

ORIGINAL ARTICLE

Ameliorative Effect of Thymoquinone on Ovalbumin-induced Allergic Conjunctivitis in Balb/c Mice

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ABSTRACT

Objective: Thymoquinone (TQ) is an active and potent compound in the oil of *Nigella sativa*, which has anti-inflammatory properties. The aim of this study was to evaluate the effects of TQ on ovalbumin (OVA)-induced allergic conjunctivitis (AC) in Balb/c.

Methods: The mice were divided into seven experimental groups; PBS Con, OVA Con, Conj, 0.05%TQ, 0.1%TQ, 0.5%TQ and Dex group. The mice were immunized and exposed to OVA, and eyes were treated with TQ and dexamethasone. Ocular symptoms were observed after last exposure to OVA. Eosinophils count in blood and Ophthalmic lavage fluid (OLF), recruitment of inflammatory cells in conjunctiva, serum IgE and OVA-specific IgE were evaluated by Giemsa and HE staining, and Enzyme-Linked Immunosorbent Assay (ELISA) respectively. High Performance Liquid Chromatography (HPLC) was used to determine the histamine level in OLF. The mRNA expression and protein level of cytokines were examined by real time RT-PCR and ELISA respectively.

Results: Ocular symptoms of AC and other characteristics of allergic inflammation including IgE and OVA-specific immunoglobulin E (IgE) level, recruitment of eosinophils, histamine level, mRNA expressions and protein level of cytokines were remarkably increased in OVA-exposed mice compared with the control groups. Administration of TQ suppressed the ocular symptoms, inflammatory cell infiltration in conjunctiva, blood and OLF, increased level of serum IgE and OVA-specific IgE, and OLF histamine level in OVA-exposed mice. Furthermore, TQ abrogated the mRNA expression and serum level of interleukin including IL-4, IL-5, IL-13 and transforming growth factor beta (TGF- β) in mice immunized and exposed to OVA.

Conclusion: Administration of TQ significantly reduced the ocular symptoms in AC by attenuating the recruitment of eosinophils, level of IgE, histamine and cytokines. Our findings suggest that TQ might be a useful intimation for the treatment and future research for AC.

Keywords: Allergic conjunctivitis, Eosinophils, Interleukins, Histamine, Thymoquinone

INTRODUCTION

Among intensively researched plants, *Nigella sativa* (*N. Sativa*) is one of the medically promising plants commonly known as black cumin. Both seeds and oil of black cumin have been used in herbal medicine by various cultures and civilizations to treat and prevent a number of diseases, and as a supplemental nutrition to

maintain good health.¹ Thymoquinone (TQ) is a major active compound of *N. Sativa* oil which has anti-oxidant, anti-infective, anti-tumor and anti-inflammatory properties. A recent study showed that *N. Sativa* oil, with its active principal TQ, significantly diminished the allergic symptoms in patients with allergic rhinitis, atopic eczema, and bronchial asthma.^{2,3} Allergic conjunctivitis (AC) is characterized by antigen-specific

IgE in the serum, and eosinophils infiltration into the conjunctiva together with typical clinical signs and symptoms such as formation of papillae and itching, redness, tearing, chemosis, and eyelid swelling.⁴ Most of the AC cases are seasonal or perennial. Seasonal allergies are brought about by environmental triggers such as pollen, grasses, or ragweed, while perennial allergies are caused by dust, molds, or animal dander. Both seasonal and perennial AC are type I hypersensitivity allergic reaction.⁵ AC is mediated by the activation of mast cells by an allergen. Further, mast cells release chemical mediators such as histamine, prostaglandins and leukotrienes. It is known that histamine is synthesized from L-histidine by histidine decarboxylation in specific cell types, such as mast cells, basophils, enterochromaffin-like cells, and neurons. Histamine is known to be one of the major mediators of acute allergic reactions including allergic conjunctivitis.^{6,7} Infiltration of eosinophils into the conjunctiva is mediated by various chemokines, such as eotaxins which are also regulated by Th2 cells.⁸ Several studies have described that Th2 cells play a major role in the initiation and maintenance of allergic reaction through IgE, eosinophils and cytokines (IL-4, IL-5, and IL-13) production.⁹ IL-4 is critical for the initiation of allergic disease by driving differentiation of uncommitted Th cells toward a Th2 phenotype and inhibiting Th1 differentiation.¹⁰ Millions of allergic patients are trying to find suitable therapies for allergies, and over half of these patients present with ocular symptoms.¹¹ Depending on the severity of these symptoms, patients with ocular allergies experience discomfort that may affect their quality of life significantly.¹²

