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Zeaxanthin attenuates OVA-induced allergic asthma in mice by regulating the p38 MAPK/β-catenin signaling pathway

Xiaosheng Jin[a], Weijing Jin[b]*, Guoping Li[a], Jisheng Zheng[a]

[a]*Department of Respiration, Tongde Hospital of Zhejiang Province, Hangzhou, China*
[b]*Department of Neonatology, Hangzhou Children's Hospital, Hangzhou, China*

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Abstract
Background: Asthma is a heterogeneous and complex chronic airway disease with a high incidence rate, characterized by chronic airway inflammation. Although the anti-inflammatory effect of zeaxanthin has been demonstrated in various disease models, its explicit role in allergic asthma remains elusive.
Methods: An allergic asthma model was established by ovalbumin (OVA) stimulation in BALB/c nude mice. The pathological examination, collagen deposition and expression of α-smooth muscle actin (α-SMA) in lung tissues were determined by hematoxylin and eosin (H&E), MASSON and immunofluorescence staining, respectively. Besides, the effect of zeaxanthin on inflammation and oxidative stress was assessed by the enzyme-linked immunosorbent assay (ELISA) and spectrophotometry measure. Moreover, the underlying mechanism was analyzed by detecting the expression of phosphorylated p38 (p-p38), p38, β-catenin, p-c-Jun N-terminal kinase (p-JNK) and JNK with western blot assays.*Results*: The distinct infiltration of inflammatory cells was observed in the OVA-induced asthma mice model with significantly increased concentrations of immunoglobulin E (IgE), interleukin-4 (IL-4), IL-5, IL-13 and eotaxin ($p<0.001$), which were prominently reversed by zeaxanthin treatment ($p<0.001$). In addition, zeaxanthin treatment decreased the OVA-induced collagen deposition and α-SMA expression. A similar inhibitory effect of zeaxanthin on the oxidative stress was also observed in the OVA-induced asthma mice model, as evidenced by the prominent decrease of malondialdehyde (MDA) concentration and the remarkable increase of superoxide dismutase (SOD), glutathione S transferase (GST) and Glutathione (GSH) concentrations ($p<0.001$). Moreover, zeaxanthin introduction markedly reduced the relative expressions of p-p38/p38, β-catenin and p-JNK/JNK in the OVA-induced asthma mice model ($p<0.001$), indicating that zeaxanthin suppressed the p38 mitogen-activated protein kinase (p38 MAPK)/β-catenin signaling pathway in the OVA-induced asthma mice model.
Conclusions: Zeaxanthin attenuated OVA-induced allergic asthma in mice via modulating the p38 MAPK/β-catenin signaling pathway.

***Corresponding author:** Weijing Jin, Department of Neonatology, Hangzhou Children's Hospital, Hangzhou, No. 195 Wenhui Road, Gongshu District, Hangzhou, Zhejiang, China. *Email address:* jinweijing0506@163.com

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Introduction

Asthma is one of the most prevalent chronic airway syndromes that has affected approximately 12.6% of individuals with 358.2 million sufferers worldwide.[1,2] Therein, a 4.2% asthma morbidity has been reported in adults based on the China Pulmonary Health (CPH) study.[3] As the most common subtype of asthma, allergic asthma accounts for about sixty percent of all asthma, characterized by inflammation, reversible airway obstruction, hyperresponsiveness and airway remodeling.[4,5] Inflammation is an outstanding hallmark of asthma, in which type 2 cytokines facilitate its characterizations, including eosinophilia, IgE generation, bronchial hyperresponsiveness (BHR), mucus hypersecretion and sensibility of exacerbations.[6] In addition, innate cells, such as basophils[7] and mast cells[8] are implicated in the immune-pathogenesis of eosinophilic asthma, which has been validated *in vivo*. Thus, drugs targeting chronic airway inflammatory pathways can contribute to the development of asthma therapies.[9]

Zeaxanthin (ZE) is a type of xanthophyll found abundantly in kale, spinach, yellow corn, eggs and orange and red peppers, which plays an important role in human development, metabolism and health.[10] Pharmacologically, zeaxanthin has been demonstrated to own anti-tumor, anti-antioxidant, and anti-inflammation activities. For instance, El-Akabawy et al.[11] showed that zeaxanthin improved colitis induced by acetic acid through the regulation of pro-inflammatory mediator and cytokine activity as well as the antioxidative effects in rats. Yu et al.[12] reported that lutein combined with zeaxanthin isomers diminished endoplasmic reticulum stress and oxidative stress to prevent retinopathy. Zeaxanthin has been demonstrated to protect the proteasome from inactivation and reduce the expression of inflammation-related genes in retinal pigment epithelial cells, thereby reducing the incidence of age-related macular degeneration.[13] Zeaxanthin can also improve diabetes-related anxiety and depression by suppressing inflammation in diabetic rats.[14] Moreover, it has been verified that there was a positive correlation between zeaxanthin and lung function and the respiratory system.[15,16] Therefore, we speculated that zeaxanthin might be a candidate for asthma treatment.

