

Ameliorative effect of acetylshikonin on ovalbumin (OVA)-induced allergic rhinitis in mice through the inhibition of Th2 cytokine production and mast cell histamine release

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Acetylshikonin has long been known as an anti-inflammatory and antioxidative reagent. However, the anti-allergic effect has not been studied. The aim of this study was to evaluate the effect of acetylshikonin on allergic rhinitis (AR) in mice. Mice were sensitized by intraperitoneal injection of OVA and aluminum hydroxide and challenged with intranasal instillation of OVA. Acetylshikonin was administered orally after nasal cavities challenge. Severity of allergic rhinitis was assessed according to nasal symptoms; serum OVA-specific immunoglobulin E (IgE), IgG1, and IgG2a level; and interleukin (IL)-4, IL-10, IL-5, IL-13, TNF- α , IL-12, and interferon (INF)- γ levels in nasal lavage fluid (NALF). Additionally, the histological change and the release of histamine in serum and nasal lavage fluid were evaluated by acid-Schiff stain and ELISA. Acetylshikonin attenuated manifestation of nasal symptoms in sensitized mice and inhibited production of Th2-related OVA-specific IgE, IgG1, and Th2 cell-produced IL-4, IL-5, IL-13, and mast cell produced histamine; however, it had no effect on Th1 cell-produced cytokines, like INF- γ . In addition, the degree of inflammatory cell infiltration and goblet cell hyperplasia was attenuated by acetylshikonin treatment. Our results suggest that acetylshikonin effectively reduces allergic inflammation in a mouse model of allergic rhinitis by its anti-allergic and anti-inflammatory properties.

Key words: Acetylshikonin; allergic rhinitis; cytokine; IL-4; histamine.

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Common allergic reactions, such as hay fever, certain types of asthma, and hives, are linked to an antibody produced by the body called immunoglobulin E (IgE). Each IgE antibody can be very specific, reacting against certain pollens and other allergens. When a susceptible person is exposed to an allergen, the body starts producing a large quantity of similar IgE antibodies. The next exposure to the same allergen may result in an allergic reaction. Allergic rhinitis (AR) is one of the world's most common chronic illnesses among children and young adults, and its

prevalence is estimated to range from approximately 10%–50% (1). Furthermore, five hundred and million people worldwide suffer from AR and its prevalence is known to increase at alarming rate (2). Symptoms of AR include rhinorrhea, nasal obstruction, nasal itching, and sneezing, and it is often associated with ocular symptoms (3). Impairment of quality of life is observed in most AR patients, because these patients may suffer from sleep disorders and emotional problems and experience impairments in completing activities and proper social function. Despite many treatments' options for AR, there is a demand for novel therapeutic options which has the similar

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anti-inflammatory effect of glucocorticoids, but with fewer side effects.

As a type I allergic disease, AR was caused by an IgE-mediated adaptive immune response (4,5). The complex interactions between B cells, T cells, mast cells, and basophils and the presence of IL-4, IL-13, and IL-18 cytokines result in the production of IgE (6–8). The infiltration of eosinophils and Th2 cells, producing IL-4, IL-5, and IL-13, is the main feature of AR (9–13).

Zi Cao (purple gromwell) had been used for the treatment of throat sore, burns, cut, and skin diseases in China, including macular eruption, measles, and carbuncles (8,9). Acetylshikonin, the active principle component of tzu ts'ao (purple gromwell) (14–16), has been found to possess anti-inflammation (17,18) and antioxidation activity (19). Meanwhile, investigators also found that acetylshikonin could regulate the expression of Nur77 (20) and HO-1 (16) in macrophages or tumor cells.

Additionally, acetylshikonin also a kind of plant-derived natural compound with immunomodulatory effect. According to its anti-inflammatory and anti-allergy effects, it is possible that acetylshikonin could be a useful therapeutic agent against allergic asthma and rhinitis.

Therefore, it is hypothesized that acetylshikonin may be capable of immunomodulatory effects on the allergic inflammation of the airways. The aim of the present study was to evaluate whether acetylshikonin had effect on inflammation of the nasal mucosa, as well as on the immune response in an AR mouse model.

