



Anti-inflammatory effect of epigallocatechin gallate in a mouse model of ovalbumin-induced allergic rhinitis



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ABSTRACT

Currently, a variety of studies have demonstrated that green tea has anti-allergic properties, and the major polyphenolic compound, epigallocatechin gallate (EGCG), plays a significant role. Some research indicates that EGCG reduces the production and expression of allergy-related substances. Therefore, EGCG has a potential effect of reducing allergic rhinitis (AR). In this study, the effect of EGCG on allergic rhinitis in an ovalbumin (OVA)-induced mouse model was investigated. After administration of EGCG, the number of sneezes and the occurrence of nasal rubbing were significantly decreased, the concentrations of immunoglobulin E (IgE) and histamine were suppressed in AR mouse serum, the levels of interleukin (IL)-1 β , IL-4, and IL-6 were reduced in AR mice nasal lavage fluid (NLF), and the nasal mucosa mRNA and protein expression of cyclooxygenase 2 (COX-2), IL-1 β , IL-4, and IL-6 were inhibited. The data indicate that EGCG has a beneficial effect of reducing allergic rhinitis.

1. Introduction

AR, a common disease in Rhinology, is a type I hypersensitivity immune reaction due to the sensitization of specific allergens, such as mites, domestic animals, dusts and pollens. AR, as an IgE-mediated inflammatory response, was characterized by sneezing, nasal pruritis, nasal congestion, postnasal drainage and hyposmia [1]. Air pollution and lifestyle changes lead to a sharp increase in AR prevalence [2]. Epidemiological data indicate that 9–38% of the Chinese population suffers from AR; these cases are generally complicated by atopic dermatitis, asthma and eczema [3]. Symptoms of allergic rhinitis lead to sleep disturbances, fatigue and depressed mood, which seriously reduce the quality of life and work [4]. The inflammatory symptoms are mainly triggered by inflammatory mediators, such as histamine, IL-1 β , IL-4, IL-6, and COX-2, released from IgE stimulated mast cells, eosinophils, basophils and T lymphocytes [5].

Recently, various agents, such as antihistamines, corticosteroids, antileukotrienes, nasal decongestants and mast cell stabilizers, are broadly used for the treatment of AR [6]. However, the prolonged use of those methods results in a variety of undesirable side effects, including anaphylactic risk and prolonged duration. Consequently, natural products characterized as being more effective and safe, have attracted more attention [7]. EGCG is the major polyphenolic constituent of green tea catechins [8]. Recently, a great deal of research has

demonstrated the anti-allergic effects of green tea. Kyoji et al. confirmed that C-2 epimeric isomers of tea catechins can be effective against Type I allergy and Type IV allergy [9]. Hassanain et al. confirmed that green tea can suppress B cell production of IgE [10]. Kou et al. have found that an EGCG fraction of green tea extract can inhibit the production of antigen-specific IgE via an increased proportion of CD4⁺CD25⁺ regulatory T lymphocytes in the spleen [11]. A study shows that tea polyphenol EGCG has a suppressive effect of the expression on the Fc epsilon receptor I (Fc ϵ RI) by highly associating with plasma membrane microdomains and lipid rafts [12]. IgE and Fc ϵ RI are essential in the development of allergic rhinitis. Antigens invade the body, and stimulate B cells to produce the antigen specific IgE that binds to the Fc ϵ RI receptor expressed on mast cells and circulating basophils. When antigens enter the body again, they stimulate mast cell degranulation to produce inflammatory mediators by cross-linking of IgE-bound Fc ϵ RI molecules. Eventually, those inflammatory mediators cause nasal pruritis, nasal congestion and other symptoms of allergic rhinitis [13,14]. Moreover, numerous studies indicated that EGCG has a positive effect on asthma, whose pathogenesis is similar to rhinitis. For example, Smith-Norowitz et al. suggest that EGCG suppressed *Chlamydia pneumoniae*-mediated IgE responses in peripheral blood mononuclear cells from a patient with asthma [15]. Choi et al. confirmed that EGCG attenuated the mucus production and the MAPK expression in an asthma model-mouse [16].

