• Basic Research •

Chinese medicine formula "Qingxuan Runmu Yin" alleviating ocular surface inflammation in a rat model of dry eye disease by modulating the TLR4/TAK1/p38MAPK pathway

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Abstract

● **AIM:** To investigate the effects of a Chinese medicine formula "Qingxuan Runmu Yin" (QRY) on ocular surface inflammation in a rat model of dry eye, and its mechanism *via* the toll-like receptor 4 (TLR4)/transforming growth factor kinase 1 (TAK1)/p38 mitogen-activated protein kinase (p38MAPK) signaling pathway.

● **METHODS:** Seventy-two Sprague-Dawley rats were randomly divided into six groups (n=12 each): the control group, model group, 3 groups of QRY (with low-, medium-, and high-doses), and SB203580 group. Dry eye was induced using benzalkonium chloride. Schirmer's test (SIT) and corneal fluorescein staining (CFS) were performed every 14d throughout the experiment. Histopathological changes in corneal and conjunctival tissues were observed using hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining. Protein expression levels of key inflammatory markers and signaling pathway targets were assessed *via* immunohistochemistry, ELISA, and Western blotting.

● **RESULTS:** Compared to the control group, the model group showed significant reductions in SIT and increases in CFS scores, alongside structural disorganization of corneal/conjunctival tissues, decreased conjunctival goblet cell (CGC) numbers, and elevated expression of inflammatory markers [interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α), matrix metalloproteinase-9 (MMP9)] and pathway proteins (TLR4, p-TAK1, p-p38MAPK; P<0.05). Treatment with QRY (low, medium, and high doses) and SB203580 significantly improved SIT scores, reduced CFS scores, restored corneoconjunctival structure, increased CGC numbers, and decreased expression levels of IL-1β, IL-6, TNF-α, MMP9, TLR4, p-TAK1, and p-p38MAPK proteins compared to the model group (P<0.05).

● **CONCLUSION:** QRY may alleviate ocular surface inflammation associated with dry eye by inhibiting the TLR4/TAK1/p38MAPK signaling pathway, highlighting its potential therapeutic efficacy for dry eye.

● **KEYWORDS:** Chinese medicine formula "Qingxuan Runmu Yin"; dry eye; ocular surface inflammation; TLR4/TAK1/p38MAPK signaling pathway
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INTRODUCTION

Dry eye disease (DED) is one of the most prevalent ophthalmic disorders, affecting 5% to 50% of the global population[1]. DED manifests with symptoms such as ocular surface dryness, pain, itching, and burning, often impairing visual acuity and significantly diminishing patients' quality of life and psychological well-being[2-3].

The pathogenesis of DED is multifaceted, with tear film instability as its central mechanism. This instability leads to increased tear osmolarity, which triggers immunoinflammatory damage to ocular surface tissues. Immunoinflammation acts both as a cause and a consequence of ocular surface damage, creating a self-perpetuating cycle that exacerbates the condition[4].

Among the various inflammatory pathways implicated in DED, the toll-like receptor 4 (TLR4)/transforming growth factor-β-activated kinase 1 (TAK1)/p38 mitogen-activated

protein kinase (MAPK) signaling pathway is particularly critical. Environmental imbalances in the ocular surface activate the TLR4 pathway, inducing the secretion of inflammatory mediators[5] and triggering neuroinflammation and neuropathic pain[6]. p38MAPK, a key member of the MAPK family, mediates inflammation, proliferation, and apoptosis, while TAK1 acts as an upstream regulator linking TLR4 to downstream MAPK signaling[7-8].

The p38MAPK inhibitor SB203580 has shown significant anti-inflammatory effects in experimental models[9-10], effectively suppressing hyperosmolarity-induced elevation of inflammatory factors in DED[11]. At the same time, traditional Chinese medicine (TCM) has gained attention as an alternative therapeutic strategy due to its efficacy and minimal side effects[12]. Qingxuan Runmu Yin (QRY), a TCM formulation derived from the classical prescription "Humor-Increasing Decoction", has been clinically effective in alleviating DED symptoms[13]. While QRY's anti-inflammatory and tear film-stabilizing effects have been demonstrated in both clinical and experimental studies[14-15], its precise mechanisms of action remain incompletely understood.

This study investigates the hypothesis that QRY mitigates ocular surface inflammation in DED by inhibiting the TLR4/TAK1/p38MAPK signaling pathway. Using a benzalkonium chloride (BAC)-induced rat model of DED[16], we examined the effects of QRY on clinical symptoms, histopathological changes, inflammatory cytokine expression, and pathway-specific protein activity, aiming to elucidate its mechanisms of action and therapeutic potential.

MATERIALS AND METHODS

Ethical Approval All animal care and experimental procedures were approved by the Laboratory Animal Ethics Committee of Heilongjiang University of Traditional Chinese Medicine (Ethics No.HLJUTCM2023090601; Approval date: 22/Sep/2023).