MATERIAL AND METHODS

Animals

BALB/c mice (6–8 weeks) were purchased from the Shanghai Research Center for Model Organisms (Shanghai, China). Mice were housed at a mean temperature of 22 ± 2 °C with a 12-h light/dark cycle with access to standard pellet chow and water. All experiments were carried out with the approval of the local animal use committee. Efforts were made to minimize animal suffering.

Materials

Acetylshikonin was purchased from ChemFaces Company (ChemFaces, Wuhan, China) in the form of powder. Acetylshikonin can be dissolved in chloroform, dichloromethane, ethyl acetate, DMSO, acetone, etc. The purity of this product is $\geq 98\%$, formula is $C_{18}H_{18}O_6$ (Fig. 1).

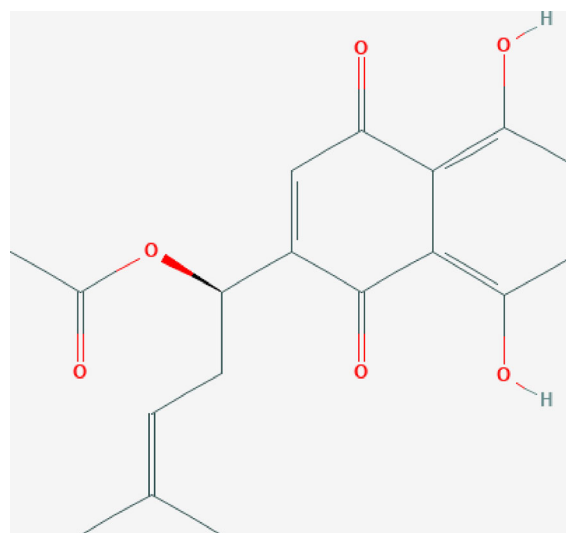


Fig. 1. The structure of acetylshikonin.

Experimental design

A total of 50 mice were randomly divided into five groups, and each group contained 10 mice: control group (normal control + saline), AR group (OVA-induced AR + saline), AR + 20 mg group (OVA-induced AR + 20 mg/kg acetylshikonin treatment), AR + 40 mg group (OVA-induced AR + 40 mg/kg acetylshikonin treatment), and AR + 80 mg group (OVA-induced AR + 80 mg/kg acetylshikonin treatment). Acetylshikonin was diluted with PBS to the final concentration before treatment.

OVA-induced AR

As shown in Fig. 2, mice were sensitized by administration of an intraperitoneal injection of OVA (75 µg) (Sigma, St. Louis MO, USA) in 200 µL of phosphate buffer solution (PBS) containing 2 mg of aluminum hydroxide (Sigma Aldrich, St. Louis MO, USA) in a total volume of 200 µL on days 0, 7, 14, and 21. On days 23, 25, and 27 after initial sensitization, mice were challenged with nasal instillation of OVA 500 µg in 20 µL of PBS into bilateral nasal cavities. The control group was challenged with PBS instead of OVA.

Acetylshikonin was given orally to mice after nasal cavities challenge on days 28–42. The control group was treated with PBS.

Measurement of nasal symptoms

At 20 min after the final OVA/PBS challenge on Day 27, the number of sneezing and nose rubbing motions was recorded and compared with that of the control group.

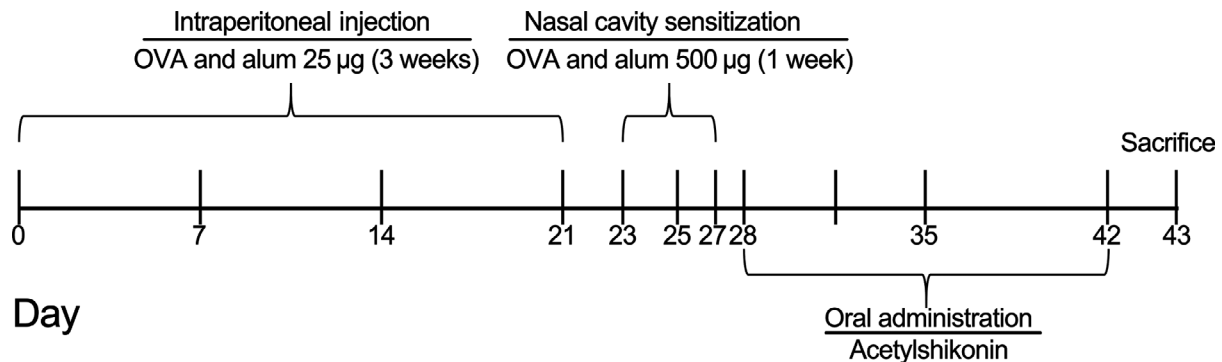


Fig. 2. Schematic diagram of the experimental group design and protocol for mouse ovalbumin-induced allergic rhinitis mouse model.