Hence, this study aimed to explore the effect of zeaxanthin on ovalbumin-induced asthma in BALB/c mice and the underlying molecular mechanism. We hope the results can establish a theoretical foundation for the development of asthma therapy.

Materials and methods

Animals

All the procedures were performed sternly following the Guide for the Care and Use of Laboratory Animals.[17] Moreover, all the experiments were conducted with the approval of ethical standards as determined by the ethical committee of Tongde Hospital of Zhejiang Province. Six weeks-old BALB/c nude mice (15±2 g) were bought from the Animal Experimental Research Center of Zhejiang Chinese Medicine University (Zhejiang, China). Mice were fed in an SPF animal room with the 12/12-h cycle of light/dark at 23-25°C. Twenty mice were randomly divided into four groups (n=5), including the Control group, OVA group, ZE (50 mg/kg) group and OVA+ZE (50 mg/kg) group. The allergic asthma model was induced by ovalbumin (OVA) according to the previous reports.[18-20] Briefly, 10 μg OVA (01641, 97%-100% (HPLC), Sigma, St. Louis, MO, USA), as an allergen, and 2 mg aluminum hydroxide (239186, CAS: 21645-51-2, reagent grade, Sigma), as an adjuvant in 200 μl phosphate buffer saline (PBS, Solarbio, Beijing, China) were intraperitoneally injected into BALB/c nude mice for sensitization on day-0, day-7, and day-14. Subsequently, mice were intranasally exposed to 3.75 μg OVA in 50 μL PBS on day-22, day-24, and day-26. Mice in OVA group were challenged as above-description, while mice in Control group experienced the same procedure by treatment with PBS alone. Mice in ZE (50 mg/kg) group were intragastrically administrated with 50 mg/kg ZE (14681, CAS: 144-68-3, ≥95.0% (HPLC), Sigma), while mice in OVA+ZE (50 mg/kg) group were intragastrically administrated with 50 mg/kg ZE 24 h before the inhaled OVA induction as mentioned above. Mice were sacrificed with an intraperitoneal injection of sodium pentobarbital (100 mg/kg) 24 h post the last OVA exposure. The blood was collected for the separation of serum through centrifugation. Lung tissues were also obtained for the following detection and the bronchoalveolar lavage fluid (BALF) was gathered by using 1 ml 1×HBSS (H1025, Solarbio) to lavage lung tissues.

Hematoxylin and eosin (H&E) stain

Lung tissues were dissected and fixed into 4% paraformaldehyde overnight. Then, tissues were dehydrated with ethyl alcohol, embedded with paraffin, and successively cut into slices (5 μm). After being dewaxed and hydrated, slices were stained with hematoxylin and eosin for 5 min and 3 min, respectively. Next, slices were mounted with neutral resin and captured by a digital trinocular camera microscope (CX23, Olympus, Tokyo, Japan).

Masson stain

Lung tissues were separated, fixed, dehydrated, embedded and cut into slices (5 μm) in sequence. After being dewaxed and hydrated, slices were stained with Wiegert's solution for 10 min and differentiated with acidic ethanol for 10 s. Subsequently, slices were treated with Masson bluing buffer for 5 min, ponceau-fuchsin solution for 10 min, phosphomolybdic acid solution for 3 min, aniline blue solution for 5 min, and weak acid solution for 30 s in turn. Finally, slices were dehydrated with 95% ethanol and absolute ethanol, transparentized with dimethylbenzene, mounted with neutral resin, and imaged under a digital trinocular camera microscope (CX23, Olympus).

