



Against NF- κ B/thymic stromal lymphopoietin signaling pathway, catechin alleviates the inflammation in allergic rhinitis

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ABSTRACT

Background: The prevalence of allergic rhinitis has risen sharply. Previous work has demonstrated the anti-inflammatory effect of catechin, including in models of allergic disease. However, the molecular mechanisms underlying this therapeutic effect remain unclear. Thymic stromal lymphopoietin (TSLP), as a molecule from epithelial cell, has been identified that plays a significant role in the development of allergic disease, and the production of TSLP is related to activation of the NF- κ B signaling pathway. For that, we try to research the treatment of catechin for allergic rhinitis and found out possible mechanism under this effect, which was based on TSLP factor.

Materials and methods: Here, the anti-inflammatory effects of catechin were studied in an ovalbumin-induced allergic rhinitis murine model and a vitro experiments using poly(I:C)-stimulated human nasal epithelial cells (HNEpCs). The pharmacological effects of catechin in allergic rhinitis mice were assessed by observing the allergic symptoms, performing hematoxylin and eosin staining and Giemsa staining of the nasal tissues. Additionally, the TSLP expression in epithelial cells was tested by enzyme-linked immunosorbent assays, immunohistochemistry, and western blots. The serum levels of interleukin-5, interleukin-13, and ovalbumin-specific immunoglobulin-E were detected by enzyme-linked immunosorbent assays, and the balance between T helper type 1 and T helper type 2 cells was assessed by flow cytometry. The expression levels of phospho-NF- κ Bp65, I κ B α , and NF- κ Bp65 proteins were further investigated by western blots or immunofluorescence.

Results: Our results reveal that catechin, in doses of 75, 150, or 300 mg/kg, remitted the allergic symptoms in mice with allergic rhinitis, like sneezing and nasal rubbing. Catechin could reduce the levels of interleukin-5, interleukin-13, and ovalbumin-specific immunoglobulin-E in the serum and restored the T helper type 2/T helper type 1 cell balance and also had anti-thymic stromal lymphopoietin effects. Moreover, as an upstream regulator of TSLP, the NF- κ B signal pathway was also suppressed after catechin treatment, which was demonstrated by the observed decrease in phospho-NF- κ Bp65 and NF- κ Bp65 levels and the reduction of I κ B α degradation and NF- κ Bp65 nuclear translocation.

Conclusions: Catechin effectively reduced the inflammation in allergic rhinitis. The underlying mechanism is that catechin inhibited the expression of TSLP in epithelial cells by influencing NF- κ B/TSLP pathway.

1. Introduction

The prevalence of allergic diseases, including asthma, allergic rhinitis, and food allergies, has risen sharply in recent years. Allergic rhinitis as an inflammatory disease of the nasal mucosa induced by an immunoglobulin E-mediated reaction in allergen-sensitized individuals [1], is associated with numerous loci and candidate genes [2, 3].

Thymic stromal lymphopoietin (TSLP) has recently been reported to be one of the molecules closely related with the development of allergic disease [4, 5].

TSLP is an interleukin-7-like cytokine expressed mainly by epithelial cells [6]. Previous studies have provided compelling evidence that TSLP can activate dendritic cells to promote a T helper type 2 inflammatory response in allergic diseases [7, 8]; for example, TSLP can directly

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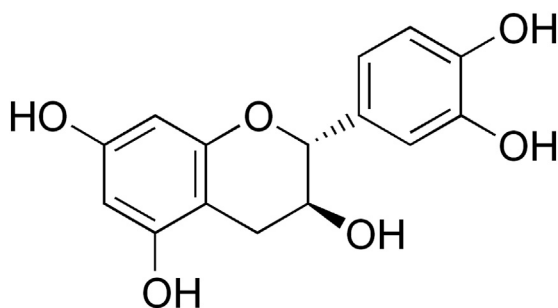


Fig. 1. The chemical structure of catechin.

promote the T helper type 2 differentiation of naïve CD4⁺ T cells and the release of T helper type 2 inflammatory factors [9], such as interleukin-4, interleukin-5, and interleukin-13. Further work indicates that inflammation factors such as interleukin-1 β , interleukin-13, and allergens can also induce the release of TSLP by stimulating the activation of NF- κ B signaling [10–12]. Moreover, in vivo experiments revealed that the toll-like receptor agonists like poly(I:C), could induce epithelial cells to produce TSLP, and this was associated with the activation of NF- κ B but not of IRF3 [13].

Catechin, the chemical structure of which is shown in Fig. 1, is a main effective component in green tea that has displayed anti-inflammation biological activity in both in vitro and vivo experiments [14, 15]. One study report that catechin could inhibit allergic inflammation in allergic keratitis and suppress NF- κ B activation following lipopolysaccharide-induced inflammation in BV-2 microglial cells [16]. However, neither the ability of catechin to inhibit allergic rhinitis inflammation nor the associated molecular mechanisms underlying this therapeutic effect of catechin had yet been determined.