Histopathological evaluation of nasal cavity

Nasal tissues were removed 24 h after the last challenge with OVA/PBS, fixed in 10% neutral buffered formalin and decalcified. The coronal nasal section (5 µm) was stained with periodic acid-Schiff (PAS). And the goblet cell numbers were quantified at $\times 200$ magnification. All tissue sections were examined blindly with respect to the source of the tissue, and counts were determined at three different mucosal areas for each of the three sections per mouse.

The epithelial thickness was directly measured on a scale of $\times 400$ magnification, and average number of goblet cells was counted at four different areas per 1 mm² of nasal mucosa by eyepiece reticule.

Nasal lavage fluid (NALF) collection

Nasal lavage fluid was collected 24 h after the last treatment with different concentrations of acetylshikonin. Nasal lavage by an 18-gauge catheter was performed after partial tracheal resection. The catheter was inserted into the tracheal opening in the direction of the upper airway and into the nasopharynx. Nasal passages were gently perfused with 1 mL cold PBS and collected in a tube. NALF was centrifuged at 2000 rpm for 7 min at 4 °C, and the supernatant was stored at -70 °C.

Measurement of OVA-specific IgE, IgG1, and IgG2a in serum

Blood specimens were collected from the inferior vena cava 24 h after the last intranasal provocation. Serum was obtained by centrifugation and stored at -70 °C. OVA-specific IgE, IgG1, and IgG2a level in serum was measured using the ELISA kit (Pharmingen, San Diego, CA, USA).

Detection of cytokines in NALF

The NALF levels of IL-4, IL-5, IL-13, IL-10, IL-12, TNF-GATA-3, and IFN- γ were detected using specific

enzyme-linked immunosorbent assay kits (R&D System, USA), according the manufacturers' instructions.

Measurement of histamine in serum and NALF

The concentrations of histamine in serum and NALF from each mouse using the appropriate ELISA (MyBioSource, USA) were measured according to the manufacturer's instructions.

Statistical analysis

The results are presented as the mean $\pm$ standard error (SD) of the mean. A Mann-Whitney *U* test was used to compare results between different groups. Statistical analyses were performed by the SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA). *p*-values of < 0.05 were considered as indicating statistically significant associations.

RESULTS

Effect of acetylshikonin on body weight and food intake

During this study, the mice body weight and food intake were measured. As shown in Fig. 3A, no significant difference between control, AR, AR + 20 mg/kg, AR + 40 mg/kg, and AR + 80 mg/kg groups of mice was observed (Fig. 3A). These observations indicated that acetylshikonin did not show any toxicity on keeping mice body weight.

Acetylshikonin treatment caused a significant improve of allergic symptoms induced after allergen challenge

The main symptoms of AR are sneezing, runny nose, itching, rhinorrhea, and/or nasal congestion. After OVA challenge, the total number of sneezing and nasal rubbing motions during a period of