Previously it was shown that the ocular administration of TQ had therapeutic potential in the treatment of corneal neovascularization and other allergic disease.¹³ TQ inhibited the TNF- α -mediated activation of NF- κ B in pancreatic ductal adenocarcinoma (PDA) cells¹⁴ and eosinophils infiltration.¹⁵ Here, we hypothesized that TQ might be able to attenuate the AC in mice. Therefore, we used ovalbumin (OVA) induced AC Balb/c mice to investigate the effects of TQ.

MATERIALS AND METHODS

Animals

Balb/c mice were obtained from the animal center, College of Medicine, Xi'an Jiaotong University, China. Male mice (20–25 g) were kept in the animal house in a pathogen-free environment with a controlled temperature ($24 \pm 2^\circ\text{C}$) and humidity ($52 \pm 10\%$), and continuous air renovation. OVA-free food and water were provided to the mice. All experiments were approved by the Institutional Animal Ethics Committee of Xi'an Jiaotong University.

Sensitization and Challenge with Ovalbumin

The animals (Balb/c mice) were randomly allocated into seven groups; PBS Con, OVA Con, Conj, TQ 0.05%, TQ 0.1%, TQ 0.5% and Dex group. Each group contained 10 animals. Mice were sensitized by intraperitoneal injection of 1 mg OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 50 mg aluminum hydroxide (AlumImject; Pierce Biotechnology, Rockford, IL, USA) in 1 ml phosphate buffered saline (PBS) at day 0 as well as day 7. One week after the second immunization, OVA solution (10 mg/ml) was instilled into the eyes of the conj, TQ, and Dex groups twice daily for 7 days to cause antigen-induced conjunctivitis. The animals in the PBS Con group were sensitized and exposed to PBS while the mice in the OVA Con group were immunized with OVA and their eyes were exposed to PBS.

Treatment with Thymoquinone and Dexamethasone

TQ powder (2-isopropyl-5-methylbenzoquinone) was purchased from Sigma-Aldrich (St. Louis, MO) and was suspended in 0.8% tween 80 with three different doses: 0.05%, 0.1% and 0.5%. A pilot experiment was performed in mice to check the deleterious effect of different doses of TQ on the lacrimal drainage system and did not find any adverse effect. Tween 80 solution (0.8%) served as a Control. After 1 h of OVA exposure, the eyes of the animals in the 0.05%, TQ 0.1%, TQ 0.5% and Dex groups were treated with different concentrations of Thymoquinone (0.05%, 0.1% and 0.5%) and 0.1% dexamethasone respectively, once daily for 7 days preceding the second OVA instillation. Mice from PBS Con, OVA Con and Conj group were treated with Tween 80 solution. Twelve hours after the final exposure of OVA, the eyeballs were removed from the mice of all groups, after euthanasia. Blood was taken from the eyes and the serum was separated out. Histological examination was performed using one eye from each animal, and other examinations were done by using the fellow eye.

Evaluation of Clinical Symptoms

Clinical symptoms including swelling, hyperemia, and discharge were observed after the last exposure of OVA. Ocular symptoms were evaluated: swelling in the palpebra, hyperemia in the bulbar conjunctiva, and discharge from the eyes. Each symptom was scored as follows: 0, no change; 1, slight change; 2, moderate change; 3, severe change by a well-trained observer to minimize the variation in score.