Here, we used an allergic rhinitis murine model induced by ovalbumin(OVA) to evaluate the TSLP and inflammation response factors during allergic rhinitis and to assess the effect of catechin treatment on this condition. Our study also stimulates human nasal epithelial cell line (HNEpCs) with TLR3 agonist poly(I:C) in vitro to determine the effect of catechin on the NF- κ B signal and TSLP.

2. Materials and methods

2.1. Animals

Female BALB/c mice (aged 6–8 weeks, weighing 18–22 g) were obtained from the Medical Experimental Animal Center of Guangdong Province (Guangdong, China), where mice were maintained under specific pathogen-free SPF conditions. The experimental protocols were conducted under SPF conditions in the Laboratory Animal Center of Guangzhou University of Traditional Chinese Medicine, according to the guidelines approved by the Ethics Committee of Guangzhou University of Traditional Chinese Medicine.

2.2. Murine allergic rhinitis model and allergic symptoms

The mice were randomly divided into control, model, catechin treatment (in the doses of 75, 150, or 300 mg/kg), and loratadine (5 mg/kg) groups ($n = 6$ per group). On days 1, 8, and 15, the mice were sensitized by intraperitoneal injection of 25 μ g of OVA (Sigma, USA) and 1 mg of alum (Thermo, USA) in 300 μ l of phosphate-buffered saline. One week after the last OVA sensitization (day 22), the mice received intranasal challenges daily with OVA (500 μ g) [17], after which they treated with catechin (Sigma, USA) or loratadine for 30 min (Fig. 2). After the final nasal challenge with OVA, sneezing and rubbing events were recorded for each mouse by blinded observers, over a 10-min interval. The mice were then sacrificed at 24 h after the last nasal challenge for further analyses.

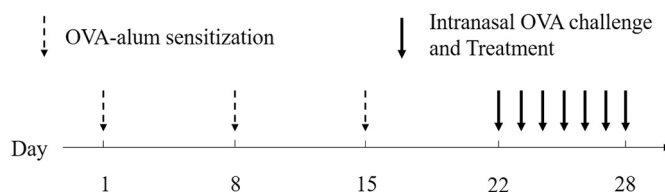


Fig. 2. Allergic rhinitis mouse model and drugs treatment.

2.3. Enzyme-linked immunosorbent assay

Twenty-four hours after the final OVA nasal challenge, the mice were sacrificed, and blood samples were harvested by cardiac puncture and collected in sterile 1.5-ml Eppendorf tubes. The blood samples were left at room temperature for 30 min before being centrifuged at 1000 $\times$ g at 4 $^{\circ}$ C for 10 min to obtain sera. The serum interleukin-5, interleukin-13, ovalbumin-specific immunoglobulin-E or TSLP levels in the cell supernatants were assayed using specific Enzyme-linked Immunosorbent Assay kits (Biolegend, USA; Arigo, China; Multi Sciences, China).

2.4. FCM of mouse lymphocytes in peripheral blood

The mice were sacrificed by cervical dislocation at the end of OVA administration. To obtain single cell suspensions, we layered the peripheral blood mixed with heparin sodium onto mouse lymphocyte separation medium (Daewe Biotech, China) and then centrifuged the samples at 800 $\times$ g for 20 min. Isolated cells were washed twice in phosphate-buffered saline and re-suspended with culture medium. After stimulation with PMA/ionomycin (Multi Sciences) for 6 h, the lymphocytes were stained with fluorescein isothiocyanate-anti-CD4 (eBioscience, USA) antibody at room temperature for 20 min. To detect T helper type 1 and T helper type 2 cells, we stained the lymphocytes with R-phycoerythrin-anti-interleukin-4 (eBioscience, USA) and allophycocyanin-anti-interferon- γ (eBioscience, USA) antibodies for 20 min, after processing the lymphocyte cells using a FIX&PERM Kit (Multi sciences, China) and BFA/Monensin (Multi Sciences, China). Finally, the samples were fixed with fixation solution and analyzed on a FACS Cantoll flow cytometer (BD Biosciences, USA) with FACSDiva™ software.

2.5. Paraffin sections of nasal tissue and histology

Twenty-four hours after the final OVA nasal challenge, the mice were sacrificed. The nasal tissues were fixed with 4% paraformaldehyde for 2 days and decalcified in 10% ethylene diamine tetra acetic acid for 20 days. Then the nasal tissues were embedded in paraffin, cut into 4- μ m sections, and stained with hematoxylin/eosin or Giemsa kit (Leagene Biotechnology, China). Infiltrating eosinophils and inflammatory condition in the nasal septal mucosa were counted under high magnification.

