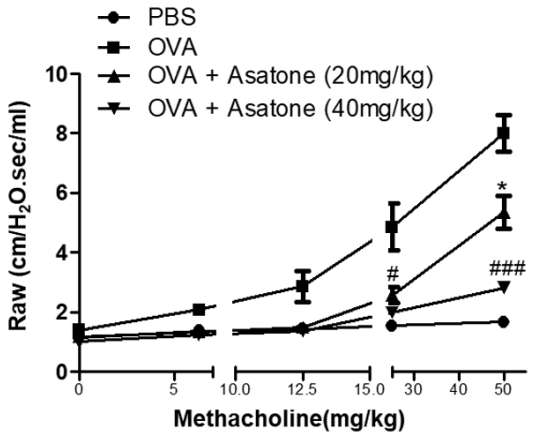

Figure 5. Effect of asatone on airway hyper-responsiveness in mice challenged with OVA. The response to increasing doses of methylcholine after asatone treatment. Seven mice were included in each study group. Experimental results are expressed as mean ± standard deviation. #P < 0.05 (OVA group VS. OVA + asatone 40 mg/kg); ###P < 0.001 (OVA group VS. OVA + asatone 40 mg/kg); *P < 0.05 (OVA group VS. OVA + asatone 20 mg/kg).

Asatone suppresses the elevation of IL-4, IL-5, IL-13 and OVA-specific IgE in OVA-induced asthmatic mice

Given the critical role of T helper 2 (Th2) cells in the initiation and progression of asthma [11-13], we examined Th2 cell derived cytokines in the BALF. Indeed, IL-4, IL-5 and IL-13 levels in BALF were significantly higher in OVA-induced asthmatic mice than those in the controls (**Figure 4A-C**). IL-4, IL-5 and IL-13 levels in BALF from asatone-treated mice, however, were markedly lower than those in OVA-induced asthmatic mice. Moreover, asatone at a concentration of 40 mg/kg caused a more significant decrease compared with the 20 mg/kg asatone group. Similar to the cytokine results, OVA-specific IgE levels were significantly lower in asatone-treated mice than those of untreated OVA-induced asthmatic mice in a dose-dependent manner (**Figure 4D**).

Airway hyperreactivity is inhibited by asatone treatment

To further evaluate the effects of asatone on asthma, we examined airway hyperreactivity by assessing Raw in response to increasing concentrations of methylcholine in mechanically ventilated mice. Interestingly, administration of asatone was identified to attenuate the development of airway resistance compared with OVA groups (**Figure 5**).

Assessment of liver function and renal function

The toxicity of asatone to the organism was also observed. Importantly, asatone had no significant toxicity to mice, as manifested by the plasma concentrations of aspartate aminotransferase (AST, **Figure 6A**), alanine aminotransferase (ALT, **Figure 6B**), urea (**Figure 6C**), and creatinine (Cr, **Figure 6D**).