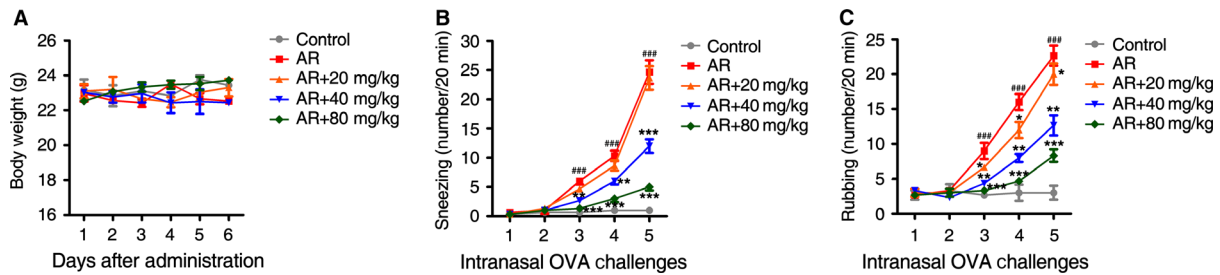


Fig. 3. Effect of acetylshikonin on the body weight and the nasal symptom score of ovalbumin (OVA)-induced allergic rhinitis mouse. Mice body weight (A), nose sneezing (B), and rubbing motions (C) of the normal ($n = 10$), AR ($n = 10$), and acetylshikonin-treated AR groups with different concentration (20, 40, 80 mg/kg, $n = 10$) were measured for a 20-min period. Results are expressed as absolute number of sneezing and nose rubbing motions. Normal group: control; OVA-induced AR group: AR; 20 mg/kg acetylshikonin-treated AR group: AR + 20 mg; 40 mg/kg acetylshikonin-treated AR group: AR + 40 mg; 80 mg/kg acetylshikonin-treated AR group: AR + 80 mg. The values presented are the mean $\pm$ SEM ($n = 10$ in each group). $\#p < 0.05$ compared with control group; $\#\#p < 0.01$, compared with control group; $\#\#\#p < 0.001$, compared with control group; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, significantly different from the value of OVA-induced AR group.

20 min was examined to evaluate the effect of acetylshikonin on the behavior of OVA-induced allergic rhinitis. As shown in Fig. 3B,C, the symptom scores of nasal sneezing (Fig. 3B) and rubbing (Fig. 3C) in the OVA-induced AR group were significantly increased when compared with those in the control group mice. Compared to the AR group mice, the scores were obviously lower in the groups treated with acetylshikonin in different dosages. These results indicated that acetylshikonin treatment could improve the symptoms of OVA-induced allergic rhinitis *in vivo*.

Effect of acetylshikonin on serum levels of OVA-specific IgE, IgG1, and IgG2a in OVA-induced AR mice

Allergic response is mainly caused by the production and effect of IgE, IgG1, and IgG2a antibodies. To further examine the underlying anti-allergic mechanism of acetylshikonin on the secretion of Th1- and Th2-related immunoglobulin, the Th2-related immunoglobulin, like OVA-specific IgE and IgG1, and the Th1-related immunoglobulin, like OVA-specific IgG2a, were measured using ELISA method. As shown in Fig. 4, compared to control group of mice, the OVA-specific IgE (Fig. 4A),