2.6. Immunohistochemistry

Immunohistochemistry was performed using an Immunohistochemistry Application Solutions Kit (Rabbit), purchased from CST company (USA). Paraffin sections of nasal tissue were baked at 60 $^{\circ}$ C and then dipped successively in dimethylbenzene, a series of concentrations of ethyl alcohol, and ultrapure water to remove the paraffin. These sections were then blocked with 10% bovine serum albumin prior to incubation overnight with rabbit anti-thymic stromal lymphopoietin monoclonal antibody (Abcam, USA) or rabbit anti-NF- κ Bp65 monoclonal antibody (CST, USA) at a 1:400 dilution, followed by incubation with secondary antibody (1:3000 dilution) for 1 h. After being reacted with DAB working solution, the tissues were observed

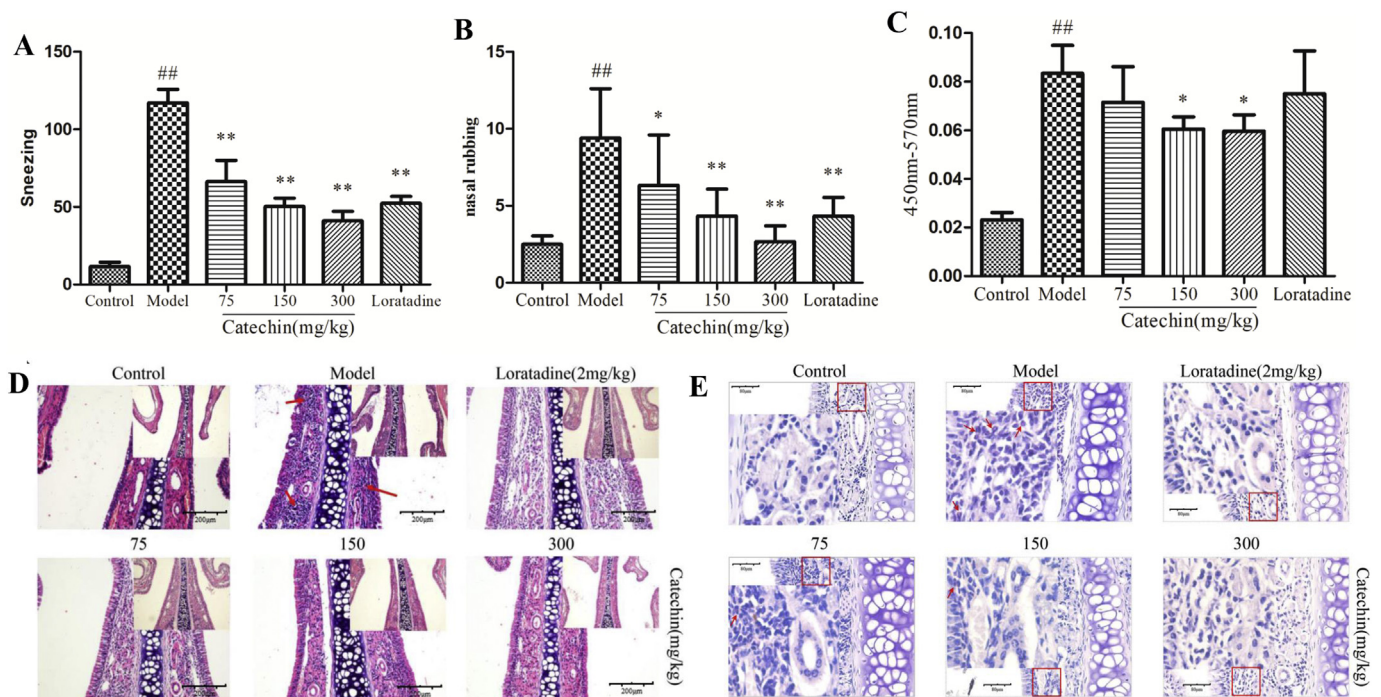


Fig. 3. The effect of catechin on allergic symptoms and nasal histology in allergic rhinitis model mice.

Allergic rhinitis model mice were treated with catechin (75, 150, or 300 mg/kg), control, or loratadine (5 mg/kg) or left untreated. A–C: The number of sneezing (A) and nose rubbing (B) occurrences, and the serum level of ovalbumin-specific immunoglobulin-E (C) in the mice. D–E: Representative images of hematoxylin/eosin (D) and Giemsa staining (E) of the nasal mucosa of the mice (200 × and 400 ×). [#]*p* < 0.05 vs. control; ^{*}*p* < 0.05, ^{**}*p* < 0.01 vs. model.