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Collectively, it is conceivable that EGCG has the potential function of anti-allergic rhinitis. There has been a series of animal models of AR that has been used in research. In this study, we explore the anti-allergic rhinitis effect of EGCG at the dose of 25 mg/kg, 50 mg/kg, and 100 mg/kg. We choose the dose range according to the oral LD₅₀ of EGCG in mouse which is 2170 mg/kg, and which has been reported in a database of the Toxicology Data Network of the U.S. National Library of Medicine. Recently, the OVA-induced mouse model of AR has been found to have a similar pathology and symptomology as human AR and can be used in preliminary profiling therapies [17,18]. Hence, we evaluated the effect of EGCG in the mouse model of OVA-induced AR.

2. Materials and methods

2.1. Preparation of animals and reagents

Female BALB/c mice weighing 18–22 g (4 weeks old) were obtained from the Laboratory Animal Center of Jilin University. The mice had ad libitum access to food and water and were housed under 12-hour light/12-hour dark conditions in an air-conditioned room (temperature $23 \pm 2^\circ\text{C}$, relative humidity $55 \pm 5\%$). The procedures in this study were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Jilin University (20160306). OVA and EGCG with a purity of 98% were obtained from Sigma (St. Louis, MO, USA). The following items were also obtained: IL-1 β , IL-4 and IL-6 ELISA Quantitation Kit (R&D Systems, Inc., Minneapolis, MN, USA); IgE ELISA Kit and Histamine ELISA Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); TRiGene (GenStar, Beijing, China); PrimeScript RT-PCR Kit and DL2000 DNA Marker (TaKaRa, Dalian, China); RIPA Lysis Buffer (GenStar, Beijing, China); BeyoECL Star Kit (Beyotime, Shanghai, China); Color Prestained Protein Marker (Beyotime, Shanghai, China); and BCA Protein Assay Kit (GenStar, Beijing, China). Anti-COX-2, anti-IL-1 β , anti-IL-4, and anti-IL-6 antibodies were purchased from ZSGB-BIO (ZSGB-BIO, Beijing, China). Goat anti-mouse IgG1-HRP was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. OVA-induced allergic rhinitis

The experimental protocol for allergic sensitization was performed as previously described with slight modification [19,20]. Female BALB/c mice were sensitized by administering an intraperitoneal injection of OVA (50 μg) in 0.1 ml of saline containing aluminum hydroxide once daily for two days. Then, local immunization was performed once a day from day 15 to day 24 by dripping the OVA into the nasal vestibule bilaterally. The mice in the normal control group were given saline alone instead of saline containing OVA. EGCG (25, 50 and 100 mg/kg, BW) and Loratadine (2 mg/kg, BW) were administered orally before the local immunization once daily. Normal and OVA-induced AR mice received distilled water alone on the same schedule.

2.3. Evaluation of nasal symptoms in mice

Before the experiment, mice were placed into an observation cage for approximately 15 min for acclimatization. Then, 10 μl of solution was dropped into the bilateral nasal cavities of the mice. Thirty minutes after administration, the frequencies of sneezing and nose rubbing were then counted for 10 min to evaluate early allergic responses according to the method of Narita et al. [21], by three observers blinded to treatment and compared with that of the AR group.

2.4. Measurement of the levels of IL-1 β , IL-4 and IL-6 in NLF

The mice were euthanized under ether anesthesia 24 h after the last immunization, the nasal cavity was gently perfused with 1 ml cold

phosphate-buffered saline and collected the lavage fluid in a tube. The nasal lavage fluid was centrifuged at $700 \times g$ for 10 min at 4°C , and supernatant was collected and stored at -80°C until analysis. The amounts of IL-1 β , IL-4 and IL-6 in NLF were measured with ELISA, using a mouse IL-1 β , IL-4 and IL-6 ELISA quantitation kit.

2.5. Serological analysis

Blood specimens were taken from ophthalmic veins in each mouse under ether anesthesia 24 h after the last immunization on day 24. Serum was collected by centrifugation and was kept at -80°C until analysis. The concentrations of IgE, histamine in the serum of mice were measured using ELISA Kits according to the manufacturer's recommendations, and the concentrations of these materials were calculated from the equations obtained from the standard curve plots.