Determination of IgE and OVA-specific IgE

Enzyme-linked immunosorbent assay (ELISA) was used to determine the level of immunoglobulin E (IgE) and OVA-specific IgE in the serum of experimental mice. Serum was obtained from mice 12 hours after the last treatment on day 22. The analyses of OVA-specific IgE (Pharmingen, San Diego, CA, USA) were carried out as described,¹⁶ using rat anti-mouse IgE capture monoclonal antibody (Pharmingen). Using a standard curve for mouse anti-OVA IgE (AbD Serotec, Oxford, UK), we calculated IgE and OVA-specific IgE concentrations (ug/ml). Data for OVA-specific serum IgE are presented as titer values derived from analysis of OD values vs. factors of serum dilution series with a logarithmic curve fitting model.

Histological Examination

Eyes along with conjunctival tissue were infused with 10% neutral buffered formalin and subjected to hematoxylin and eosin (HE). Tissues were embedded in paraffin, and cut into 5-mm thick sections. The sections were stained with hematoxylin and eosin. Infiltrations of inflammatory cells were evaluated on a graded scale: 0, none or a few inflammatory cells observed in the conjunctiva; 1, some inflammatory cells observed in the conjunctival epithelium and lamina propria; 2, inflammatory cells infiltrating the posterior region along the sclera from the conjunctival region; 3, more inflammatory cells infiltrating than score 2. The number of eosinophils in the blood and ophthalmic lavage fluid (OLF) were evaluated by Giemsa staining.

Histamine Level

OLF was collected after the last OVA exposure. Physiologic saline (10 µl) was applied to the eye using a micropipette without touching the eye. After 2 or 3 forced blinks, the OLF was collected. The lavage was repeated 5 times in each eye. OLF was centrifuged at 1000 rpm for 10 min and the supernatant was separated. The level of histamine was determined in the supernatant of OLF by high performance liquid chromatography (HPLC).¹⁷

Extraction of RNA and Real-Time PCR

Total RNA from conjunctiva tissue was isolated by using TRIZOL and quantified by measuring the optical density (CECIL Instrument, England). According to the manufacturer's protocol (RevertAid™, Fermentas Life Sciences, International INC, Canada), total cellular RNA was reverse transcribed by reverse transcription (RT). Resulting cDNA was amplified by real-time PCR

with SYRB Premix Ex Tag II (TaKaRa, Japan). By using the primers for the genes such as IL-4, IL-5, IL-13 and TGF-β real-time RT-PCR was performed at 95°C for 10 sec, 62°C for 15 sec, and 72°C for 30 sec. β-actin was used as a reference gene.

Level of Cytokines

The sera of experimental animals were screened for the presence of cytokines such as IL-4, IL-5 and IL-13 by ELISA with commercially available kits purchased from Endogen, MA, following the instructions of the manufacturer. Color reaction was measured as OD units at 450 nm on an ELISA plate reader (Thermo Electron Corporation, Finland). The concentration of cytokines was expressed in ng/ml, by kit's standard curve.

Statistical Analysis

All data were expressed as means ± SEM. We analyzed the data by using one way ANOVA followed by Bonferroni's post test. Graph pad Prism5 Demo analyzing software was used to determine the significant differences among groups. Differences were considered statistically significant when the *p*-value was less than 0.05.

RESULTS

TQ Inhibits the Ocular Symptoms in the Allergic Conjunctivitis Mice

The effect of TQ on ocular symptoms in mice was evaluated after last exposure to OVA (Figure 1). Scores for swelling, discharge and hyperemia in the AC mice were significantly suppressed after the ocular administration of TQ. The effect of TQ gradually enhanced with increasing concentration. The 0.5% TQ showed more convincing tendency to decrease the ocular symptoms in AC than 0.1% and 0.05% TQ. On the other hand, dexamethasone obviously attenuated the swelling, hyperemia and discharge symptoms. No significant difference was observed among the groups in the experimental mice for Edema (data not shown). The tendency of 0.5% TQ to decrease the AC symptoms was almost the same as dexamethasone.