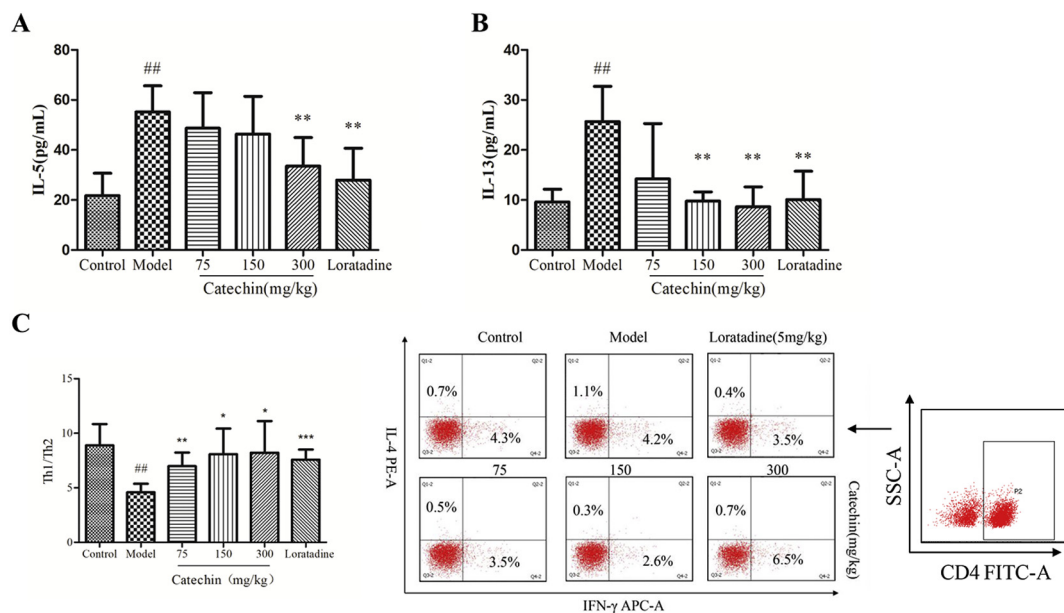


Fig. 4. The effect of catechin on the balance between T helper type 1 cells and T helper type 2 cells in allergic rhinitis model mice.

Allergic rhinitis model mice were treated with catechin (75, 150, or 300 mg/kg), control, or loratadine (5 mg/kg) or left untreated. A–B: The serum level of interleukin-5 (A) and interleukin-13 (B) in the mice as measured by enzyme-linked immunosorbent assays. C: The ratio of T helper type 1 cells to T helper type 2 cells in the peripheral blood lymphocytes of the mice as measured by flow cytometry. [#]*p* < 0.05 vs. control; ^{*}*p* < 0.05, ^{**}*p* < 0.01 vs. model.

under a microscope at a magnification of 400 ×.

2.7. Cell culture and cytotoxicity assay

The human nasal epithelial cell line (HNEpCs), which was ordered from Guangzhou Jini Ou Biological Ltd. (China), was cultured in formulated Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml of

penicillin, and 100 mg/ml of streptomycin at 37 °C in a humidified incubator with an atmosphere containing 5% CO₂. The culture medium was changed daily. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay to measure the cytotoxic response to catechin. In brief, 200 μl per well of HNEpCs suspension was cultured in 96-well plates for 24 h after treatment with each final concentration of catechin (10, 20, 40, and 80 μM), and the samples were assessed following the conventional