2.6. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

The experimental protocol for RT-PCR was performed as previously described in [22]. Total RNA samples were isolated from nasal mucosa using TRiGene. Then, the purity of RNA was reflected by the OD₂₆₀/OD₂₈₀. The first strand complementary DNA (cDNA) was synthesized, and polymerase chain reaction was performed to analyze the expression of mRNA for COX-2, IL-1 β , IL-4, IL-6 and GAPDH (internal control) using a PrimeScript RT-PCR Kit according to the manufacturer's protocol. PCR was carried out with the following primers: COX-2 (F 5'-CCA GCA CTT CAC CCA TCA GT-3'; R 5'-ACC CAG GTC CTC GCT TAT GA-3'); IL-1 β (F 5'-CCG GAT CCA TGG CAC CTG TAC GAT CA-3'; R 5'-GGG GTA CCT TAG GAA GAC ACA AAT TG-3'); IL-4 (F 5'-TCA ACC CCC AGC TAG TTG TC-3'; R 5'-CGA GCT CAC TCT CTG TGG TG-3'); IL-6 (F 5'-CCA ATT TCC AAT GCT CTC CT-3'; R 5'-ACC ACA GTG AGG AAT GTC CA-3') and GAPDH (F 5'-CAA AAG GGT CAT CAT CTC TG-3' and R 5'-CCT GCT TCA CCA CCT TCT TG-3'). The amplified products were electrophoresed on 1.5% agarose gel containing ethidium bromide.

2.7. Western blotting

The experimental protocol for western blot was performed as previously described in [23]. The nasal mucosa tissue was lysed with RIPA Lysis Buffer supplemented with protease inhibitors for obtaining the proteins. The protein concentrations were detected using the BCA Protein Assay Kit. The proteins were separated using 10% SDS-PAGE electrophoresis and transferred to PVDF membranes. Then, the membranes were blocked using 5% skim milk power. The blocked membrane was incubated with primary antibodies at 4°C for 12 h, and then with goat anti-mouse IgG₁-HRP secondary antibodies at room temperature for 1 h. Signals were visualized using an enhanced chemiluminescence reagent.

2.8. Statistical analysis

The data were presented as the means $\pm$ standard error of mean. Statistical analyses were performed by one-way analysis of variance using SPSS statistical software. The differences between the two groups were considered statistically significant, when $P < 0.05$.

3. Results

3.1. Effect of EGCG on clinical symptoms in the AR model

To demonstrate the effect of EGCG by administration on nasal symptoms, the frequency of occurrences of sneezing and nasal rubbing were counted for 10 min after OVA sensitization. As shown in Fig. 1, the number of occurrences of nasal rubbing and sneezing in the OVA-

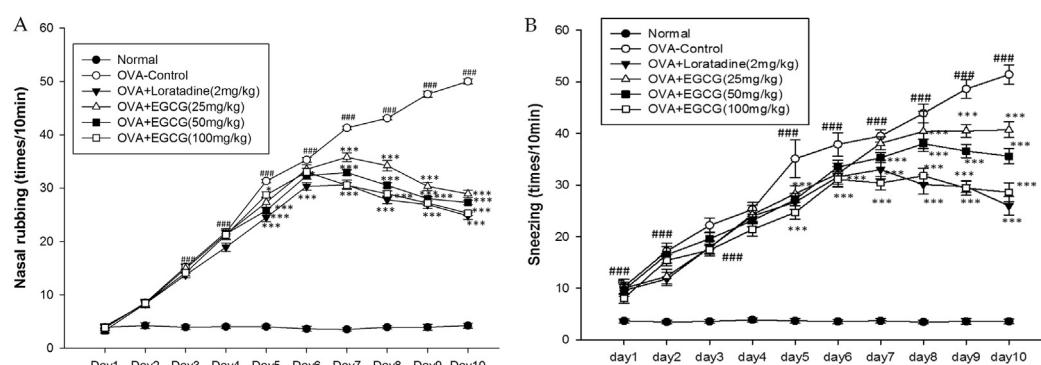


Fig. 1. Effect of EGCG on clinical symptoms in the AR model. The number of occurrences of (A) nasal rubbing and (B) sneezing counted 10 min after OVA intranasal provocation. Each data value represents the mean $\pm$ S.E.M. # Significantly different from the Normal (###: $p < 0.001$), and * significantly different from the Control value (*: $p < 0.05$, ***: $p < 0.001$), $n = 10$ /group.

sensitized mice was significantly higher than the non-sensitized mice. Further, the OVA-sensitized mice were used to confirm the effect of EGCG on the nasal symptoms. As expected, the increased nasal rubbing and sneezing numbers in the OVA-sensitized mice were dose-dependently inhibited by administration of EGCG at 25, 50, and 100 mg/kg, and Loratadine also resulted in a significant inhibition of the number of occurrences of nasal rubbing and sneezing at a dose of 2 mg/kg by oral administration.