Immunofluorescence (IF) detection for α-smooth muscle actin (α-SMA)

Mice were transcardially perfused with ice-cold 0.1 M PBS (Solarbio) followed by ice-cold 4% buffered paraformaldehyde (Solarbio). Lung tissues were then fleetly removed, fixed in 4% buffered paraformaldehyde overnight, embedded into OCT (SAKURA, CA, USA) and consecutively cut into 5-μm coronal sections with Leica CM 1950 Frozen slicer (Leica microsystems, Wetzlar, Germany). Subsequently, sections were sequentially immersed in blocking buffer (PBS including 3% bovine serum albumin (BSA, Solarbio) and 0.2% Triton X-100 (Solarbio)), and the antibody against α-SMA (1:500, ab124964, Abcam, Cambridge, UK) at 4°C overnight. After being rinsed with 0.1 M PBS three times, sections were incubated with Goat anti-mouse IgG-Alexa Fluor 647 (1:1000, ab150115, Abcam) at room temperature for 1 h. Sections were conclusively mounted with Mounting Medium, antifading (with DAPI) (S2110, Solarbio), and observed under fluorescence microscopy (Olympus, IX71, Olympus Co., Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Blood was allowed to form a clot for 30 min at room temperature and then centrifuged for 10 min at 4000 rpm to separate the serum. The serum concentration of immunoglobulin E (IgE) was measured with a mouse IgG ELISA kit (PI480, Beyotime, Shanghai, China)) following the operating manual. In addition, the concentrations of interleukin-4 (IL-4), IL-5, IL-13 and eotaxin in BALF were examined by mouse IL-4 ELISA kit (PI612, Beyotime), mouse IL-5 ELISA kit (PI620, Beyotime), mouse IL-13 ELISA kit (ab219634, Abcam) and mouse eotaxin ELISA kit (ab201277, Abcam) according to instructions.

Determination of oxidative stress biomarkers level

The concentrations of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione S transferase (GST) and Glutathione (GSH) in lung tissues were detected by the commercial malondialdehyde (MDA) assay kit (A003-1-1), total superoxide dismutase (T-SOD) assay kit (A001-1-1), Glutathione S transferase (GSH-ST) assay kit (A004-1-1) and Glutathione (GSH) assay kit (A006-1-1, all from Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on the manufacturer's specifications. The absorbance was read at 532 nm (MDA), 560 nm (SOD), and 412 nm (GSH) by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) severally.

Western blot

The western blot assay was conducted as previously described.[21] In brief, total proteins from lung tissues were extracted by RIPA buffer (Solarbio) and quantified with the BCA protein quantification kit (Abcam, Cambridge, UK) following the operating instruction. Twenty micrograms of protein samples were separated and electrically transferred onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). After being blocked with 3% bovine serum albumin (BSA, Solarbio) at room temperature for 1 h, membranes were hatched with primary antibodies against diverse proteins, containing p38 (1:1000, ab68515), phosphorylated p38 (p-p38, 1:2000, ab196495), ß-catenin (1:1000, ab16051), c-Jun N-terminal kinase (JNK, 1:1000, ab32072), p-JNK (1:1000, ab32072) and GAPDH (1:2500, ab9485; all from Abcam) at 4°C overnight. Membranes were then treated with corresponding secondary antibodies at room temperature for 3 h and visualized by an ECL assay (Beyotime). The gray value was calculated by QUANTITY ONE software.

Statistical analysis

All the results were expressed as mean ± standard deviation (SD). Data differences were assessed by the one-way analysis of variance (ANOVA) among multiple groups followed by the *post hoc* Bonferroni test by SPSS 26.0 software (IBM, Armonk, New York, USA). $p<0.05$ was identified as a significant difference.

Results

ZE reduced the infiltration of inflammatory cells in OVA-induced asthma mice model

To explore the effect of ZE on asthma, an allergic asthma model was induced by OVA in BALB/c nude mice. HE results presented in Figure 1 showed the complete structure of bronchial wall tissue, neat-arranged epithelial cells, airway wall with moderate thickness, and no non-treated cup-shaped cells in Control mice. However, the bronchial wall was thickened and damaged, and the airway lumen was narrowed. In addition, the infiltration of eosinophils increased in the OVA-induced asthma mice model, which was decreased by ZE treatment. Therefore, ZE diminished the inflammatory cells infiltration in the OVA-induced asthma mice model.