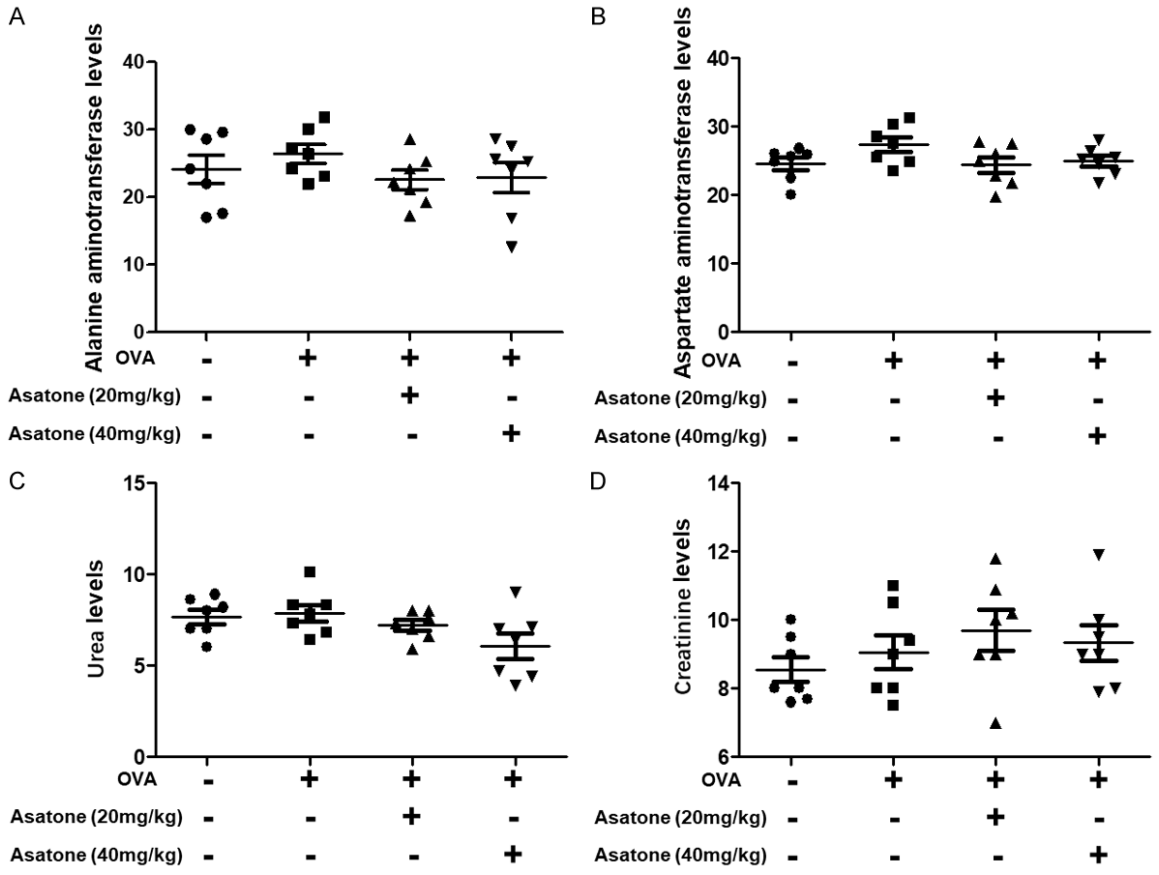

Figure 6. Toxic effect of asatone in mice. A, B. Liver function. C, D. Seven mice were included in each study group. Experimental results are expressed as mean ± standard deviation.

Discussion

Currently, corticosteroids are still regarded as the classic method to treat asthma [14, 15]. However, prolonged use of corticosteroids has several adverse effects [16]. Therefore, it is urgent to seek novel therapeutic options safe and effective in asthma management. In the present study, we explored the therapeutic effects of asatone on asthma in OVA-induced mice models and found that asatone attenuated histopathologic changes in the lung, accompanied by reduced airway hyperreactivity, decreased inflammatory cell infiltration, and suppression of the elevation of IL-4, IL-5 and IL-13 in BALF.

Previous studies showed that airway inflammation played a critical role in the initiation, progression, and persistence of asthma [1, 13, 17], especially, Th2-related immune response [18]. Th2 cells are the main producers of the Th2 cytokines IL-4, IL-5, and IL-13. IL-4 is known to mediate the isotype B cell production of IgE, eosinophil recruitment, and the differentiation of Th2 cells [19], while IL-5 plays a key role in the differentiation, development, and survival of eosinophils [20]. Additionally, IL-13 promotes eosinophilia in mouse models, and induces eotaxin production, a chemokine responsible for selective eosinophil chemotaxis [21]. Herein, our data showed asatone could successfully reduce the production of the Th2 cytokines IL-4, IL-5 and IL-13, infiltration of eosinophils, and levels of IgE in a dose-dependent manner. Similarly, previous data demonstrated that asatone could attenuate the inflammatory response induced by LPS [5].

It was noted that infiltration of eosinophils caused airway hyperreactivity [22]. Therefore, we evaluated the effects of asatone on airway hyperreactivity in asthmatic mice. Indeed, airway hyperreactivity significantly increased with increasing concentrations of methylcholine in OVA-challenged mice. Conversely, airway hyper-

responsiveness was abolished by asatone, which provides further evidence for the protective effect of asatone against asthma, particularly in the 40 mg/kg asatone group. Mucus hypersecretion is common in asthmatic patients [23]. In our mouse model, we observed high mucus production, as evidenced by PAS staining. Surprisingly, asatone had the ability to reduce goblet cell hyperplasia and reduce excessive secretion of mucus in a concentration-dependent manner.

Asatone is a pharmacologically active component in the traditional Chinese herb Radix et Rhizoma Asari (xixin) [5]. As one of the most important traditional Chinese medicines, xixin was used to treat several different diseases according to the Chinese Pharmacopoeia for thousands of years [24-26]. Previous study indicated that large dose of Xixin may cause liver cell injury in rats, including increasing cell membrane permeability, the desmoenzyme leakage entering the blood, and increasing serum liver enzymes [27]. Herein, we measured liver function and renal function to assess the toxicity of asatone in mice. Importantly, no significant differences in the levels of blood alanine aminotransferase, aspartate aminotransferase, serum urea and creatinine were observed between asatone-treated mice and the control mice, indicating that asatone may be safe, but more studies were need to confirm this result.

Together, asatone could reduce the production of the Th2 cytokines, the infiltration of inflammatory cells, mucus production, and airway hyperreactivity in a dose-dependent manner. These results suggest that asatone may play a multifunctional role in controlling progression of asthma, and is thus worthy of further attention for anti-asthma therapy.

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Disclosure of conflict of interest

None.

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