3.2. Effect of EGCG on the serum levels of IgE and histamine in the AR model

To investigate the regulatory effect of EGCG on allergic responses, the levels of IgE and histamine in the serum were evaluated by enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 2, the levels of IgE and histamine after the OVA sensitization were markedly elevated compared to those in the normal control group. At the dose of 25, 50, and 100 mg/kg, administration of EGCG antagonized the effect of the increased serum levels of IgE and histamine in OVA-sensitized mice, and those mediators in serum were decreased in the Loratadine-administered AR mice.

3.3. Effect of EGCG on the levels of IL-1 β , IL-4 and IL-6 in NLF

To evaluate the effect of EGCG in allergic rhinitis, we measured the levels of IL-1 β , IL-4 and IL-6 in NLF on goat AR model. As shown in Fig. 3, the levels of IL-1 β , IL-4 and IL-6 in NLF were increased in the AR mice compared to those levels in the normal mice. However, increased levels of IL-1 β , IL-4 and IL-6 were significantly reduced by EGCG

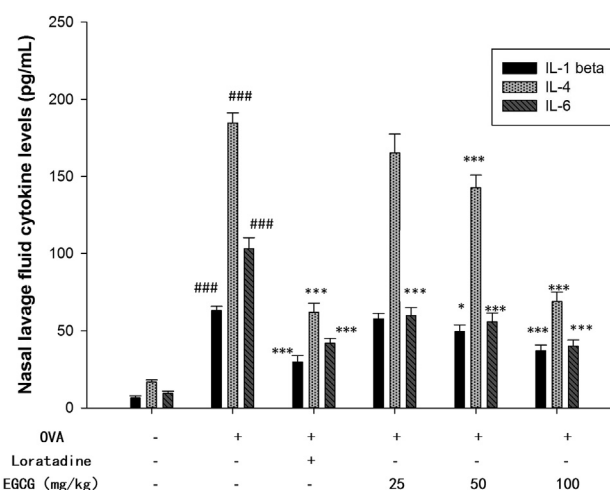


Fig. 3. Effect of EGCG on the levels of IL-1 β , IL-4 and IL-6 in the AR model. The levels of IL-1 β , IL-4 and IL-6 were measured in mouse NLF by ELISA. Each data value represents the mean $\pm$ S.E.M. # Significantly different from the Normal (###: $p < 0.001$), and * significantly different from the Control value (*: $p < 0.05$, ***: $p < 0.001$), $n = 10$ /group.

administration.

3.4. Effect of EGCG on inflammation-related mRNA levels in the nasal mucosa

To confirm the effect of EGCG in the AR model, the nasal mucosa

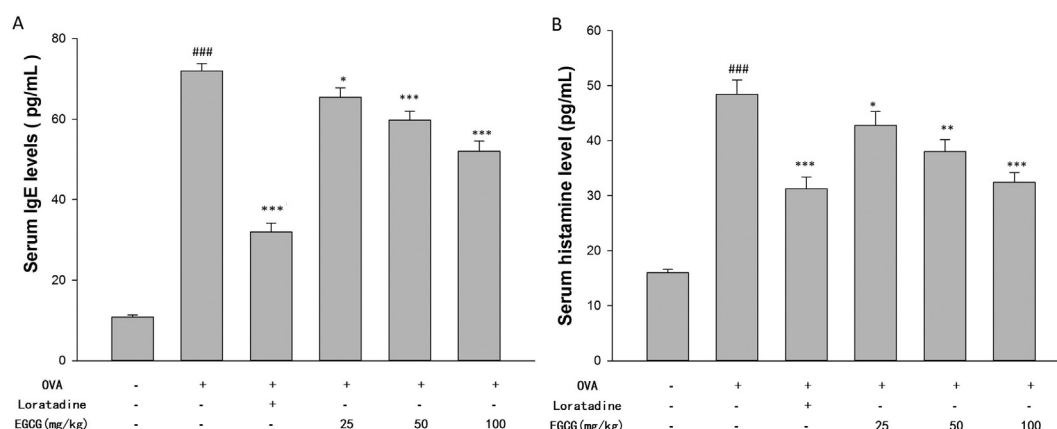


Fig. 2. Effect of EGCG on the levels of IgE and histamine in the AR mouse serum. The levels of (A) IgE and (B) histamine were measured in mouse serum by ELISA. Each data value represents the mean $\pm$ S.E.M. # Significantly different from the Normal (###: $p < 0.001$), and * significantly different from the Control value (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$), $n = 10$ /group.