TQ Suppressed the Total IgE, OVA-specific IgE and Recruitment of Inflammatory Cells

The efficacy of TQ on the level of IgE and OVA-specific IgE was assayed by using ELISA (Figure 2). Recruitment of inflammatory cells in conjunctiva, and eosinophils in blood and OLF were evaluated by HE and Giemsa staining respectively (Figure 3). The level of total IgE and OVA-specific IgE in the serum,

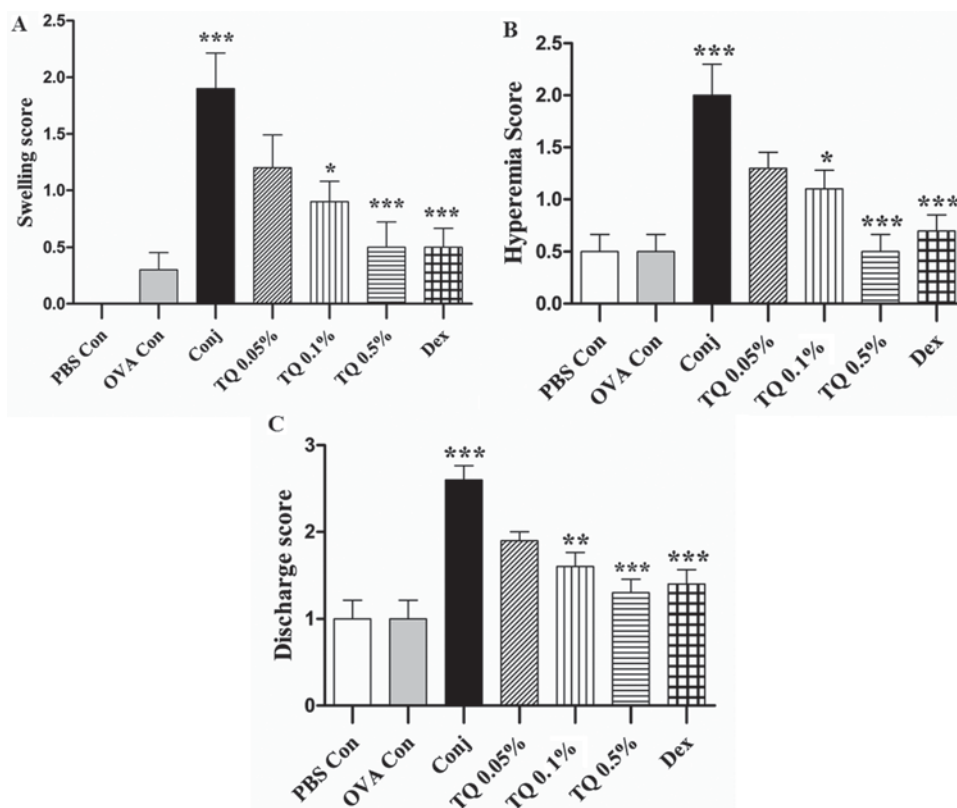


FIGURE 1 Effect of TQ on ocular symptoms of AC. (A) Swelling score, (B) Hyperemia score, and (C) Discharge score. AC was induced in mice by OVA exposure. Ocular symptoms were observed in mice after 12h of last OVA exposure. Data are represented as mean $\pm$ SEM. * p < 0.05, ** p < 0.01 and *** p < 0.001 show the difference between the two groups.

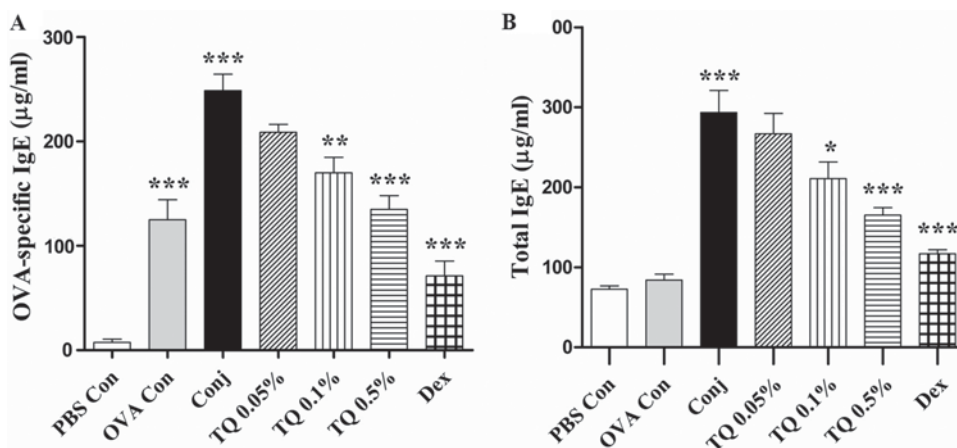


FIGURE 2 Effect of TQ on the total IgE and OVA-specific IgE in serum. (A) Represents total IgE level while (B) represents OVA-specific IgE level in serum. Data are represented as mean $\pm$ SEM for 10 mice in each group. * p < 0.05, ** p < 0.01 and *** p < 0.001 compare the differences between the two groups.