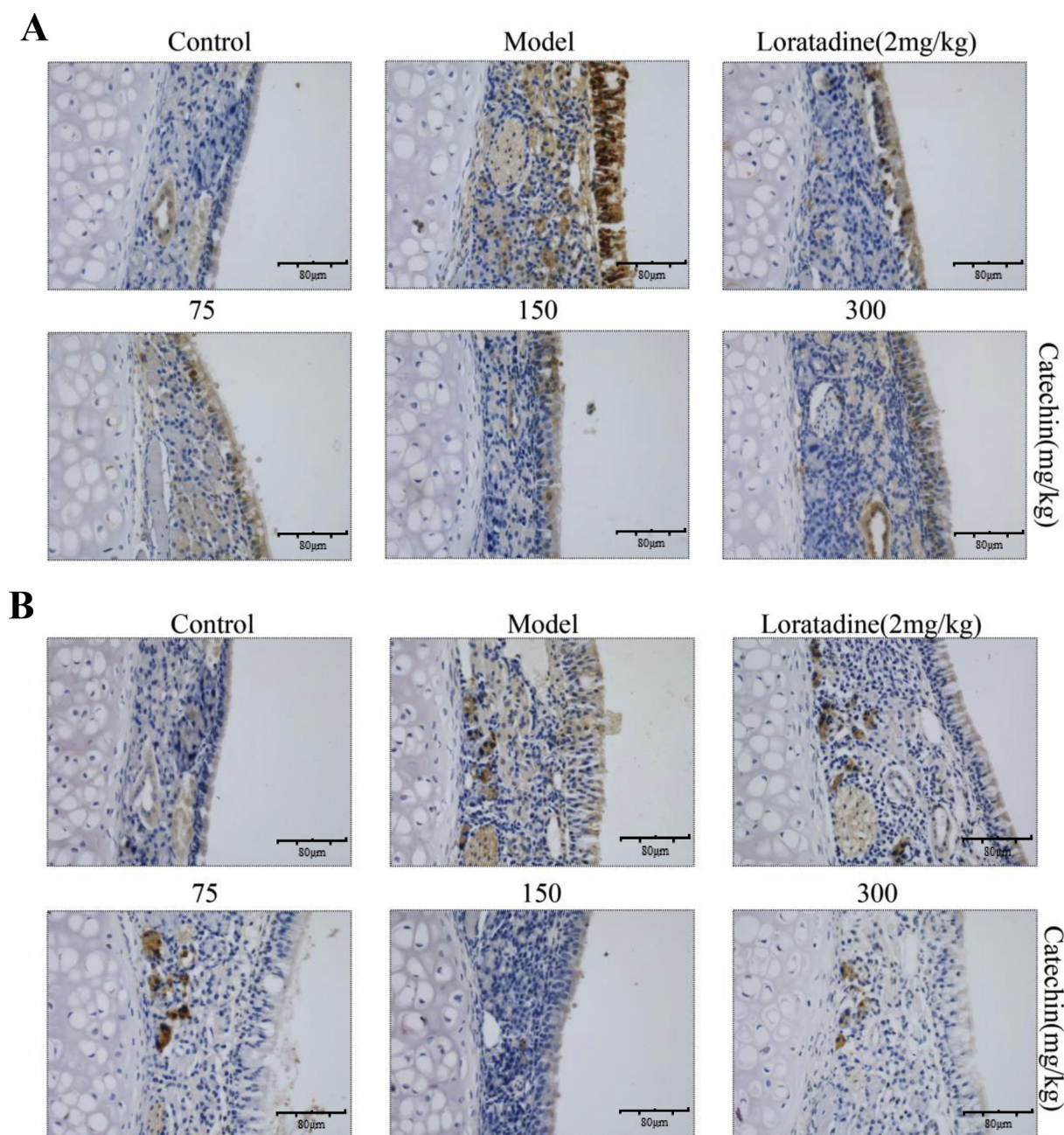


Fig. 5. The expression of NF- κ Bp65 and thymic stromal lymphopoietin protein in the nasal mucosa.

Allergic rhinitis model mice were treated with catechin (75, 150, or 300 mg/kg), control, or loratadine (5 mg/kg) or left untreated, and immunohistochemistry was performed on nasal tissue sections of these mice. A–B: Representative images of the thymic stromal lymphopoietin protein expression (A) and NF- κ Bp65 protein expression (B) in the nasal mucosa (400 $\times$).

procedure. The optical density of 96-well culture plates was measured at 490 nm and 630 nm.

2.8. Poly(I:C) stimulation and catechin treatment

HNEpCs were grown and maintained in 96- or 6-well dishes with Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin at 37 °C in a humidified incubator with an atmosphere containing 5% CO₂. After the cells were cultured to 80% confluence in 12- or 6-well plates, the cells were treated with catechin and 5 μ M toll-like receptor-3 antagonist (Millipore, USA) for 1 h before stimulation with 20 μ g of poly(I:C) (Sigma, USA). For the thymic stromal lymphopoietin, I κ B α , and NF- κ Bp65 measurements, the cells were challenged with catechin or

control for 24 h, and the resulting supernatants were stored at –20 °C until analysis. For phospho-NF- κ Bp65 measurements, the cells were challenged for 12 h rather than 24 h. Each experiment was performed with biological triplicates.

2.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blots

Cell lysates were prepared in ethylene diamine tetra acetic acid-free lysis buffer containing complete protease inhibitors and were centrifuged at 12,000 $\times$ g for 10 min. After being boiled with Laemmli sample buffer for 5 min, the cell lysates (20 μ g of protein) were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and blocked

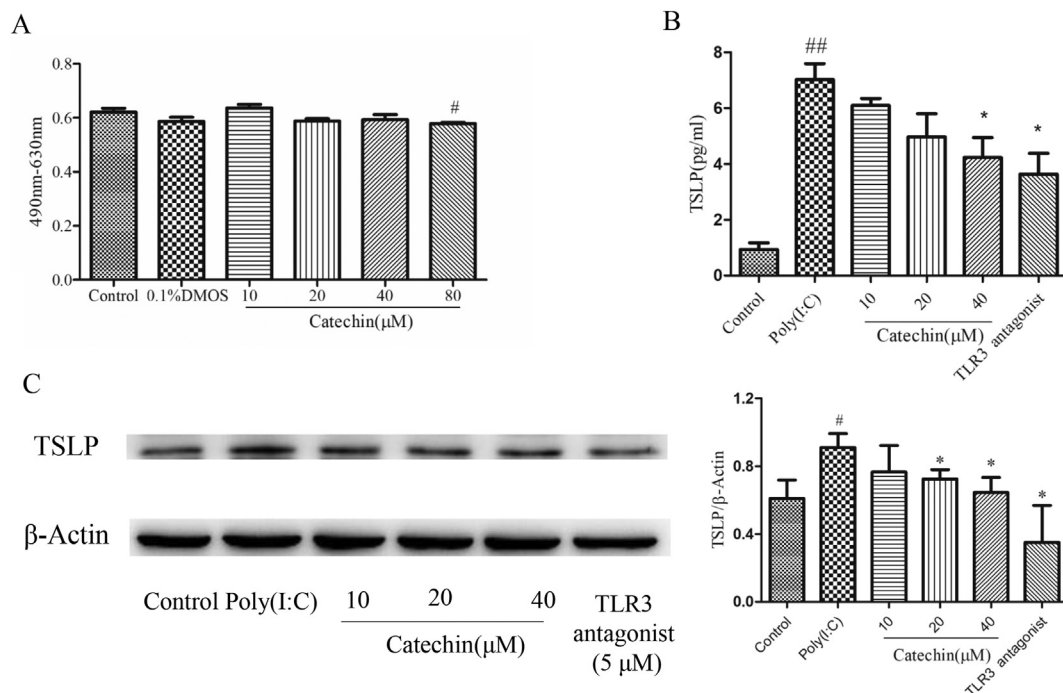


Fig. 6. Catechin cytotoxicity and thymic stromal lymphopoietin expression in HNEpCs.

