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Protective effects of aloin on asthmatic mice by activating Nrf2/HO-1 pathway and inhibiting TGF- β /Smad2/3 pathway

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Abstract

Background: Asthma is a severe chronic respiratory disease affecting all age groups with increasing prevalence. Anti-inflammatory strategies are promising options for the treatment of asthma. Although the inhibitory effect of aloin on inflammation has been demonstrated in various diseases, its effect on asthma remains unknown.

Methods: A mice asthma model was established by treating with ovalbumin (OVA). The effects and mechanism of aloin on the OVA-treated mice were determined by enzyme-linked-immunosorbent serologic assay, biochemical examination, hematoxylin and eosin and Masson's staining, and Western blot assay.

Results: OVA treatment in mice significantly increased the number of total cells, neutrophils, eosinophils, and macrophages and the concentration of interleukin (IL)-4, IL-5, and IL-13, which were attenuated with the administration of aloin. The content of malondialdehyde was enhanced in OVA-treated mice, with the decreased levels of superoxide dismutase and glutathione, which were reversed with aloin treatment. Aloin treatment reduced the airway resistance of OVA-induced mice. The inflammatory cell infiltration around small airways was accompanied by the thickening and contraction of bronchial walls and pulmonary collagen deposition in OVA-treated mice; however, these conditions were ameliorated with aloin treatment. Mechanically, aloin upregulated the expression of nuclear factor erythroid 2-related factor 2 (Nrf2)–heme oxygenase 1 (HO-1) pathway but inhibited the level of transforming growth factor beta-SMAD2/3 genes (TGF- β /Smad2/3) axis in OVA-induced mice.

Conclusion: Aloin treatment lessened airway hyperresponsiveness, airway remodeling, inflammation, and oxidative stress in OVA-treated mice, and was closely related to the activation of Nrf2/HO-1 pathway and the weakening of TGF- β /Smad2/3 pathway.

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Introduction

Asthma is a common chronic respiratory disorder that affects millions of people around the world.¹ Around 30 million people are reported to suffer from asthma in China, with an increasing prevalence of 20-50% every 10 years.^{2,3} It is estimated that by 2025, the number of asthmatic patients would be increased by 100-150 million, with a mortality of 4%.³

Asthma is also considered a pediatric disease, and its prevalence is found to be higher in children than in adults. The prevalence of pediatric asthma has increased globally in the past 40 years.⁴ In China, the number of children with asthma is approximately 10 million, with a prevalence of 2.0-4.2% in different areas.^{5,6}

Asthma is characterized by airway hyperresponsiveness, inflammation, and remodeling,⁷ and its clinical presentation is heterogeneous, mainly evidenced by respiratory clinical manifestations, including dyspnoea, wheezing, coughing, and chest tightness.⁸ The pathogenesis of asthma is intricate, among which the centrality of inflammation is doubtless. The hyperactivation of T-helper 2 (Th2) cells causes the dysregulation of Th1/Th2 cells, which is identified as the immunological foundation of asthma.⁹ Based on the Global Initiative for Asthma (GINA), and the Global Strategy for Asthma Management and Prevention 2022 (<https://ginasthma.org/wp-content/uploads/2022/07/GINA-Main-Report-2022-FINAL-22-07-01-WMS.pdf>), inhaled corticosteroids (ICS) with a long-acting β_2 -agonist (LABA) are recommended as the reliever therapy. Besides, biological therapies, such as omalizumab, are available for patients with asthma, even for asthmatic children.¹⁰ However, the long-term use of glucocorticoids may result in glucocorticoid-related adverse events and difficulty in administering during the onset of asthma.^{11,12} Therefore, establishment of novel strategies for the treatment of asthma is significant and essential.

Aloe vera, a member of the Liliaceae family, has been used in medicines, food products, and cosmetics for ages; it has been also used as a therapy of a variety of diseases, including asthma.¹³ Shida et al. reported effects of aloe extract on bronchial asthma in adults.¹⁴ Aloin, a category of anthraquinone, is the most active ingredient of *Aloe vera*. Various pharmacological properties, such as anti-inflammatory, antitumor, antiviral, organ-protection, anti-microbiota, antiparasitic, antiosteoporosis, and laxative, have been recognized in aloin.¹⁵ In particular, aloin has been used as an alternative anti-inflammatory agent in various diseases. Lee et al. has demonstrated that aloin inhibits vascular inflammatory responses in lipopolysaccharide (LPS)-induced endothelial cells in mice.¹⁶ Sun et al. revealed that aloin prevents myocardial ischemia/reperfusion injury by attenuating inflammation.¹⁷ The anti-inflammatory effects of aloin are also demonstrated in non-alcoholic steatohepatitis,¹⁸ traumatic brain injury,¹⁹ cardiotoxicity,²⁰ and sepsis.²¹ However, the effect and potential mechanism of aloin in asthma remained unclear.