inflammatory cell infiltration in the conjunctiva, count of eosinophils in the blood and eosinophils in OLF were attenuated after the ocular administration of TQ in the mice immunized and challenged with OVA as compared with mice in the conjunctivitis group (Conj group). The treatment tendency of TQ was enhanced gradually by increasing the concentration. The effect of 0.5%TQ on the level of IgE, OVA-specific IgE and eosinophils count was similar with the treatment of dexamethasone.

TQ Attenuated the Level of Histamine in Ophthalmic Lavage Fluid (OLF)

The HPLC method was used to determine the level of histamine in OLF of mice (Figure 4). The level of histamine was enhanced significantly in the mice sensitized and exposed to OVA as compared to mice in the PBS control and OVA control groups. The decline of histamine level was increased gradually by increasing the concentration of TQ.

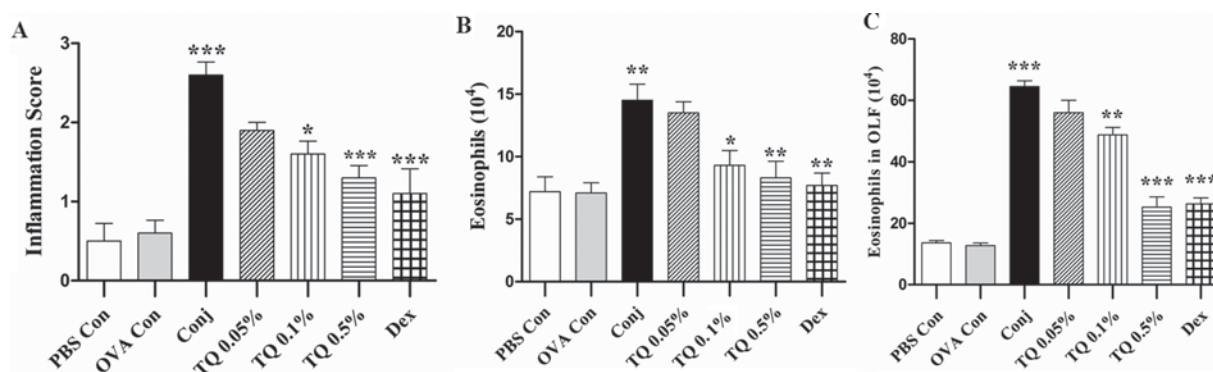


FIGURE 3 Effect of TQ on inflammatory cell infiltration in blood and OLF. (A) Inflammatory cells in conjunctiva, (B) Count of eosinophils in blood and (C) Count of eosinophils in OLF. Data are represented as mean $\pm$ SEM for 10 mice in each group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compare the differences between the two groups.

TQ Decreased the mRNA Expression and the Level of Cytokines

To determine the molecular basis of AC in OVA-exposed mice, we examined the mRNA level of cytokines by real-time PCR (Figure 5). The mRNA showed low levels of IL-4, IL-13, IL-5 and TGF- β . However, after sensitization and exposure with OVA, mRNA expression of IL-4, IL-13, IL-5 and TGF- β were greatly induced. By comparison, ocular treatment with the TQ had markedly decreased the OVA-induced mRNA expression of cytokines. The level of cytokines in the sera of experimental mice was determined by ELISA (Figure 6). The increased level of IL-4, IL-13 and IL-5 was significantly diminished in the mice exposed to OVA and treated with TQ, while the level of TGF- β and IFN- γ did not show any significant change (data not shown). The effect of TQ increased gradually with concentration, while 0.5% of TQ showed the same tendency to decrease the level and mRNA expression of cytokines as the efficacy of dexamethasone.