A: The cytotoxicity of catechin (10, 20, and 40 μM) in HNEpCs. B–C: After incubation with catechin (10, 20, or 40 μM) or toll-like receptor-3 antagonist (5 M), the HNEpCs were stimulated with 20 μg of poly(I:C). B: The level of thymic stromal lymphopoietin in cellular supernatant. C: The expression of thymic stromal lymphopoietin from HNEpCs. [#]*p* < 0.05 vs. control; ^{*}*p* < 0.05, ^{**}*p* < 0.01 vs. poly(I:C) group.

with TBST containing 5% skim milk. The membranes were then incubated overnight with primary antibodies (1:1000 dilution) followed by incubation with secondary antibodies (1:3000 dilutions) for 1 h at room temperature. The blots were developed using an ECL Chemiluminescence kit (Millipore, USA), and the integrated density of pixels in each membrane was quantified using Image J software. Rabbit anti-IκBα antibody, rabbit anti-p-NF-κBp65 monoclonal antibody, and rabbit anti-histone H3 monoclonal antibody were purchased from CST(USA), mouse anti-β-actin antibody was purchased from Boster Biological Technology (USA), and secondary antibodies was purchased from Tianjin Sungene Biotech (China).

2.10. Immunofluorescence

HNEpCs were cultured in glass-bottomed dishes for confocal microscopy. After treatment with 20 μg/ml poly(I:C) or catechin for 24 h, the cells were washed twice in phosphate-buffered saline, fixed with 4% paraformaldehyde for 20 min, and treated with 3% Triton X-100 for 20 min. The sections were then treated with blocking solution (phosphate-buffered saline with 10% goat serum) for 30 min at room temperature and incubated overnight with rabbit anti-NF-κBp65 antibody (1:400 dilution in 2% bovine serum albumin) at 4 °C. Finally, the sections were incubated with secondary antibody (1:1000 dilution in blocking buffer) for 1 h, stained by 4',6-diamidino-2-phenylindole, mounted, and examined under a confocal microscope.

2.11. Statistical analysis

The experimental data between two groups were analyzed using Student's *t*-tests, and data among several groups were analyzed by one-factor ANOVAs in SPSS (version 17.0). A *p*-value of ≤0.05 was considered significantly different.

3. Results

3.1. Catechin remits allergic symptoms and nasal inflammation in allergic rhinitis mice

To assess the effect of catechin administration on nasal symptoms of allergic rhinitis, the frequency of sneezing and nasal rubbing occurrences was counted for 10 min after ovalbumin sensitization. The mice were treated with catechin at doses of 75, 150, or 300 mg/kg before ovalbumin challenge. Catechin treatment clearly decreased the frequency of occurrences of sneezing (Fig. 3A) and nasal rubbing (Fig. 3B) as well as the level of ovalbumin-specific immunoglobulin-E in the serum of allergic rhinitis model mice (Fig. 3C). Moreover, histology staining revealed that catechin treatment also lessened infiltrating eosinophils (Fig. 3D) and inflammatory condition (Fig. 3E) in the nasal mucosa.

3.2. Catechin restores the balance between T helper type 1 and T helper type 2 cells in allergic rhinitis mice

Allergic rhinitis is accompanied by an increase in T helper type 2 inflammatory factors. Our enzyme-linked immunosorbent assay results show that catechin treatment decreased the levels of interleukin-5 (Fig. 4A) and interleukin-13 (Fig. 4B) in the serum of allergic rhinitis model mice. To further confirm the anti-inflammatory effect of catechin, flow cytometry was performed to assess the expression of target intracellular factors for T helper type 1 or T helper type 2 cells. We found that the ratio of T helper type 1 to T helper type 2 cells in the catechin treatment group was higher than that in the model group (Fig. 4C).