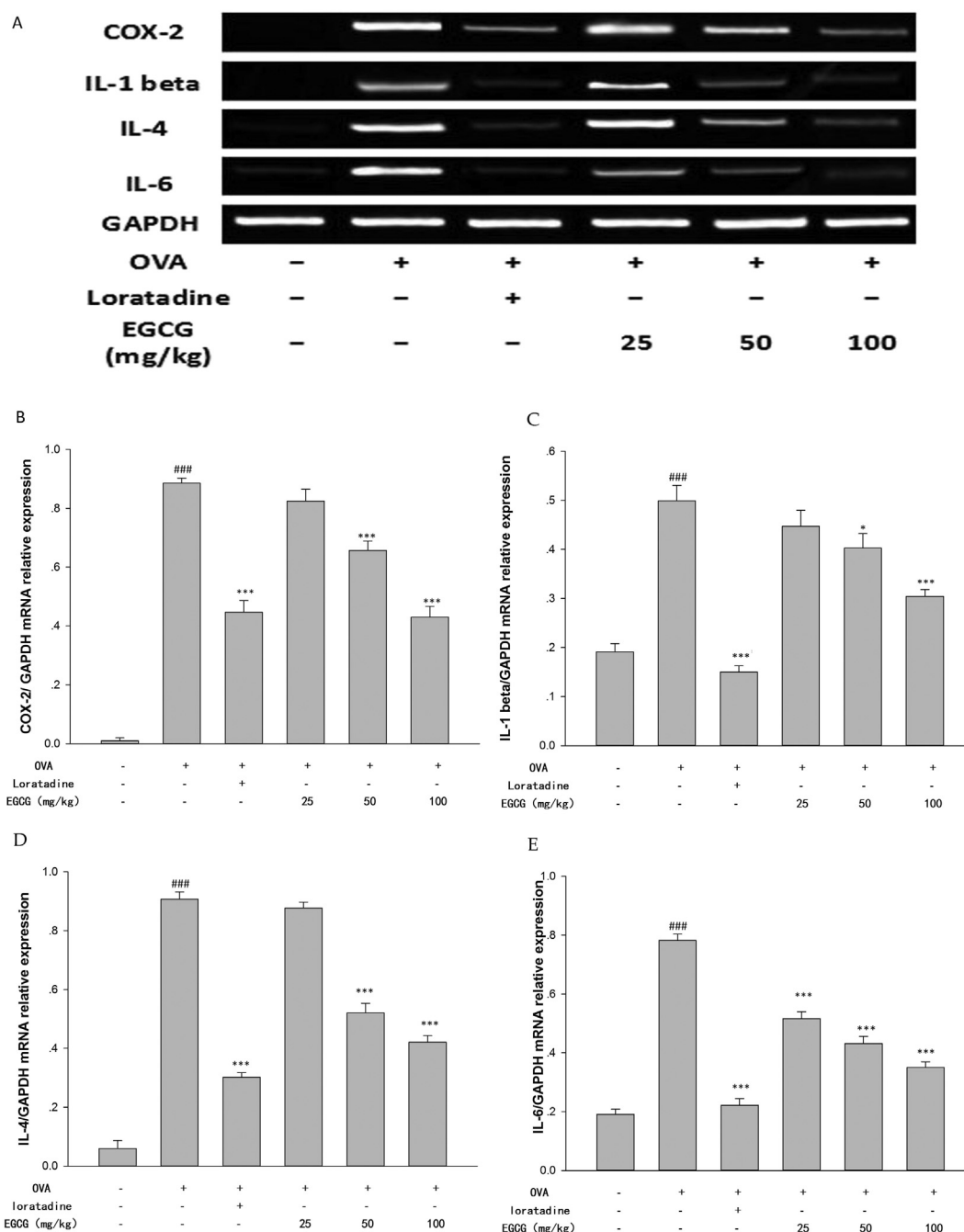


Fig. 4. Effects of EGCG on inflammation-related mRNA levels in the AR model. (A) The nasal mucosa mRNA levels of COX-2, IL-1 β , IL-4 and IL-6 were analyzed by RT-PCR. The mRNA relative expression of (B) COX-2, (C) IL-1 β , (D) IL-4 and (E) IL-6 were calculated. Each data value represents the mean $\pm$ S.E.M. # Significantly different from the Normal (###: $p < 0.001$), and * significantly different from the Control value (*: $p < 0.05$, ***: $p < 0.001$), $n = 10$ /group.

mRNA levels of COX-2, IL-1 β , IL-4 and IL-6 were analyzed by RT-PCR. As shown in Fig. 4, OVA-induced allergic rhinitis increased mRNA levels of COX-2, IL-1 β , IL-4 and IL-6. In contrast, EGCG markedly diminished mRNA levels of those inflammatory mediators.