DISCUSSION

We have discovered in our study that TQ significantly attenuates AC induced by the exposure of OVA in Balb/c mice. We also have shown the potential TQ effects which abate allergen-induced clinical symptoms through suppressing IgE, histamine and cytokine levels.

Mast cells degranulate and release vasoactive amines such as histamine or serotonin after the exposure of allergen to the eye, provoking itching, eyelid swelling, conjunctival swelling, and mucus secretion. It is well known that both seasonal and perennial AC is type I hypersensitive allergic reaction together with clinical signs and symptoms.⁶ Thus, exacerbation of the symptom scores may reflect the phase of an allergic reaction both in human and animal models.^{5,18} Ocular symptoms such as swelling, hyperemia and discharge were found in mice after allergen exposure

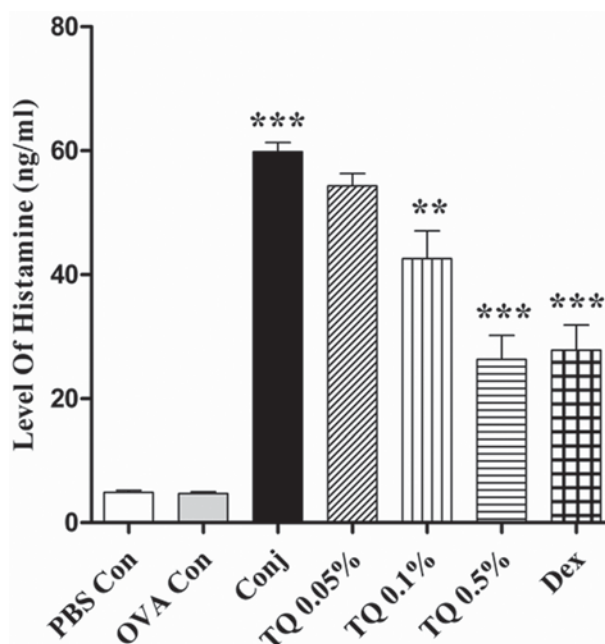


FIGURE 4 Effect of TQ on histamine level in OLF. Histamine level was determined by HPLC in the mice. Data are represented as mean $\pm$ SEM for 10 mice in each group. ** $p < 0.01$ and *** $p < 0.001$ show the differences between the two groups.

compared with the control group. TQ suppressed the ocular symptoms significantly in the mice with AC disease. Furthermore, repeated instillation of TQ did not show any deleterious effects on the lacrimal drainage system in mice. Edema was not exhibited in mice after immunization and exposure to OVA (data not shown). In terms of etiologic factors, increased levels of IgE and cytokines released from infiltrated lymphocytes play a pivotal role in AC.^{19,20} High levels of IgE have been detected in the patients with vernal Keratoconjunctivitis (VKC), while mild levels of IgE were found in the tears of patients with perennial AC.^{20,21} Mimura et al.²³ found a positive correlation between tear and serum IgE in the patients with AC. Increased level of serum IgE was also determined in other allergic disorders such as bronchial asthma and atopic dermatitis.^{22–24} IgE presents binding sites for

antigen and the surface of basophils and mast cells. This cross-linking of the IgE surface to antigen on mast cells promotes the production of mediators.²⁵ We found that the level of total IgE and OVA-specific IgE

was reduced significantly in the mice exposed to OVA on ocular administration of TQ.

Activated mast cells released histamine in AC disease and other allergic diseases that recruits eosinophils,