ZE inhibited collagen deposition and α-SMA expression in OVA-induced asthma mice model

Then, the effect of ZE on the collagen deposition and α-SMA expression in the OVA-induced asthma mice model was also evaluated by Masson staining and IF examination. As shown in Figure 2A, the collagen deposition was prominently increased in the OVA-induced asthma mice model compared with that in control mice, which was notably restored by ZE administration. Consistently, ZE feed also distinctly decreased the OVA-induced expression of α-SMA (Figure 2B). No evident difference was observed in both collagen deposition and α-SMA expression between the Control group and ZE (50 mg/kg) group (Figures 2A and B). Thus, these results suggested that ZE suppressed collagen deposition and α-SMA expression in the OVA-induced asthma mice model.

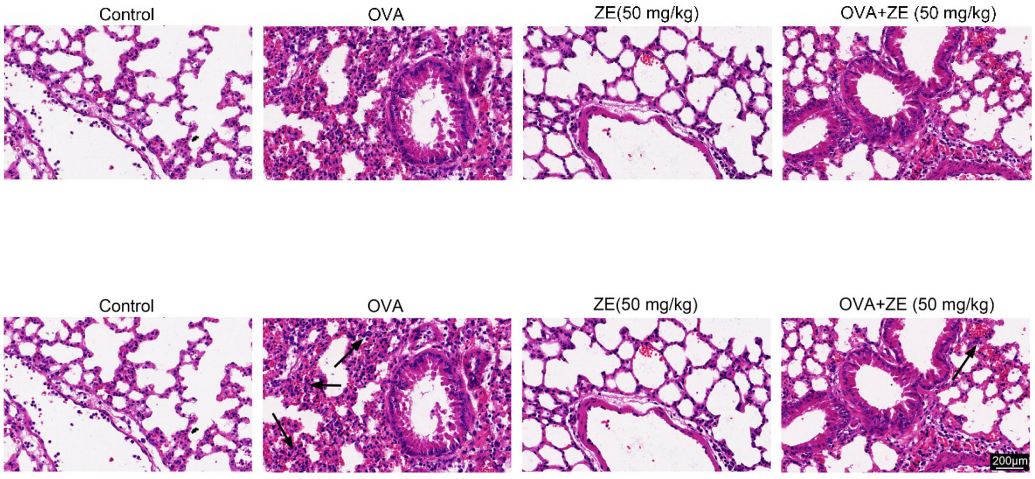

Figure 1 ZE decreased the inflammatory cells infiltration in the OVA-induced asthma mice model. An allergic asthma model was induced by OVA in BALB/c nude mice. Mice were orally received with 50 mg/kg ZE. The pathology of lung tissues was analyzed by HE staining. ×200.