3.5. Effect of EGCG on inflammation-related protein levels in the nasal mucosa

We also examined the effect of EGCG on nasal mucosa protein expression of inflammatory mediators in the AR model using western blot. Sensitization of OVA induced a predominant increase in protein expressions of COX-2, IL-1 β , IL-4 and IL-6. EGCG and Loratadine administration suppressed the OVA-induced protein expression of

COX-2, IL-1 β , IL-4 and IL-6 (Fig. 5).

4. Discussion

Allergic rhinitis is an immediate type I allergic inflammatory response to inhaled allergens [24]. The immune reactions include the recruitment of inflammatory cells to nasal mucosa as well as the product and release of inflammatory mediators. The first step in the development of AR is when an allergen invades the nasal mucosa, and antigen presenting cells (APCs) recognize, uptake, and process the antigen to T lymphocytes. Simultaneously, APCs secrete the IL-4 and other mediators, which promote the transformation of naïve T helper cells into Th2 lymphocytes [25,26]. Mature Th2 cells produce cyto-

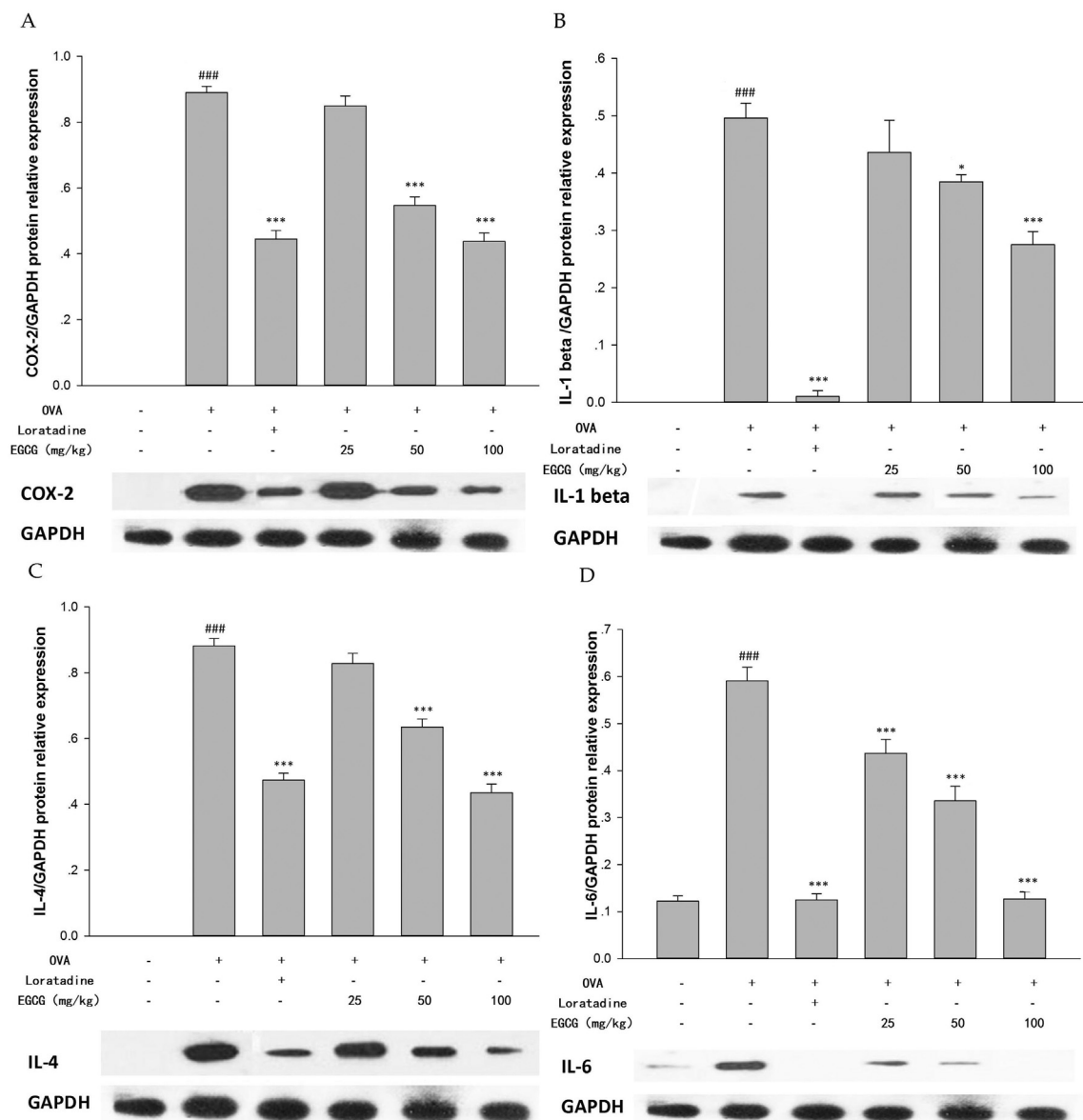


Fig. 5. Effect of EGCG on inflammation-related protein levels in the AR model. The nasal mucosa protein levels and relative expression of (A) COX-2, (B) IL-1 β , (C) IL-4 and (D) IL-6 were evaluated by Western blot. Each data value represents the mean $\pm$ S.E.M. # Significantly different from the Normal (###: $p < 0.001$), and * significantly different from the Control value (*: $p < 0.05$, ***: $p < 0.001$), $n = 10$ /group.

kines, which contribute to the production of eosinophiles, and trigger the isotype switching from IgM to IgE class antibodies in B lymphocytes [27]. When the same antigen re-invades the body, the antigen memorial B lymphocytes could produce antigen-specific IgE and bind to the Fc ϵ RI receptors. The antigen cross-linked IgE bound Fc ϵ RI complex results in the aggregation of Fc ϵ RI receptors, which lead to the phosphorylation of Src family kinase. The phosphorylated Src family kinase activates mitogen-activated protein kinases (MAPKs), which causes