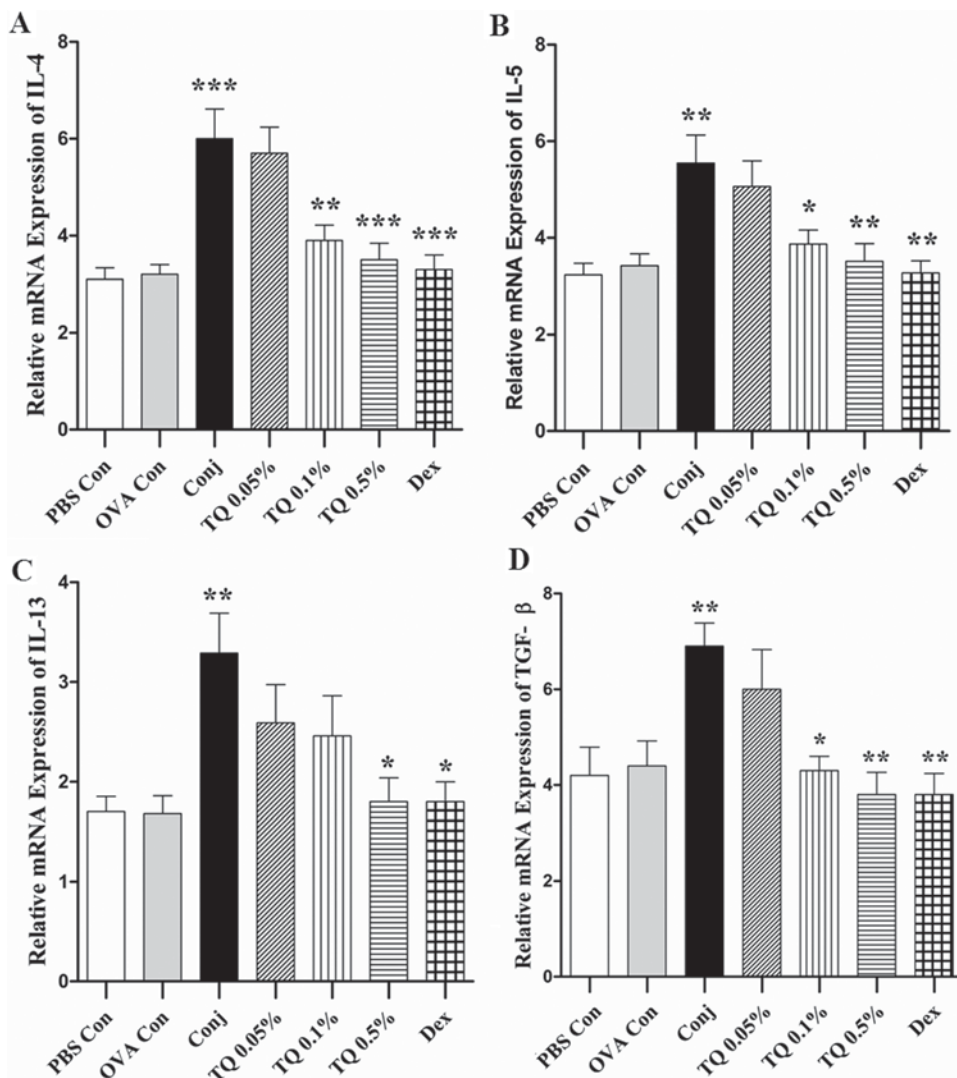


FIGURE 5 Effect of TQ on the mRNA expression of cytokines. (A) Relative mRNA expression of IL-4, (B) Relative mRNA expression of IL-5, (C) Relative mRNA expression of IL-13, and (D) Relative mRNA expression of TGF- β . Data are represented as mean $\pm$ SEM for 10 mice in each group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compare the differences between the two groups.