Therefore, this study first constructed an asthma model comprising mice by the treatment of ovalbumin (OVA). The effect of aloin on asthma was then investigated in OVA-induced mice. Moreover, the related mechanism was also addressed *in vivo*.

Material and Methods

Animal

Male C57BL/6 mice (6-8-week old, 20 ± 2 g), were acquired from Junke Biological Co. (Nanjing, China). The animals were kept under temperature-controlled laboratory conditions with a 12-h day-night cycle, and freely provided with rodent chow. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals²² and the Animal Research Ethics Committee of the Second Affiliated Hospital of Harbin Medical University.

Animal model and treatment

Mice were randomly divided into the following four groups ($n = 6$): control group, aloin alone group, OVA group, and OVA + aloin group. The asthma model of mice was established by the administration of OVA according to Lu et al. (2019).²³ In brief, 2-mg aluminum hydroxide ($Al(OH)_3$) was mixed in 200- μ L phosphate buffer saline (PBS, pH = 7.4; P1020; Solarbio, Beijing, China), which was then used to emulsify OVA (20 μ g). The emulsified OVA were intraperitoneally injected into mice for sensitization on days 0 and 14. On days 25-31, mice were challenged with 1% OVA (OVA and OVA + aloin groups) or PBS (control group) for 30 min. Subsequently, mice in the aloin alone and OVA + aloin groups were intragastrically treated with 20-mg/kg aloin whereas mice in the control and OVA groups were affected with the same amount of PBS. OVA (01641; 97-100% [HPLC]), aluminum hydroxide (239186), and aloin (B6906; purity $\geq 97\%$) were bought from Sigma (St. Louis, MO, USA). One day following the last administration, mice were intraperitoneally anesthetized with sodium pentobarbital (60 mg/kg) to harvest bronchoalveolar lavage fluid (BALF), and then administered 100-mg/kg sodium pentobarbital for sacrifice to yield lung tissues.

Cell counts

The collected BALF was centrifuged at 3000 rpm for 10 min at 4°C to harvest precipitates. Then, the precipitates were resuspended in PBS (100 μ L), and total cell numbers and numbers of inflammatory cells, containing lymphocytes, macrophages, eosinophils, and neutrophils, were counted for morphological determination with Wright-Giemsa staining solution (G1020; Solarbio). More than 200 cells were counted on each slide.

Enzyme-linked immunosorbent serologic assay (ELISA)

The contents of interleukin (IL)-4, IL-5, and IL-13 in BALF were measured by commercial ELISA kits, including Mouse IL-4 ELISA KIT (SEKM-0005; Solarbio), Mouse IL-5 ELISA kit (SEKM-0006; Solarbio) and Mouse IL-13 ELISA kit (SEKM-0014; Solarbio), based on the manufacturer's instructions. In addition, the concentration of transforming growth factor-beta 1 (TGF- β 1) in BALF was determined by TGF-beta-1

mouse ELISA kit (BMS608-4; Thermo Fisher Scientific, Waltham, MA, USA). In brief, all required reagents and standards at working concentrations were prepared according to the instruction manual; 100 μ L of standards or samples were added into the well of the plate and incubated at 37°C for 90 min. After four washings of the plate, 100 μ L of biotinylated antibody working solution was added into the well and incubated at 37°C for 60 min. Enzyme conjugate working solution, 100 μ L, was then added into the well and incubated at 37°C for 30 min after four washings of the plate. After five washings of the plate, 100 μ L of chromogenic substrate tetramethylbenzidine (TMB) was added into each well and protected from light for 15 min at 37°C. Subsequently, 50 μ L of stop solution was added into each well, and optical density was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

Quantification of oxidative stress indexes

The levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH) in lung tissues were examined by malondialdehyde content assay kit (BC0025, Solarbio), superoxide dismutase activity assay kit (BC0175, Solarbio), and reduced glutathione content assay kit (BC1175; Solarbio) according to the instruction for use.