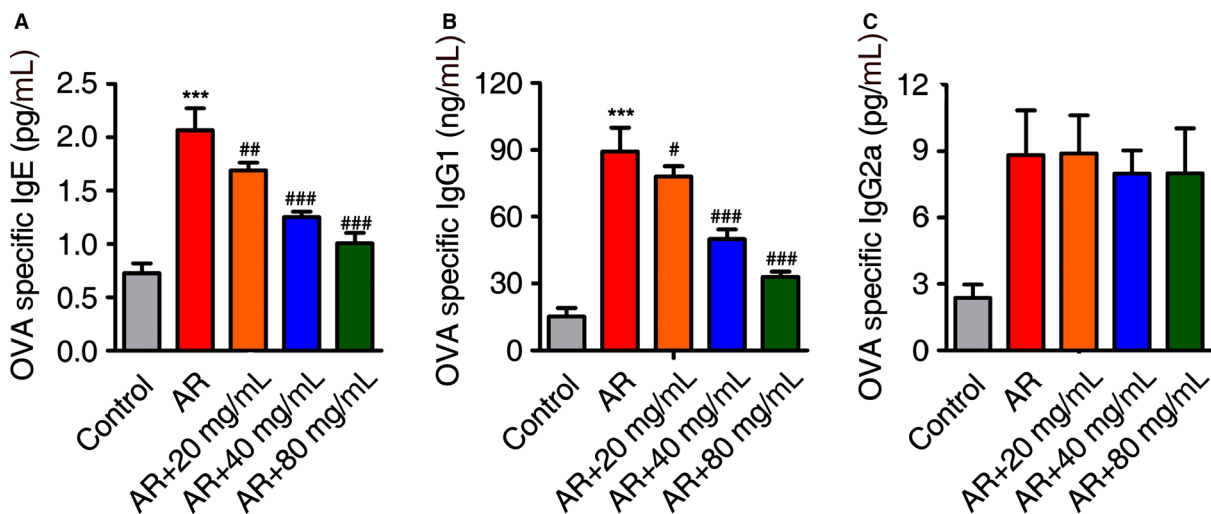


Fig. 4. Ovalbumin (OVA)-specific IgE, IgG1, and IgG2a levels in serum of each study group. The content of (A) Serum IgE, (B) IgG1, (C) IgG2a of different group of mice. Normal group: control; OVA-induced AR group: AR; 20 mg/kg acetylshikonin-treated AR group: AR + 20 mg; 40 mg/kg acetylshikonin-treated AR group: AR + 40 mg; 80 mg/kg acetylshikonin-treated AR group: AR + 80 mg. The values presented are the mean $\pm$ SEM ($n = 10$ in each group). $***p < 0.001$, compared with control group; $\#p < 0.05$, compared with OVA-induced AR group; $\#\#p < 0.01$, compared with OVA-induced AR group; $\#\#\#p < 0.001$, compared with OVA-induced AR group.

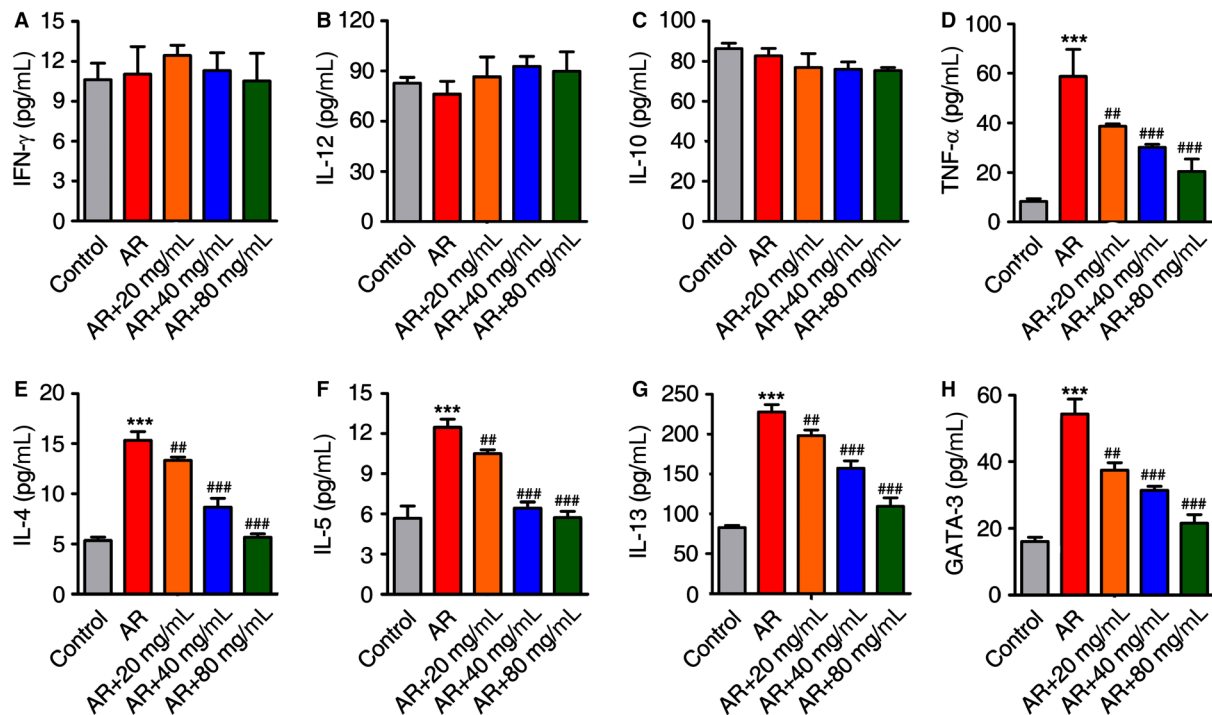


Fig. 5. Effect of acetylshikonin on the production of IFN- γ (A), IL-12 (B), IL-10 (C), TNF- α (D), IL-4 (E), IL-5 (F), IL-13 (G), and GATA-3 (H) in the NALF. The values presented are the mean $\pm$ SEM ($n = 10$ in each group). *** $p < 0.001$, compared with control group; # $p < 0.05$, compared with OVA-induced AR group; ## $p < 0.01$, compared with OVA-induced AR group; ### $p < 0.001$, compared with OVA-induced AR group.