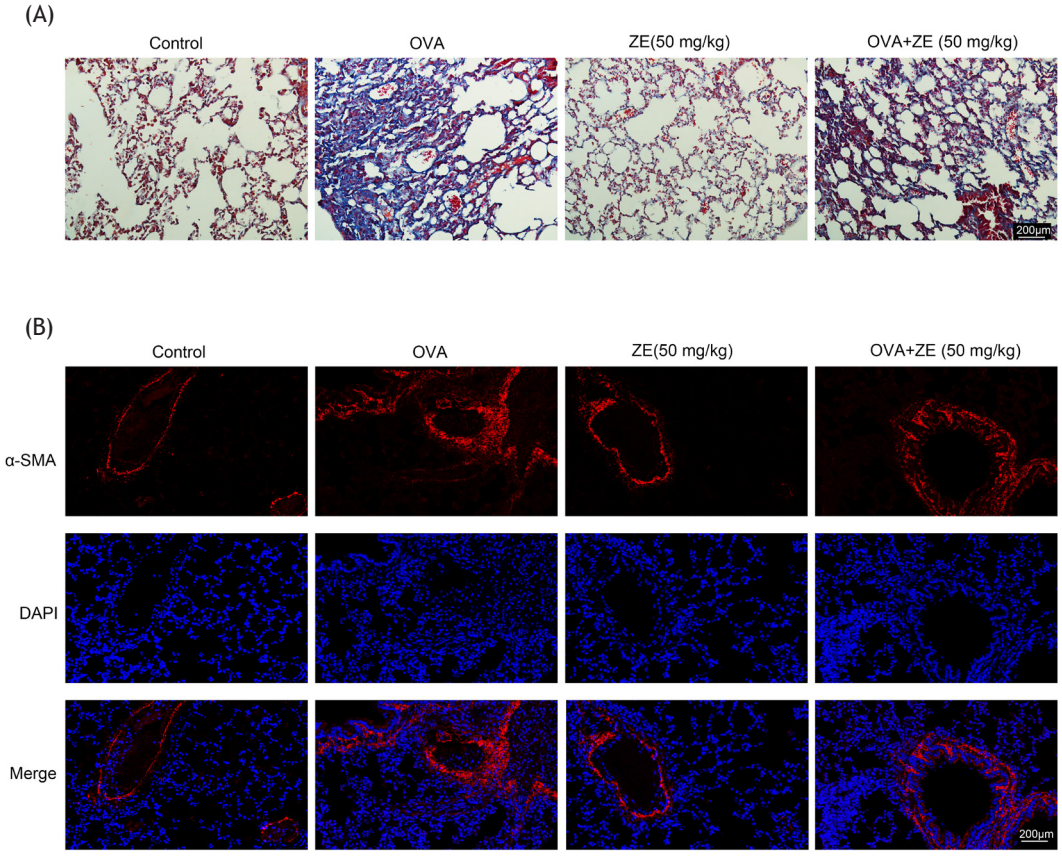

Figure 2 ZE restrained collagen deposition and α-SMA expression in OVA-induced asthma mice model. An allergic asthma model was induced by OVA in BALB/c nude mice. Mice were orally received with 50 mg/kg ZE. (A) The collagen deposition was assessed by Masson staining. (B) The expression of α-SMA was detected by IF assays.

ZE dampened the production of IgE and pro-inflammatory factor in the OVA-induced asthma mice model

Considering the increased infiltration of eosinophils in the OVA-induced asthma mice model and the important role of inflammation in asthma, the levels of IgE and several conventional pro-inflammatory factors, including IL-4, IL-5, IL-13 and eotaxin were measured with ELISA. The results revealed that a major increase in the concentrations of IgE, IL-4, IL-5, IL-13 and eotaxin was observed in the OVA-induced asthma mice model ($p<0.001$), which was significantly counteracted by ZE treatment ($p<0.001$) (Figure 3). In addition, the concentrations of IgE, IL-4, IL-5, IL-13 and eotaxin were also observably enhanced in OVA+ZE (50 mg/kg) group relative to ZE (50 mg/kg) group ($p<0.05$) (Figure 3).

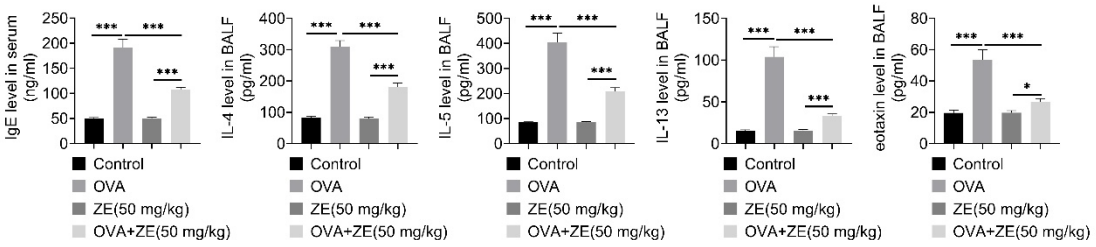

Figure 3 ZE inhibited the generation of IgE and pro-inflammatory cytokines in the OVA-induced asthma mice model. An allergic asthma model was induced by OVA in BALB/c nude mice. Mice were orally received with 50 mg/kg ZE. The serum concentration of IgE, as well as the concentrations of IL-4, IL-5, IL-13 and eotaxin in BALF was detected by ELISA. Data differences were determined by the one-way analysis of variance (ANOVA) followed by the *post hoc* Bonferroni test by SPSS 26.0 software. *p<0.05 and ***p<0.001.