Airway hyperresponsiveness assessment

The airway hyperresponsiveness was evaluated by the forced oscillation technique based on the study conducted by Jia et al. (2021).²⁴ Briefly, a catheter concatenated with an MS-impulse oscillometry (IOS) pulmonary function detector (JAEGER, Lüdenschied, Germany) was interposed into mice trachea and ventilated mechanically. The airway responsiveness was examined by enhancing doses of inhaled methacholine chloride. Data were collected every 1 min and the lung function values of airway resistance (cm H₂O/mL/s) were determined.

Pathological staining

Lung tissues were immersed into 4% paraformaldehyde (P1110; Solaibio) for fixation overnight and then were embedded in paraffin (YA0012; Solaibio). Embedded tissue samples were cut into 5- μ m-thick sections. Sections were dewaxed twice in xylene for 10 min each, and hydrated in ascending series of graded alcohol concentrations 100%, 95%, 85%, and 75% for 3 min each. Then, sections were stained with Hematoxylin and Eosin (H&E) staining kit (G1120; Solaibio) and Masson's Trichrome stain kit (G1340; Solaibio). Subsequently, sections were mounted with neutral resin (G8590; Solaibio), and images were acquired under a digital trinocular camera microscope (CX23; Olympus, Tokyo, Japan) based on the previous reports.^{25,26}

Western blot analysis

The relative protein levels of nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase 1 (HO-1), *Smad2*,

phosphorylated *Smad2* (p-*Smad2*), *Smad3*, and p-*Smad3* in lung tissues were quantified with Western blot analysis following the previous studies.^{27,28} Total protein was collected by radioimmunoprecipitation assay (RIPA) lysis buffer (R0010; Solarbio) and the protein concentration was measured with bicinchoninic acid (BCA) assay kit (PC0020; Solarbio). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 10%, was applied to isolate the proteins, which were immediately transferred electrically on a polyvinylidene fluoride (PVDF) membrane (IPVH00010; EMD Millipore, Billerica, MA, USA). Membranes were hatched with Western blocking buffer (SW3010; Solarbio) for 30 min at room temperature and then treated with primary antibodies at 4°C overnight. Subsequently, membranes were administrated with goat anti-rabbit immunoglobulin G (IgG) H&L (horseradish peroxidase [HRP]; 1:50,000; 31460; Thermo Fisher Scientific) for 60 min at 37°C after rinsed thrice. The bands were developed with 3,3'-diaminobenzidine (DAB) kit (DA1010; Solarbio). The protein expressions were normalized to histone H3 or β -actin.^{29,30} The primary antibodies included anti-Nrf2 (1:500, PA5-119633), anti-histone H3 (1:4000; PA5-16183), anti-HO-1 (1:1000; PA5-77833), anti-*Smad2* (1:1000; 51-1300), anti-p-*Smad2* (1:1000; 44-244G), anti-*Smad3* (1:1000; PA5-17378), anti-p-*Smad3* (1:1000; MA5-14936), and anti- β -actin (1:2000; PA1-183). ALL primary antibodies were obtained from Thermo Fisher Scientific.

Statistical analysis

Data were displayed as mean \pm standard deviation (SD). First, complete data were tested for normality, and the results showed that the data satisfied normal distribution. The statistical differences were determined by one-way analysis of variance (ANOVA), followed by *post hoc* Bonferroni test. All statistical analyses were conducted with the SPSS 26.0 software (IBM, Armonk, New York, USA). Significant difference was defined at $P < 0.05$.

Results

Aloin decreased the amount of inflammatory cells in BALF from asthmatic mice

In order to investigate the effect of aloin on inflammation in asthma, the number of inflammatory cells was first counted in BALF. No statistical difference was observed in the number of total cells (0.69 ± 0.05 vs 0.65 ± 0.06), neutrophils (0.03 ± 0.01 vs 0.03 ± 0.01), eosinophils (0.02 ± 0.01 vs 0.02 ± 0.01), macrophages (0.55 ± 0.06 vs 0.58 ± 0.07), and lymphocytes (0.02 ± 0.01 vs 0.03 ± 0.01) between the control and aloin alone groups ($P > 0.05$). As shown in Figure 1, the total number of cells was significantly elevated in BALF obtained from OVA-induced mice, compared to the same in BALF obtained from control mice (5.67 ± 0.55 vs 0.69 ± 0.05 , $P < 0.001$), which was markedly counteracted by the treatment of aloin (4.45 ± 0.63 vs 5.67 ± 0.55 , $P < 0.01$). In addition, administration of aloin significantly neutralized OVA-induced elevation in the number of neutrophils (0.94 ± 0.11 vs 1.41 ± 0.13 , $P < 0.001$), eosinophils (1.24 ± 0.15