IgG1 (Fig. 4B), and IgG2a (Fig. 4C) in serum were significantly increased in OVA-induced AR group of mice. However, this AR caused increasing of the Th2-related OVA-specific IgE and IgG1 was obviously decreased by acetylshikonin treatment with a dosage-dependent pattern (Fig. 4A,B). No changes of Th1-related OVA-specific IgG2a were observed between AR group and acetylshikonin-treated AR group of mice (Fig. 4C). These results suggested that acetylshikonin attenuates AR symptoms by suppressing the production of Th2-related OVA-specific IgE and IgG1, but no effect on Th1-related OVA-specific IgG2a.

Effect of acetylshikonin on the production of Th1- and Th2-related cytokines in NALF

To further evaluate the effect of acetylshikonin treatment on AR, the Th1- and Th2-related cytokines in NALF were detected using ELISA method. As shown in Fig. 5, we observed that compared to control group of mice, both of the Th1 and Th2-related and inflammation-related cytokines and molecules, including IFN- γ (Fig. 5A), IL-12 (Fig. 5B), IL-10 (Fig. 5C), IL-4 (Fig. 5E), IL-5 (Fig. 5F), IL-

13 (Fig. 5G), GATA-3 (Fig. 5H), and TNF- α (Fig. 5D) in NALF, were significantly increased in OVA-induced AR group of mice, while acetylshikonin treatment obviously restored this OVA-induced elevation of Th2-related and inflammation-related cytokines and molecules, like IL-4, IL-5, IL-13, GATA-3, and TNF- α , with a dosage dependent pattern compared to no treated AR group of mice. Meanwhile, no significant changes of these Th1- and Treg-related cytokines, like IFN- γ (Fig. 5A), IL-12 (Fig. 5B), and IL-10 (Fig. 5C), were observed between AR group and acetylshikonin-treated AR group of mice. Taken together, these results indicated that acetylshikonin might regulate Th2 immune response to attenuate OVA-induced AR in mice.

Effect of acetylshikonin on the level of histamine in the serum and NALF of OVA-induced AR mice

Accumulated studies indicated that mast cells secreted histamine play main roles in the pathogenesis of allergic reaction to AR (4,21). After antigen/IgE stimulation, activated mast cells release allergic mediators including histamine, pro-inflammatory

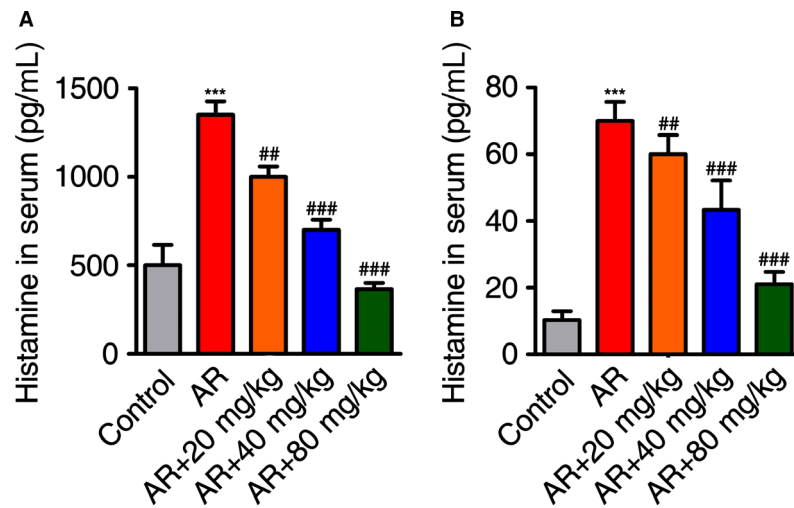


Fig. 6. Effect of acetylshikonin on the release of histamine in serum (A) and NALF (B). The values presented are the mean $\pm$ SEM (n = 10 in each group). ***p < 0.001, compared with control group; #p < 0.05, compared with OVA-induced AR group; ###p < 0.01, compared with OVA-induced AR group; ###p < 0.001, compared with OVA-induced AR group.