3.3. Catechin reduces the expression of NF-κBp65 and TSLP proteins in the nasal mucosa

TSLP is a main factor that induces T helper type 2 inflammation. To

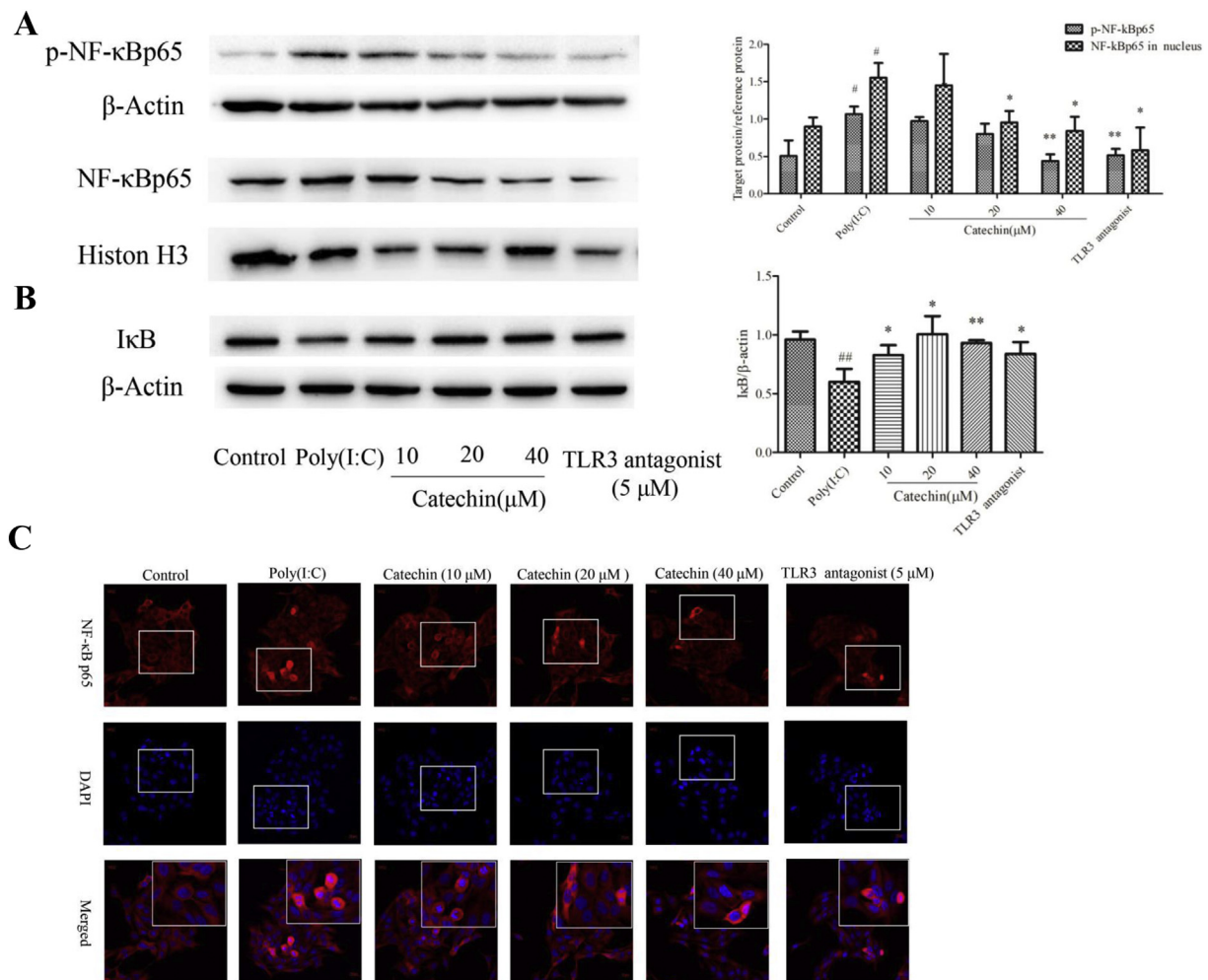


Fig. 7. The effect of catechin on NF-κBp65 activation

HNEpCs were treated with catechin or control. A–B: The expression of phospho-NF-κBp65 and NF-κBp65 (A) and of IκB (B) in HNEpCs as assessed by western blot. C: Representative immunofluorescence images of the localization of NF-κBp65 (200×). ##*p* < 0.01 vs. control; **p* < 0.05, ***p* < 0.01 vs. poly(I:C) group.

confirm the anti-inflammation mechanism of catechin in our allergic rhinitis model mice, we used immunohistochemistry to detect the expression of TSLP and its upstream regulator, NF-κBp65, in the nasal mucosa. The results show that catechin effectively reduced the expression levels of NF-κBp65 (Fig. 5A) and TSLP (Fig. 5B) proteins in the nasal mucosa.

3.4. Catechin inhibits poly(I:C)-induced TSLP expression in vitro

For in vitro studies investigating the mechanism responsible for the anti-inflammatory effects of catechin, we stimulated HNEpCs with the toll-like receptor agonist poly(I:C) to induce thymic stromal lymphopoietin production. First, we assessed the cytotoxicity of catechin to HNEpCs by performing MTT assays. The results confirmed that treatment with catechin at concentrations of 10, 20, or 40 μM did not affect the cell viability of HNEpCs (Fig. 6A). Thus, catechin was used at concentrations of 10, 20, and 40 μM during the follow-up experiments. After incubation with catechin (10, 20, or 40 μM) or toll-like receptor-3 antagonist (5 M) for 1 h, the HNEpCs were stimulated with 20 μg of poly(I:C). Our results show that poly(I:C) stimulated the HNEpCs to release TSLP in vitro (Fig. 6B) and that catechin reduced the TSLP level in cellular supernatants (Fig. 6C).