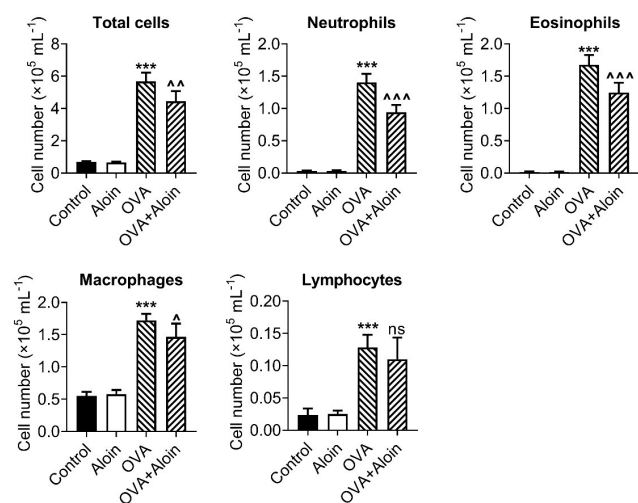


Figure 1 Aloin decreases the amount of inflammatory cells in BALF. The cells in BALF were stained with Wright-Giemsa solution, and the total cell number as well as the number of inflammatory cells (neutrophils, eosinophils, macrophages, and lymphocytes) was counted by morphological determination. *** $P < 0.001$ vs control; $\hat{P} < 0.05$, $\hat{\hat{P}} < 0.01$, and $\hat{\hat{\hat{P}}} < 0.001$ vs OVA.

vs 1.68 ± 0.15 , $P < 0.001$), and macrophages (1.47 ± 0.21 vs 1.72 ± 0.1 , $P < 0.05$) in BALF. Besides, the number of lymphocytes was markedly enhanced in BALF from OVA-induced mice, compared to that in BALF from control mice (0.13 ± 0.02 vs 0.02 ± 0.01 , $P < 0.001$), but no prominent decrease was observed after the introduction of aloin (0.11 ± 0.03 vs 0.13 ± 0.02 , $P > 0.05$). Altogether, aloin reduced the number of inflammatory cells in BALF.

Aloin inhibited inflammation and oxidative stress in asthmatic mice

Moreover, the contents of inflammatory mediators, including IL-4, IL-5, and IL-13, in BALF were quantified by ELISA. There was no statistical difference in the concentrations of IL-4 (27.56 ± 2.78 vs 26.68 ± 2.9), IL-5 (33.74 ± 6.12 vs 36.46 ± 5.84), and IL-13 (7.99 ± 0.61 vs 8.27 ± 0.93) between the control and aloin alone groups ($P > 0.05$). The concentrations of IL-4 (100.57 ± 6.02 vs 27.56 ± 2.78), IL-5 (81.06 ± 7.13 vs 33.74 ± 6.12), and IL-13 (158.04 ± 13.03 vs 7.99 ± 0.61) were markedly enhanced in OVA-treated mice, compared to control mice ($P < 0.001$), and were significantly attenuated by the supplementation of aloin (IL-4: 31.08 ± 4.91 vs 100.57 ± 6.02 ; IL-5: 40.19 ± 6.98 vs 81.06 ± 7.13 ; and, IL-13: 104.83 ± 14.79 vs 158.04 ± 13.03 ; $P < 0.001$; **Figure 2A**). In addition, to assess the effect of aloin on oxidative stress in OVA-induced mice, the levels of oxidative stress indicators, including malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione (GSH), were examined in BALF. No statistical difference in the concentration of MDA (0.63 ± 0.07 vs 0.7 ± 0.1), SOD (54.67 ± 3.44 vs 53.19 ± 4.11), and GSH (7.7 ± 0.34 vs 7.84 ± 0.64) was found between the control and aloin alone groups ($P > 0.05$). A significant elevation in the concentration of MDA (1.39 ± 0.18 vs 0.63 ± 0.07)