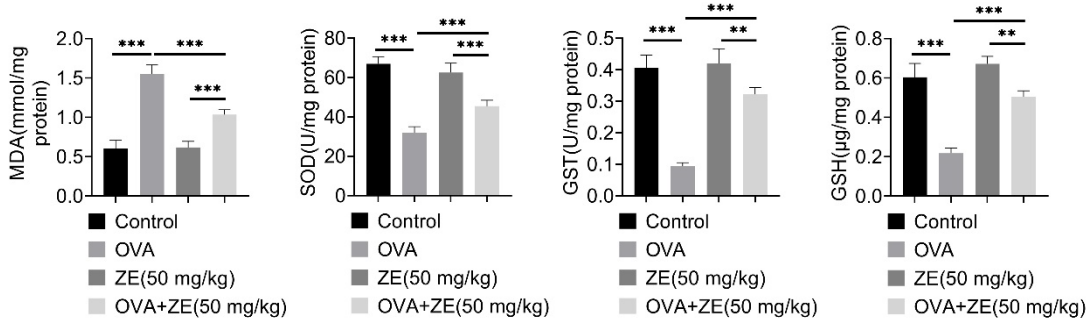

Figure 4 ZE refrained the oxidative stress in the OVA-induced asthma mice model. An allergic asthma model was induced by OVA in BALB/c nude mice. Mice were orally received with 50 mg/kg ZE. The concentrations of MDA, SOD, GST and GSH in lung tissues were measured using commercial kits. Data differences were determined by the one-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni test by SPSS 26.0 software. **p<0.01 and ***p<0.001.

Hence, these data indicated that ZE inhibited the generation of IgE and pro-inflammatory factors in the OVA-induced asthma mice model.

ZE antagonized the oxidative stress in the OVA-induced asthma mice model

In addition, the effect of ZE on oxidative stress was also investigated by examining MDA, SOD, GST and GSH levels in lung tissues. The concentration of MDA was prominently increased in the OVA-induced asthma mice model (p<0.001), which was neutralized by ZE treatment (p<0.001) (Figure 4). On the contrary, the ZE treatment markedly enhanced the concentration of SOD, GST and GSH (p<0.001) (Figure 4). A remarkable upregulation in MDA concentration and a distinct downregulation in SOD, GST and GSH concentrations were observed in OVA+ZE (50 mg/kg) group compared to ZE (50 mg/kg) group (p<0.01) (Figure 4). Thus, these outcomes manifested that ZE inhibited oxidative stress in the OVA-induced asthma mice model.

ZE regulated the p38 MAPK/β-catenin signaling pathway in the OVA-induced asthma mice model

Moreover, the molecular mechanism of ZE in the OVA-induced asthma mice model was also clarified by detecting the relative protein expressions of p38, p-p38, β-catenin, JNK and p-JNK in lung tissues. The relative expressions of p-p38/p38, β-catenin and p-JNK/JNK were significantly upregulated in the OVA-induced asthma mice model, notably reversed by ZE administration (p<0.001) (Figure 5). There was also a significant increase in the relative expressions of p-p38/p38, β-catenin and p-JNK/JNK observed in the OVA+ZE (50 mg/kg) group compared with those in the ZE (50 mg/kg) group (p<0.05) (Figure 5). Therefore, these results indicated that ZE inhibited the activation of the p38 MAPK/β-catenin signaling pathway in the OVA-induced asthma mice model.

Discussion

Asthma is a heterogeneous and complex chronic airway disease with a high incidence rate, characterized by chronic airway inflammation.[6] Although the anti-inflammatory effect of zeaxanthin has been verified on colitis,[11] age-related macular degeneration[13] and diabetes-related anxiety and depression.[14] its explicit role in allergic asthma remains elusive. In this study, an allergic asthma model was induced by OVA in BALB/c nude mice. The distinct infiltration of inflammatory cells was observed in the OVA-induced asthma mice model with significantly increased concentrations of IgE, IL-4, IL-5, IL-13 and eotaxin, which were prominently reversed by zeaxanthin treatment. In addition, zeaxanthin treatment decreased the OVA-induced collagen deposition and α-SMA expression. A similar inhibitory effect of zeaxanthin on oxidative stress was also observed in the OVA-induced asthma mice model, as evidenced by decreased MDA concentration and increased