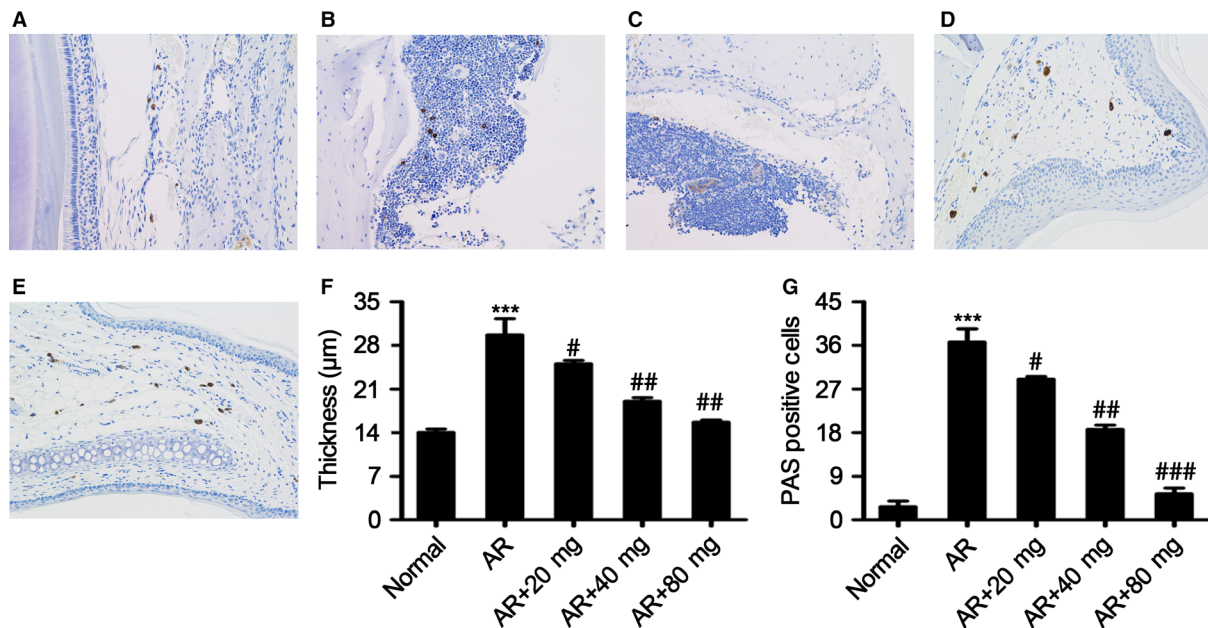


Fig. 7. Photographs of periodic acid-Schiff (PAS) stain of nasal mucosa. (A) Representative histological changes of nasal mucosa from control group mice; (B) Representative histological changes of nasal mucosa from OVA-induced AR group mice; (C) Representative histological changes of nasal mucosa from 20 mg/kg acetylshikonin-treated AR group mice; (D) Representative histological changes of nasal mucosa from 40 mg/kg acetylshikonin-treated AR group mice; (E) Representative histological changes of nasal mucosa from 80 mg/kg acetylshikonin treated AR group mice; (F) Epithelial thickness of differentially treated AR mice. (G) The number of periodic acid-Schiff (PAS)-positive cells of nasal mucosa tissue from differentially treated AR mice. The figure demonstrates a representative view ($\times 200$) from each group. Normal group: control; OVA-induced AR group: AR; 20 mg/kg acetylshikonin-treated AR group: AR + 20 mg; 40 mg/kg acetylshikonin-treated AR group: AR + 40 mg; 80 mg/kg acetylshikonin-treated AR group: AR + 80 mg. The values presented are the mean $\pm$ SEM (n = 10 in each group). *p < 0.05, compared with normal group; #p < 0.05, compared with OVA-induced AR group.

cytokines, and chemokines that are associated to induce the recruitment of inflammatory cells and eosinophils (5). To investigate the anti-allergic

mechanism of acetylshikonin on OVA-induced AR in mice, the levels of histamine in serum and NALF were measured in each group of mice. As shown in

Fig. 5, we observed that OVA induction markedly increased the secretion of histamine in OVA-induced AR mice compared with control group of mice (Fig. 6A,B). However, this OVA-induced histamine release could be significantly inhibited by acetylshikonin treatment with a dosage-dependent pattern compared with the no treated AR group of mice (Fig. 6). These data suggested that acetylshikonin might improve OVA-induced AR symptoms by blocking the release of histamine *in vivo*.