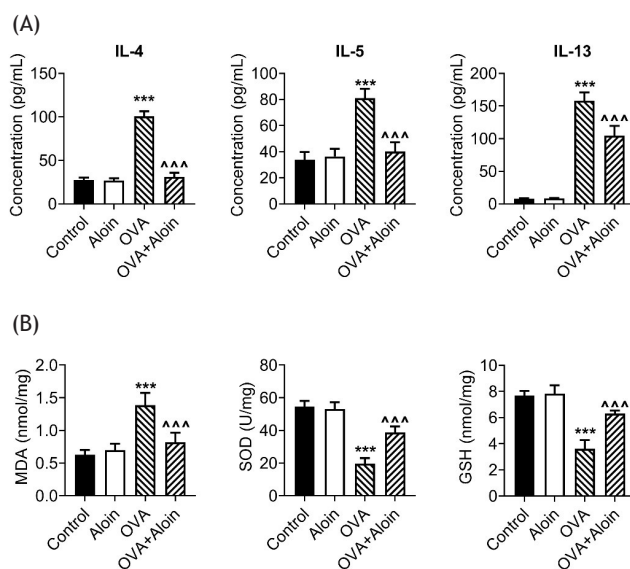


Figure 2 Aloin attenuated the release of inflammatory factors and oxidative stress in OVA-induced mice. (A) The concentrations of IL-4, IL-5, and IL-13 in BALF were examined by ELISA. (B) The contents of MDA, SOD, and GSH in BALF were determined by biochemical examination. *** $P < 0.001$ vs control; $\hat{\hat{\hat{P}}} < 0.001$ vs OVA.

and a remarkable reduction in the contents of SOD (19.77 ± 3.32 vs 54.67 ± 3.44) and GSH (3.62 ± 0.67 vs 7.7 ± 0.34) were observed in BALF from OVA-treated mice, compared to that in BALF from control mice ($P < 0.001$); the marked concentrations were markedly reversed with the administration of aloin (MDA: 0.82 ± 0.15 vs 1.39 ± 0.18 ; SOD: 38.73 ± 3.83 vs 19.77 ± 3.32 ; and GSH: 6.31 ± 0.24 vs 3.62 ± 0.67 ; $P < 0.001$; **Figure 2B**). Therefore, these results indicated that aloin suppressed the release of inflammatory factors and oxidative stress in OVA-induced mice.

Aloin improved airway hyperresponsiveness and airway remodeling in asthmatic mice

In order to address the effect of aloin on airway hyperresponsiveness, the airway resistance was monitored after mice were inhaled methacholine chloride. No obvious alterations in airway resistance were discovered with the increased inhalation of methacholine chloride in control mice (2.54 ± 0.46 vs 2.72 ± 0.3 vs 2.38 ± 0.4 vs 2.64 ± 0.32 , $P > 0.05$; **Figure 3A**). Similar results were observed in aloin alone-treated mice (2.76 ± 0.41 vs 2.48 ± 0.35 vs 2.51 ± 0.44 vs 2.53 ± 0.36 , $P > 0.05$; **Figure 3A**). Compared with control mice, the airway resistance in OVA-induced mice was significantly increased compared to control mice (4.88 ± 0.53 vs 2.54 ± 0.46 ; 6.58 ± 0.89 vs 2.72 ± 0.3 ; 9.81 ± 0.72 vs 2.38 ± 0.4 ; 15.11 ± 1.24 vs 2.64 ± 0.32 , $P < 0.001$; **Figure 3A**).

Administration of aloin markedly reduced airway resistance in OVA-treated mice exposed to the high concentration of methacholine chloride (12.5 and 25 mg/mL) (6.92 ± 0.51 vs 9.81 ± 0.72 and 8.31 ± 1.58 vs 15.11 ± 1.24 , $P < 0.001$; **Figure 3A**). In addition, the pathological examination showed that inflammatory cell infiltration existed

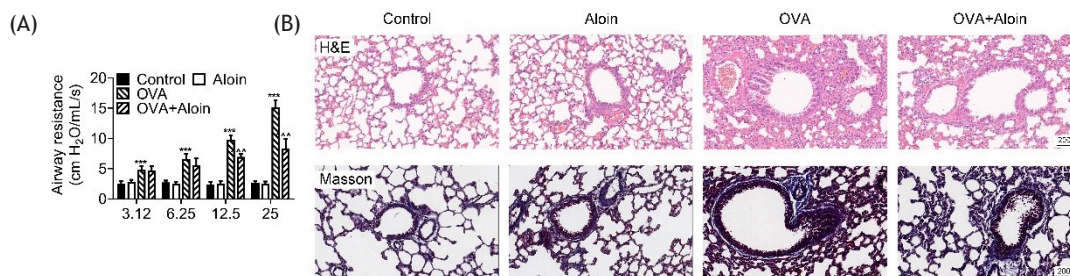


Figure 3 Aloin ameliorated airway resistance and lung histopathological manifestations in OVA-induced mice. (A) Airway resistance was monitored by forced oscillation technique in mice. $***P < 0.001$ vs control; $***P < 0.001$ vs OVA. (B) The lung tissues were conducted by H&E and Masson staining. Scale bar = 200 μ m.