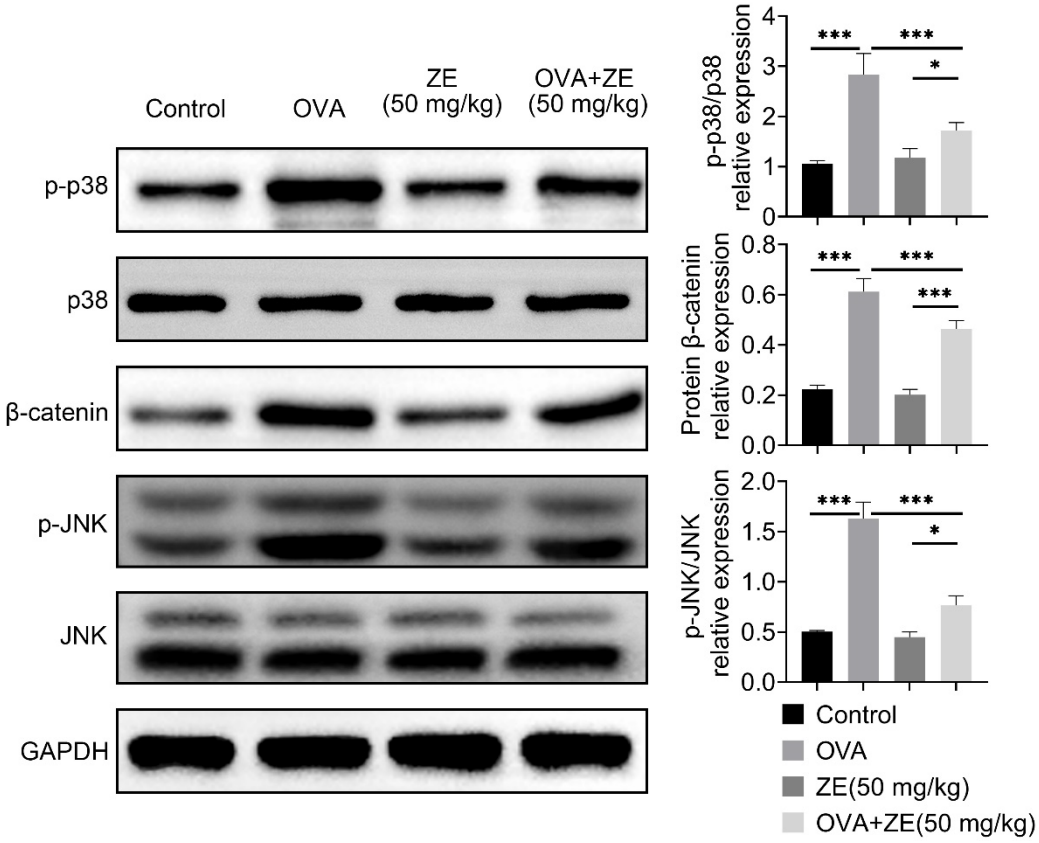

Figure 5 ZE repressed the p38 MAPK/β-catenin signaling pathway in the OVA-induced mice. An allergic asthma model was induced by OVA in BALB/c nude mice. Mice were orally received with 50 mg/kg ZE. The relative protein expressions of p38, p-p38, β-catenin, JNK and p-JNK in lung tissues were examined by western blot. GAPDH served as an internal reference. Data differences were determined by the one-way analysis of variance (ANOVA) followed by the p*ost hoc* Bonferroni test by SPSS 26.0 software. *p<0.05 and ***p<0.001.

SOD, GST and GSH concentrations. Moreover, zeaxanthin treatment markedly reduced the relative expressions of p-p38/p38, β-catenin and p-JNK/JNK in the OVA-induced asthma mice model, indicating that zeaxanthin suppressed the activation of p38 MAPK/β-catenin signaling pathway in the OVA-induced asthma mice model. Altogether, these results illustrated that zeaxanthin attenuated OVA-induced allergic asthma in mice via modulating the p38 MAPK/β-catenin signaling pathway.

Allergic asthma is characterized by chronic airway inflammation, accompanied by the enhancement of inflammatory cells infiltration, allergen-specific IgE, the allergen-reactive T helper type 2 (Th2) cells and pro-inflammatory factors.[22] Notably, Th2 cells can secrete a series of cytokines, such as IL-4, IL-5, and IL-13 to promote IgE generation, eosinophil recruitment, mucus overproduction and bronchial hyper-reactivity.[23,24] In the current study, the distinct infiltration of inflammatory cells was discovered in the OVA-induced asthma mice model with significantly increased concentrations of IgE, IL-4, IL-5, IL-13 and eotaxin, which was observably antagonized by zeaxanthin treatment. The above results indicated the anti-inflammatory effect of zeaxanthin on allergic asthma, which was in line with the previous studies.[11,25,26] In addition, plenty of studies have elaborated oxidative stress was also strongly associated with the pathogenesis of allergic asthma.[27] Thus, drugs that can inhibit oxidative stress might be the optimal candidates for treating allergic asthma. Gong et al[28] reported that yeast fermentate prebiotic suppressed the level of oxidative stress to improve allergic asthma. Abscisic acid alleviated OVA-induced allergic asthma by inhibiting oxidative stress and activating the NLRP3 inflammasome.[29] Moreover, the antioxidant activity of zeaxanthin in various organs, including the heart, eyes, skin and liver has been demonstrated.[30] Consistently, an inhibitory effect of zeaxanthin on oxidative stress was also observed in the OVA-induced asthma mice model, as evidenced by the decrease of MDA concentration and the increase of SOD, GST and GSH concentrations. The serum level of zeaxanthin is positively associated with forced expiratory volume in the first second[15] and significantly lower[31] in patients with asthma. Therefore, a diet with zeaxanthin is an optimal choice for patients with asthma. However, emerging findings reveal that oxidative stress also play an important role in the development of inflammation in allergic asthma (31). Thus, whether zeaxanthin suppressed inflammation through oxidative stress in OVA-induced allergic asthma needs to be further studied in the following investigation. These outcomes illustrated that zeaxanthin attenuated inflammation and oxidative stress in OVA-induced allergic asthma.