Effect of acetylshikonin on histological change of the nasal tissues

As shown in Fig. 7, the histological images of the nasal tissues of each group demonstrated that compared with the OVA-induced AR group mice, the eosinophil infiltration and the epithelial layer disruption in the acetylshikonin-treated AR group mice were significantly decreased (Fig. 7A–E). Additionally, PAS-positive goblet cells in nasal mucosa of OVA-induced AR mice also showed a significant increase compared with the normal control group mice. However, acetylshikonin treatment could obviously decrease the elevation of PAS-positive cells in nasal mucosa tissues caused by OVA induction (Fig. 7F). Furthermore, we also analyzed the thickness of epithelial cells in mucosa, and we observed that acetylshikonin treatment also inhibit the OVA caused increasing of epithelial cells thickness in AR mice (Fig. 7G).

DISCUSSION

Acetylshikonin, an bioactive compound isolated from tzu ts'ao (purple gromwell) (15,18), has been reported to have anti-inflammatory (17) and antioxidative activity (19). In this study, we clarified the effect of acetylshikonin on OVA-induced allergic rhinitis in mice. Our results showed that acetylshikonin treatment could inhibit the production and secretion of OVA specific IgE in serum and allergic cytokines like IL-4 and IL-10 in NALF caused by OVA induction *in vivo*. In addition, acetylshikonin also decreased the OVA induction caused PAS-positive goblet cells and the thickness of epithelial cells in nasal mucosa. Furthermore, acetylshikonin treatment also significantly improved the symptoms of rubbing and sneezing in AR mice, suggesting acetylshikonin has a potential application to attenuate OVA-induced allergic rhinitis.

Recent evidence has been accumulated to suggest that allergen-reactive type 2 helper T cells (Th2) play a triggering role in the activation and/or recruitment of IgE antibody-producing B cells,

mast cells, and eosinophils, the cellular triad involved in the allergic inflammation. Accumulated studies have documented that presentation of allergen to the lymphocytes will lead to the release of type II cytokine, like IL-4, which promote IgE production and infiltration of inflammatory cell into nasal mucosa (21,22). Our data found that the secretion of IL-4 in nasal lavage fluid is inhibited by acetylshikonin treatment. Additionally, as an anti-inflammatory cytokine produced by monocytes, IL-10 could suppress the production of type I cytokines, like IFN- γ , IL-3, and TNF- α (23–25). Th1 cells are defined by their ability to secrete the inflammatory cytokines INF- γ and are involved in cellular immunity, some autoimmune diseases, and in chronic inflammatory disorders (21,26–29). Th2-biased cells preferentially produce IL-4, IL-5, and IL-13 and participate in humoral response and some allergic inflammatory, like asthma and allergic rhinitis (30,31). Previous results demonstrated that type I cytokines, like IFN- γ , can significantly inhibit the production of type II cytokines, like IL-4 (32,33). The detailed mechanism of regulation of acetylshikonin on allergic cytokines expression was not investigated in this study, but we speculated that acetylshikonin may affect the cell activation through NF- κ B. However, our data indicated that acetylshikonin could inhibit nasal allergic inflammation by inhibition of cytokine secretion.

Previous studies have reported that allergic rhinitis exhibits sneezing, itching, and rubbing. Additionally, increased thickness of lamina prairie and epithelium was observed in allergic rhinitis mouse model. In our present study, we observed acetylshikonin treatment decreased the OVA caused thickness of epithelium in AR mouse model, and inhibit the infiltration of inflammatory cells and PAS-positive cells and result in improvement of nasal symptoms, like rubbing and sneezing. These observations suggest acetylshikonin might be a potential drug candidate for allergic rhinitis therapy.

CONCLUSIONS

Our results illustrate the potential therapeutic value of acetylshikonin in treating OVA-induced allergic rhinitis by its anti-allergic and anti-inflammatory properties. Although further study for clinical use was needed, we can suggest that acetylshikonin may be a useful therapeutic strategy for allergic diseases.

CONFLICT OF INTEREST

No conflict of interests were declared.

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