around small airways accompanied by thickening and contraction of bronchial walls in OVA-induced mice, while no distinct pathological manifestations were found in control and aloin alone-treated mice (Figure 3B). Moreover, the pulmonary collagen deposition was observed by Masson staining in OVA-treated mice (Figure 3B). However, aloin treatment significantly improved OVA-induced pathological manifestations (Figure 3B). Taken together, aloin relieved airway resistance and lung histopathological manifestations in OVA-induced mice.

Aloin regulated Nrf2/HO-1 and TGF- β /Smad2/3 pathways in asthmatic mice

In order to further explore the mechanism of aloin in OVA-induced mice, the relative expressions of proteins involved in Nrf2/HO-1 and transforming growth factor-beta-SMAD2/3 genes (TGF- β /Smad2/3) pathways were determined by Western blot analysis. No statistical difference was discovered in the relative protein expressions of Nrf2 (0.16 ± 0.01 vs 0.17 ± 0.02) and HO-1 (0.05 ± 0.00 vs 0.05 ± 0.01), the

concentrations of TGF- β 1 (175.44 ± 20.01 vs 171.04 ± 5.95), and the relative protein levels of p-Smad2/Smad2 (0.08 ± 0.01 vs 0.08 ± 0.01) and p-Smad3/Smad3 (0.25 ± 0.01 vs 0.24 ± 0.02) between the control and aloin alone groups.

The relative protein expressions of Nrf2 (0.38 ± 0.05 vs 0.16 ± 0.01) and HO-1 (0.38 ± 0.02 vs 0.05 ± 0.00) were significantly upregulated in OVA-treated mice compared to control mice ($P < 0.001$); protein expressions were further increased with the administration of aloin (Nrf2: 0.9 ± 0.08 vs 0.38 ± 0.05 ; and HO-1: 0.89 ± 0.11 vs 0.38 ± 0.02 ; $P < 0.001$; Figures 4A and 4B). On the other hand, the elevated concentrations of TGF- β 1 in OVA-induced mice (444.62 ± 70.29 vs 175.44 ± 20.01 , $P < 0.001$) were markedly decreased with the introduction of aloin (206.93 ± 33.62 vs 444.62 ± 70.29 , $P < 0.001$; Figure 4C). Besides, treatment of aloin significantly offset the OVA-induced relative protein levels of p-Smad2/Smad2 (0.31 ± 0.03 vs 1.07 ± 0.06 , $P < 0.001$) and p-Smad3/Smad3 (1.1 ± 0.06 vs 1.27 ± 0.12 , $P < 0.001$; Figure 4D). Overall, aloin upregulated the expression of Nrf2/HO-1 pathway but suppressed the level of the TGF- β /Smad2/3 genes pathway in OVA-induced mice.