the level of intracellular Ca^{2+} to increase (a key factor for granule movement and the inflammation cytokine release), and activates the nuclear factor (NF)- κ B (an essential mediator for inflammation cytokine synthesis) [28–30]. Subsequently, mast cells are activated, and secrete preformed and newly generated mediators, such as histamine, prostaglandins, leukotrienes, and cytokines, which could cause blood vessels to leak and to dilate, and can stimulate sensory nerves [6,31]. Those responses trigger the rhinitis symptoms including sneezing, rhinorrhea, nasal congestion, sore throat and itching. In the early phase inflammatory reaction, the mast cell-derived mediators advance the infiltration of eosinophils, neutrophils, and basophils in the epithelium and sub-

epithelium of the nasal mucosa, and promote the inflammatory cells' release of COX-2, IL-1 β , IL-4, IL-6, and other inflammatory cytokines [32]. Those mediators maintain the allergic reactions, and in turn reactivate much early phase inflammatory reaction, which increases the degree of nasal congestion, and lead to hyposmia [33]. Therefore, restraining the local release of histamine, IgE, IL-1 β , IL-4, and IL-6 is a target for the development of drugs for allergic rhinitis.

The aim of our study was to investigate the role of EGCG in allergic rhinitis. In our study, the serum concentration of IgE increased sharply after OVA sensitization. This is due to OVA, an exogenous antigen which stimulates B cells to produce IgE. IgE can activate mast cells, as well as play a vital role in mast cell activity amplification via upregulating the Fc ϵ RI expression in mast cells [34,35]. Therefore, reducing the level of IgE is an effective way to treat allergic rhinitis. Our research data show that EGCG can decrease the serum levels of IgE. Mast cells were activated by the OVA cross-linking IgE bound Fc ϵ RI complex. The activated mast cells triggered a series of allergy symptoms [2]. The early phase can induce sneezing, nasal itching, runny and congested nasal passages, and other symptoms. The late phase con-