3.5. Catechin inhibits the poly(I:C)-induced activation of NF-κBp65 signaling

To detect the expression of phospho-NF-κBp65, NF-κBp65, and IκBα (an inhibitor of NF-κBp65) by poly(I:C)-stimulated HNEpCs, we performed western blots. Our results indicate that catechin reduced the level of poly(I:C)-induced phospho-NF-κBp65 in the cytoplasm (Fig. 7A). At the same time, the IκBα level in the cytoplasm, which was reduced by poly(I:C) treatment, was recovered after catechin treatment (Fig. 7B). To further confirm the inhibiting effect of catechin on NF-κBp65 signaling, we performed immunofluorescence assays so we could directly observe the influence of catechin on NF-κBp65 nuclear translocation. As shown in the resulting images, catechin treatment prevented the NF-κBp65 protein from translocating into the nucleus (Fig. 7C).

4. Discussion

Allergic rhinitis is an immediate type I allergic inflammatory response to inhaled allergens. Immune reactions participate in this condition include the recruitment of inflammatory cells to the nasal mucosa as well as the production and release of inflammatory mediators, such as thymic stromal lymphopoietin [18]. TSLP is a protein belonging to the cytokine family [19] and is produced mainly by non-hematopoietic cells, such as fibroblasts, epithelial cells [20]. These cells are

located in regions where TSLP activity is required. Both allergic patients and allergen-induced animal models have been reported to express high levels of TSLP in the nasal mucosal or airway epithelial cells [21, 22].

In allergic inflammation, TSLP is known to play an important role in the maturation of T cell populations through the activation of antigen-presenting cells [23]. Specifically, TSLP from epithelial cells can promote dendritic cells to express CD11c⁺, which is a marker of mature dendritic cells. Because mature dendritic cells promote CD4⁺ T cells transform into T helper type 2 cells, but not T helper type 1 cells [24], excessive amounts of TSLP leading to an unbalance between the T helper type 2 and T helper type 1 cells. T helper type 2 inflammatory factors [25], such as interleukin-4 and interleukin-5, are well known to play a significant role in allergic inflammation. For example, interleukin-4 is associated with production of immunoglobulin-E antibody, and interleukin-5 can induce eosinophils to migrate to diseased region [26, 27].

Our results have shown that catechin treatment could abate the nasal inflammation and allergic symptoms, like sneezing and nasal rubbing, in allergic rhinitis model mice. Moreover, we also found that the high serum levels of T helper type 2 inflammatory factors, such as interleukin-5 and interleukin-13, and the unbalance between the T helper type 2 and T helper type 1 cells in allergic rhinitis model mice were restored to normal after catechin treatment, which may be associated with the observed decrease in the TSLP expression in nasal mucosa epithelial tissue.

The initial findings of this study confirm the reported anti-inflammation and anti-thymic stromal lymphopoietin effects of catechin in allergic rhinitis model mice. As such, the rest of this study aimed to determine a potential mechanism for this therapeutic effect. Previous work indicated that the production of TSLP in epithelial cells may be related to the NF- κ B signal [28]. Notably, NF- κ B-binding sites were identified in the promoters of both human and mouse TSLP genes. Additionally, pro-inflammatory mediators, such as interleukin-1 β , tumor necrosis factor- α , and allergens, could also stimulate epithelial cells to express thymic stromal lymphopoietin in a NF- κ B-dependent manner [12]. Toll-like receptor signaling pathways are well-known to activate downstream NF- κ B, so it is not surprising that various toll-like receptor agonists, as well as infections and allergens, are able to stimulate thymic stromal lymphopoietin expression in epithelial cells. For example, poly(I:C) can induce human nasal epithelial cells to produce thymic stromal lymphopoietin factor through the excitation of NF- κ B, but not IF3 [13]. Furthermore, the deletion of I κ B kinase- β or an intervention with NF- κ B signal inhibitor decreases the epithelial cell expression of TSLP [29]. Studies also have shown that catechin can alleviate liver damage in rats by inhibiting the NF- κ B signaling pathway [30].

In this study, we assessed the NF- κ Bp65 protein expression in the mouse nasal mucosa and found that catechin treatment inhibited the expression of NF- κ Bp65. To further investigate this pathway, we created an in vitro model by using poly(I:C) to induce TSLP release in a human nasal epithelial cell line and used it to examine the effect of catechin on the activation of the NF- κ B signaling pathway, the degradation of I κ B α , and the nuclear translocation of NF- κ Bp65 [31]. The results of these experiments show that catechin not only lowers the level of TSLP induced by poly(I:C), it also inhibits the activation of phospho-NF- κ Bp65 and the nuclear translocation of NF- κ Bp65 and increases the level of I κ B α protein.

5. Conclusion

Our results revealed that catechin may be effective against allergic rhinitis inflammation. The likely mechanism for this is that catechin decreases the expression of TSLP in nasal epithelial cells by inhibiting the NF- κ B/thymic stromal lymphopoietin signaling pathway.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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