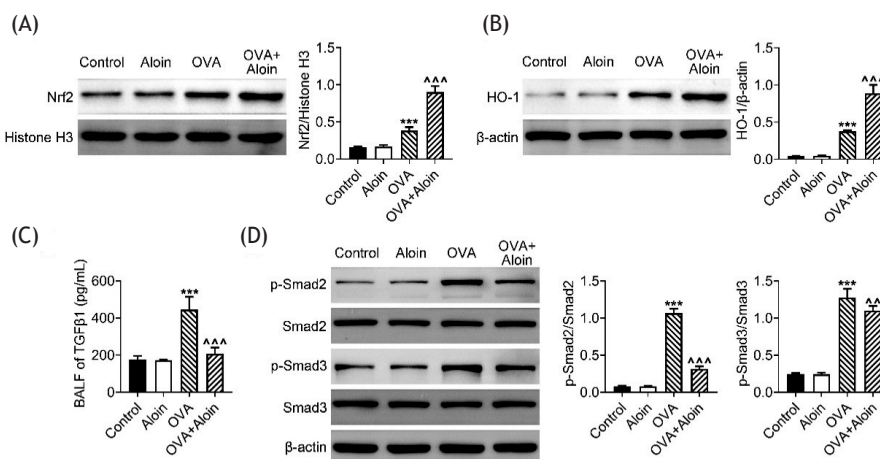


Figure 4 Aloin upregulated the expression of Nrf2/HO-1 pathway but suppressed the expression of TGF- β /Smad2/3 pathway in OVA-induced mice. (A) The relative protein expression of Nrf2 was examined by Western blot analysis. The protein expressions were normalized to histone H3. (B) The relative protein expression of HO-1 was determined by Western blot analysis. The protein expressions were normalized to β -actin. (C) The concentrations of TGF- β 1 in BALF were measured by ELISA. (D) The relative protein expressions of p-Smad2, Smad2, p-Smad3, and Smad3 were quantified by Western blot analysis. The protein expressions were normalized to β -actin. $***P < 0.001$ vs control; $***P < 0.001$ vs OVA.

Discussion

The anti-inflammatory effect of aloin was revealed in a variety of diseases, but whether aloin could improve asthma by suppressing inflammation remained unclear. This study elucidated that aloin treatment lessened airway hyperresponsiveness, remodeling and inflammation as well as oxidative stress in OVA-treated mice. Moreover, the effect of aloin on mice with OVA-induced asthma was closely related to the activation of Nrf2/HO-1 pathway and the weakening of TGF- β /Smad2/3 pathway.

Asthma is an inflammatory respiratory disorder characterized by airway remodeling and airway hyperresponsiveness.⁷ Several allergens, such as house dust mite (HDM) extract, OVA, toluene diisocyanate (TDI), and *Aspergillus fumigatus*, have been used to construct the experimental model of asthma in mice. The comparative results from the asthmatic mice induced by HDM, OVA, and TDI showed that both HDM asthma and OVA asthma mice showed typical eosinophilic asthma, while TDI asthma mice showed mixed cell asthma animal models.³¹ Basophils played an important role in asthmatic mice that responded to *Aspergillus fumigatus*.^{32,33} In the present study, an asthma mice model was constructed by the treatment of OVA according to Lu et al. (2019).²³ In the OVA-induced asthma, type 2 inflammation combined with pivotal effector cells, such as Th2 cells, group 2 innate lymphoid cells (ILC2s), dendritic cells, eosinophils, and airway epithelial cells, was strongly involved in the pathogenesis of asthma.^{34,35} In response to exogenous allergens, Th0 cells were activated to differentiate into CD4⁺ Th2 cells, which resulted in the secretion of IL-4, IL-5, and IL-13.³⁶ IL-4 played an important role in the activation, regulation, and classification of B lymphocyte function, which also promoted the modulation of immunoglobulin E (IgE) in inflammation.³⁷

IL-5 prominently influenced the formation of eosinophils and enhanced their activation, differentiation, growth, and migration.³⁸ IL-13 is a multi-effect cytokine that played the similar role as that of IL-4 under type II immune response.³⁹ Significant eosinophilia accompanied by upregulated levels of IL-4, IL-5 and IL-13 were discovered in patients with Th2-high asthma.⁴⁰ In addition, the contents of IL-4, IL-5, and IL-13 were increased in the sera of asthma patients.⁴¹

The present study showed that the number of total cells, that is, neutrophils, eosinophils, macrophages, and lymphocytes, was significantly increased in OVA-treated mice, similar to the results reported by Zhang et al.⁴² Moreover, OVA induced a prominent upregulation in the contents of IL-4, IL-5, and IL-13 in BALF, consistent with the previous reports.^{24,42} However, these effects were counteracted with the treatment of aloin. Thus, aloin inhibited inflammation in asthmatic mice.

In addition, our results showed that airway resistance was observably enhanced in OVA-induced mice, indicating an elevation in airway hyperresponsiveness. Similar results have been also reported in the previous studies.^{23,24,42} It was demonstrated that airway inflammation and remodeling resulted in airway hyperresponsiveness.⁴³ Airway remodeling could directly lead to severe airway hyperresponsiveness and pulmonary dysfunction that indicated alterations in airway structure, such as thickening of airway wall, narrowing of airway lumen, deposition of

collagen, and subepithelial fibrosis.⁴⁴ In the current study, OVA induced inflammatory cell infiltration around the small airways accompanied by the thickening and contraction of bronchial walls in mice lung tissues. Besides, the pulmonary collagen deposition was observed in OVA-treated mice, which was consistent with the previous study.⁴⁵ Both airway resistance and clinical manifestations of airway remodeling were attenuated by the administration of aloin. Therefore, these results expounded that aloin improved airway remodeling and hyperresponsiveness in OVA-induced asthmatic mice.