Airway fibrosis and remodeling are other hallmarks of asthma, in which α-SMA expression and collagen deposition are significant indicators.[32] Wang et al.[33] showed that vitamin D3 prevented asthmatic damage associated with the

reduction of collagen deposition and α-SMA expression in an OVA-induced asthma mice model. In line with these findings, results from this study also revealed that zeaxanthin treatment decreased the OVA-induced collagen deposition and α-SMA expression. Hence, these results indicated that zeaxanthin impeded airway fibrosis and remodeling in OVA-induced allergic asthma.

Furthermore, zeaxanthin treatment markedly reduced the relative expressions of p-p38/p38, ß-catenin and p-JNK/JNK in the OVA-induced asthma mice model, indicating that zeaxanthin suppressed the activation of p38 MAPK/ß-catenin signaling pathway in the OVA-induced asthma mice model. Both MAPK and ß-catenin signaling are crucial pathways involved in various physiological and pathological progress.[34,35] More importantly, Khorasanizadeh et al.[36] outlined that MAPK can act as therapeutic targets for asthma. ß-catenin has also been demonstrated to be tightly involved in asthma pathogenesis by regulating the progress of airway remodeling.[37-39] Furthermore, Jia et al.[40] found that the Wnt/ß-catenin signaling pathway modulated asthma airway remodeling by regulating the p38 MAPK-dependent pathway, suggesting an interaction between MAPK and Wnt/ß-catenin pathways in asthma. Additionally, the modulatory effect of zeaxanthin on MAPK signaling is also demonstrated in various disease models, such as melanoma[41] and gastric cancer.[42] Consistently, zeaxanthin, as one of the major carotenoids, may associate with the cancer chemoprevention via regulating the Wnt/ß-catenin signaling pathway.[43] Consistent with these reports, the current study also expounded that zeaxanthin suppressed the activation of the p38 MAPK/ß-catenin signaling pathway in the OVA-induced asthma mice model.

In conclusion, our findings showed that zeaxanthin inhibited the infiltration of inflammatory cells, secretion of inflammatory factors, collagen deposition, α-SMA expression, level of oxidative stress, and expression of proteins related to the p38 MAPK/ß-catenin signaling pathway in the OVA-induced asthma mice model. Therefore, we concluded that zeaxanthin dampened the progression of OVA-induced allergic asthma in mice by regulating the p38 MAPK/ß-catenin signaling pathway. However, there were several limitations in the current study. Emerging findings revealed that oxidative stress also played an important role in the development of inflammation in allergic asthma.[44] Thus, whether zeaxanthin suppressed inflammation through oxidative stress in OVA-induced allergic asthma needs to be further studied in the following investigation. Additionally, pharmacological blocks or other effective interferences might be applied in subsequent studies to confirm the direct role of the p38 MAPK/ß-catenin signaling pathway in the process of zeaxanthin-modulating OVA-induced asthma. Moreover, other experimental methods could provide a firmer support for our results. In brief, as far as we know, this is the first time to report the ameliorative effect of zeaxanthin on the OVA-induced asthma mice model. These outcomes establish a theoretical basis for the development of treatment of asthma.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors state that there are no conflict of interest to disclose.

Ethics approval

Ethical approval was obtained from the Ethics Committee of Tongde Hospital of Zhejiang Province.

Author's Contribution

All authors contributed to the study's conception and design. Material preparation and the experiments were performed by Xiaosheng Jin. Data collection and analysis were performed by Weijing Jin and Guoping Li. The first draft of the manuscript was written by Jisheng Zheng and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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