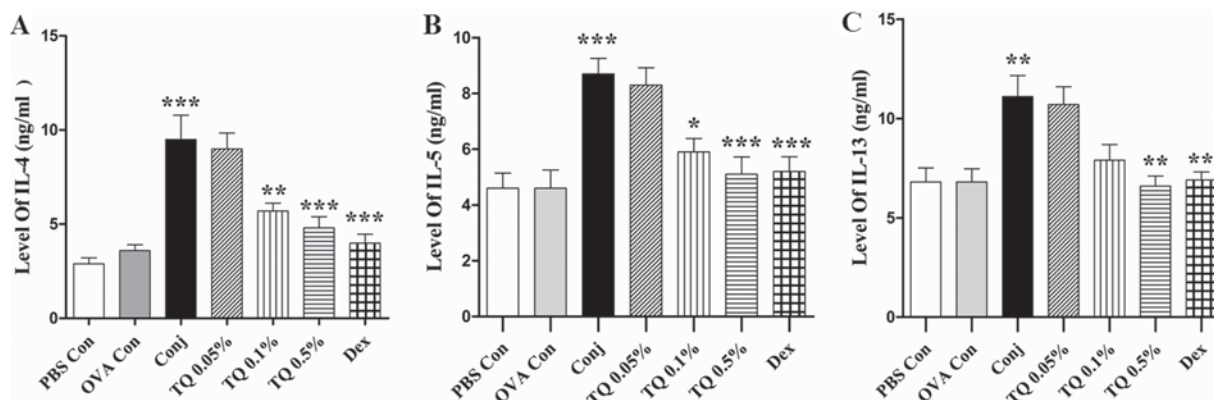


FIGURE 6 Effect of TQ on the level of cytokines in sera. (A) Level of IL-4, (B) level of IL-5, and (C) level of IL-13. Data are represented as mean $\pm$ SEM for 10 mice in each group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compare the differences between the two groups.

neutrophils and lymphocytes to the ocular surface.^{5, 26} The eosinophils are important in pathophysiology and impairment of the ocular surface due to eosinophil-derived cytotoxic proteins such as eosinophils cationic protein, major basic protein, and eosinophil peroxidase (EPO). We found that eosinophils in the blood and the eye tissues were increased significantly in the mice exposed to OVA compared with the control groups. The recruitment of eosinophils was found in the biopsy of conjunctiva and the tear fluid of patients with allergic diseases, especially SAC, PAC, or VKC.^{15,27,28} In the present study, level of histamine in tear, and eosinophils infiltration in OLF, conjunctiva and blood decreased significantly after the ocular administration of TQ in the mice immunized and exposed with OVA compared with mice in the Conj group.

An altered balance between Th1 and Th2 cells and their cytokines are thought to be responsible for the development of allergic disorders including AC. The regulation of Th2 cytokines production is known to be involved in allergic pathophysiology, and allergic immune and inflammatory responses.²⁹ IL-4 and IL-5 are essential for IgE and eosinophil production respectively,³⁰ while IL-13 and TGF- β regulate the pathogenesis of allergic illnesses.^{20,31} In this study, Th2 cytokines both in serum and tissues increased in the mice exposed to allergen compared with Control. The ocular administration of TQ suppressed the mRNA expression of IL-4, IL-5, IL-13 and TGF- β in the homogenate of eye tissues. Furthermore, increased levels of IL-4, IL-5 and IL-13 in the serum of mice exposed to OVA were diminished after the ocular instillation of TQ. However, during the active inflammatory phase of the disease multiple Th1 and Th2 cytokines are over expressed and produce typical Th1 cytokine, interferon (IFN)- γ , which probably contribute to increasing ocular inflammation similar to what has been shown in animal models.²⁹ In the present study we found that both mRNA expression and protein level of FN- γ had no significant differences between the Conj and control groups (data not shown). We found that the ameliorative effect TQ had on OVA-induced allergic conjunctivitis increased with increasing concentration and 0.05% of TQ had the same tendency to suppress the cytokines as Dexamethasone.

Currently, corticosteroids and mast cell stabilizers are being used for the management of AC, but unfortunately neither of them is sufficiently effective without leading to unpleasant side effects. Thus, many kinds of immunomodulatory and anti-mediator therapies have been investigated, but none of them has proved successful for AC. Here we conclude that the ocular instillation of TQ decreased the ocular symptoms of AC by attenuating eosinophil infiltration, level of histamine in tear fluid and cytokine level in tissues and serum of mice immunized and exposed to OVA. In this study we evaluated that TQ could reduce the symptoms of allergy in BALB/c mice, but whether TQ affected them

locally or systemically was not evaluated to the full extent. However the decreased IgE and cytokines level in the serum of mice after treatment with TQ suggest that it also has systemic effects. Nevertheless, further study is required to explore the effects of TQ on IgE production after allergen exposure in allergic conjunctivitis both *in vivo* and *in vitro* which might potentially prove useful for the treatment of AC in humans.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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