Oxidative stress, an important pathogenesis of various diseases, has been demonstrated to play a crucial role in asthma.⁴⁶ It promotes the pathological manifestations of asthmatic animal and population, such as airway inflammation, airway remodeling, excessive mucus secretion, pulmonary function reduction, and tissue injury.^{47,48} In the present study, a significant elevation in the concentration of MDA and a remarkable reduction in the contents of SOD and GSH were found in the BALF of OVA-treated mice. These results were consistent with the previous findings.^{42,45} MDA is a critical index of oxidative balance.⁴⁹ SOD and GSH are two crucial antioxidant enzymes that are reduced in allergic asthma patients.⁴⁷ However, these changes in the contents of MDA, SOD, and GSH were significantly reversed with the treatment of aloin. Several studies have elucidated that aloin inhibits oxidative stress in different disease models, such as intervertebral disc degeneration,⁵⁰ ischemia/reperfusion injury,^{17,51,52} cardiotoxicity,^{20,53} traumatic brain injury,⁵⁴ and alcoholic liver disease.⁵⁵ Collectively, these results suggested that aloin suppressed oxidative stress in asthmatic mice.

Mechanically, the levels of both Nrf2/HO-1 and TGF- β /Smad2/3 pathways were altered in OVA-induced mice, consistent with the results from Wang et al.⁴⁵ Nrf2 is a vital transcription factor that enhances the expression of cytoprotective genes, such as *HO-1*, thereby decreasing the injury caused by different stimuli, especially oxidative stress.⁵⁶ Activation of Nrf2/HO-1 pathway is reported to attenuate asthma.^{57,58} Moreover, Nrf2/HO-1 pathway is revealed to be related to oxidative stress, airway inflammation, and airway modeling during asthma.^{59,60} TGF- β /Smad2/3 pathway is an important and essential signaling pathway in the pathogenesis of asthma and acts as a key regulator of airway modeling in asthma because of its action on subepithelial fibrosis, epithelial changes, smooth muscle proliferation, and goblet cell hyperplasia.^{61,62} TGF- β /Smad2/3 pathway is markedly activated in bronchial biopsy specimens of patients with asthma and is closely associated with the thickness of basement membrane.⁶³

A recent clinical study has reported that the mRNA expression of TGF- β /Smad2/3 pathway was altered after subjects were challenged with specific and nonspecific allergens.⁶⁴ Here, aloin upregulated the expression of Nrf2/HO-1 pathway but repressed the level of the TGF- β /Smad2/3 axis in OVA-induced mice. Aloin was shown to activate Nrf2/HO-1 pathway in pathological cardiac hypertrophy,⁶⁵ and myocardial ischemia/reperfusion injury.¹⁷ Besides, the effect of aloin on Nrf2/HO-1 signaling pathway has been documented in the experimental models of other immunological diseases, such as acute colitis and non-alcoholic steatohepatitis.^{18,66} Altogether, aloin regulated the

levels of Nrf2/HO-1 and TGF- β /Smad2/3 pathways in asthmatic mice.

Conclusions

The results of the present study elaborated that aloein lessened asthma in mice, which was associated with the activation of Nrf2/HO-1 pathway and the weakening of TGF- β /Smad2/3 pathway. The findings identified the potential therapeutic effect of aloein in asthma, which provided a theoretical basis for its clinical application. However, the direct role of Nrf2/HO-1 and TGF- β /Smad2/3 pathways in asthma must be confirmed by effective blockade or activation in the future studies. Besides, owing to differences in the pathogenesis of different asthma murine models as discussed above, the effect of aloein on other allergens-induced asthma murine models should be confirmed in the future research. In addition, more preclinical trials need be performed to contribute to its clinical application.

Availability of Data and Materials

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

Competing interests

The authors declared that there were no conflict of interest to disclose.

Statement of Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information published in this article.

Author Contributions

All authors contributed to the study's conception and design. Material preparation and the experiments were performed by Siyu Wu. Data collection and analysis were performed by Yan Xia and Chengcheng Yang. The first draft of the manuscript was written by Mei Li, and all authors commented on the previous versions of the manuscript. All authors read and approved the final manuscript.

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