tributes to patient fatigue, malaise, irritability, and other symptoms [7]. Our data are a clear indication that the number of occurrences of nasal rubbing and of sneezing increased after OVA stimulation, which is a hallmark of allergic rhinitis. Ji-Eun Lee et al. also reported that nasal symptoms increased with similar treatment as previously described [36]. Increased nasal rubbing and sneezing were inhibited in a dose-dependent manner by the administration of EGCG, thus indicating that EGCG can relieve the early allergic rhinitis symptom. These symptoms are triggered by various mediators, such as histamine, IL-1 β , IL-4, and IL-6. Histamine has the effect of dilating blood vessels, increasing vascular permeability, and stimulating the peripheral nerve, which leads to the above allergic rhinitis symptom [37]. Moreover, accumulating evidence has demonstrated that histamine promotes the maturation of dendritic cells (DC), which plays vital role in the differentiation of the naive T cell and the amplification Th2 immune responses [38]. Hence, antihistamines, such as Azelastine, Loratadine, and Cetirizine, were used to treat allergic disease via reducing the serum level of histamine and suppressing rhinitis symptoms [39]. IL-1 β is a powerful pro-inflammation cytokine in inactive proenzyme (pro-IL-1 β) form in the cytoplasm. When IgE mediates the activation of mast cells, pro-IL-1 β is hydrolyzed to IL-1 β , and released into the extracellular matrix by degranulation. IL-1 β has the ability to stimulate monocytes and promote eosinophil recruitment and degranulation [40]. In addition, Hu Wei-xu et al. study demonstrated that the IL-1 β inhibitor, anti-IL-1 β IgY, could reduce pathological allergic inflammation in the nasal mucosa and lung tissues in AR guinea pigs [41]. IL-4 secreted by T helper (Th)-2 cells was initially known to induce the isotype switching from IgM to IgE class antibodies in B lymphocytes to maintain the allergic inflammatory cascade, and promote eosinophil and basophil infiltration [27,42,43]. An increasing number of studies have been published showing that IL-4 is associated with the expression of adhesion molecules. Hideto Tozawa et al. showed evidence that IL-4 could upregulate the expression of adhesion molecules, like vascular cell adhesion molecule 1 (VCAN-1) on the endothelial cells facilitating the infiltration of eosinophils, T lymphocytes and basophils into the nasal mucosa [44]. IL-6, secreted by various innate immune cells, is associated with the maturation and differentiation of B cells and mononuclear macrophage, the enhanced activity of the natural killer cell, and the expression of COX-2 [45–47]. A previous study showed that intranasal challenge with IL-6 can increase nasal mucus production. IL-6 could amplify allergic inflammation by suppression of Foxp3⁺ regulatory T-cell/IL-10 axis [48]. After allergen sensitization, IL-6 knockout (KO) significantly decreases in inflammatory cell recruitment and Th2 and Th17 cytokine production and lowered allergen specific IgG1 when compared with the wild type mice [49]. It is known that COX-2 is involved in the production of prostaglandins and thromboxane A₂, and is induced by inflammatory cytokines, including IL-1, IL-6, and TNF- α , as part of the delayed allergic reaction [50]. Moreover, Hong Li et al. used COX-2 KO mice to indicate that PGD₂ and PGE₂, produced by COX-2, regulate Th9 cell differentiation by negative regulation of IL-17RB expression via a protein kinase A (PKA)-dependent mechanism [51]. Overall, accumulating evidence indicates that the above-mentioned mediators act as a primary contributory role in the pathogenesis of AR. Consistent with previous studies [7,36,52,53], our data showed that OVA stimulation increased the level of histamine, IL-1 β , IL-4, IL-6, and IgE. Thereby, a variety of anti-allergic drugs alleviate the symptoms of allergic rhinitis by lowering the level of aforementioned mediators [54]. Our data indicated that oral administration of EGCG markedly reduce, as a function of its concentration, the serum level of histamine and IgE, as well as the NLF level of IL-1 β , IL-4, and IL-6. Furthermore, the proteins and mRNA expression of COX-2, IL-1 β , IL-4 and IL-6 in the nasal mucosa progressively decreased with increasing concentrations of EGCG.

In summary, our observations suggest that EGCG exerts anti-allergic effects by suppressing nasal symptoms, and inhibiting the production and expression of inflammatory mediators in OVA-induced allergic

rhinitis model.

Author contributions

Tie Hong, Meng Fu, ShuiLian Fu, Saihong Ni, Liyuan Zou, and Yumei Liu conceptualized and designed this study and wrote the paper. Tie Hong and Meng Fu performed most of the experiments. ShuiLian Fu and Saihong Ni analyzed the data. All authors read and approved the final manuscript.

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