Animals Seventy-two specific-pathogen-free female Sprague-Dawley rats, aged 8wk and weighing 200±20 g, were procured from Liaoning Changsheng Biotechnology Co., Ltd. The animals were housed in an SPF-grade facility at Heilongjiang University of Traditional Chinese Medicine, maintained under controlled conditions (temperature: 25°C±2°C, humidity: 50%±10%, and a 12-hour light/dark cycle).

QRY Formula QRY was formulated using 10 g each of Baizhu (*Atractylodes macrocephala Koidz.*), Maidong [*Ophiopogon japonicus* (*L.f.*) *Ker-Gawl.*], Xuanshen (*Scrophularia ningpoensis Hemsl.*), Shengdihuang (*Rehmannia glutinosa Libosch*), Fangfeng [*Saposhnikovia divaricata* (*Turcz.*) *Schischk.*], Gancao (*Glycyrrhiza glabra L.*), Jinyinhua (*Lonicera japonica Thunb.*), Shihu (*Dendrobium nobile Lindl.*), Jiegeng [*Platycodon grandiflorum* (*Jacq.*) *A.DC.*], and Lianqiao [*Forsythia suspensa* (*Thunb.*) *Vahl*]. The herbal mixture was decocted by combining it with 1000 mL of distilled water in a clay pot; the mixture was initially brought to a vigorous boil and then simmered over low heat until the volume was reduced to approximately 400 mL. All herbal powders contained in QRY are purchased from Shundechang Chinese medicine Co.

Grouping and Treatment Seventy-two rats were randomly divided into the control group (12 rats) and the BAC group (60 rats) according to the random number table method. Both eyes of the control group were treated with 5 μL of phosphate buffer drops twice a day for 14 consecutive days, while both eyes of the BAC group received 5 μL of 0.2% BAC solution twice daily for 14 consecutive days. After the 14th day, the BAC group was randomly divided into the model group, QRY low-dose group, QRY medium-dose group, QRY high-dose group, and SB203580 group, with 12 animals in each group. The medium-dose group was designed to correspond to the standard clinical dose. Using the body surface area normalization method and considering the clinical dosage of QRY (200 mL administered twice daily for adult patients), the daily equivalent dose for rats was calculated as approximately 9 g/kg. Subsequently, low and high dose groups received half and double this calculated dose, respectively, to establish the experimental dose gradient. The QRY low, medium, and high dose groups were given QRY at concentrations of 4.5 g/kg·d, 9 g/kg·d, and 18 g/kg·d, respectively, twice daily for 14d; the control, model, and SB203580 groups were given an equal volume of saline by gavage; in addition, the SB203580 group was given the SB203580 inhibitor 10 mg/kg·d by intraperitoneal injection twice daily.

Dry Eye Symptom Indicators Staining Tear production was assessed on days 0, 14, and 28 using Schirmer's test (SIT) strips. The strips were placed in the lower conjunctival sac approximately 1/3 from the lateral canthus and left for 1min. The length of the wetted portion was then recorded.

On days 0, 14, and 28 of the experiment, rats in each group were tested for SIT and corneal fluorescein staining (CFS) in both eyes. SIT: Tear secretion test paper was placed under the conjunctival sac about 1/3 of the way from the outer canthus in the lower lid and left for 1min to measure the length of the discolored portion of the paper. CFS: Corneal staining with sodium fluorescein was recorded in the cobalt-blue light of a slit lamp and scored in quadrants (0 points for no staining; 1 point for ≤30 punctate stains; 2 points for punctate stains ≥30 but no lamellar stains; 3 points for lamellar stains).

Histology Staining Two rats (4 eyes) were taken from each group, eyeballs were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Sections were treated with xylene (10min, two washes), followed by graded ethanol and tap

water rinses. Hematoxylin and eosin (Wuhan Servicebio Technology Co., LTD, Wuhan, China) staining was performed, and histopathological changes were examined under a light microscope.

Conjunctival sections from paraffin-embedded tissues were stained with periodic acid-Schiff (PAS, Beijing Solarbio Science & Technology Co., LTD, Beijing, China) reagent to visualize goblet cells. Observations were made using a light microscope.

Immunohistochemistry After antigen retrieval, paraffin sections were incubated with TLR4 primary antibodies and corresponding secondary antibodies. The diaminobenzidine chromogenic solution (Wuhan Boster Biological Technology., LTD, Wuhan, China) was used to develop the signal, and nuclei were counterstained. Stained sections were visualized at 400× magnification, and positive staining areas were quantified using Image J software.

Enzyme-Linked Immunosorbent Assay Corneal tissues from three rats (6 eyes) per group were collected, and standards were prepared following the instructions for tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and matrix metalloproteinase-9 (MMP9) enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Jianglai Biotechnology Co., Ltd, Shanghai, China). Absorbance values at 450 nm were measured using a microplate reader, and results were calculated relative to standard curves.

Quantitative Real-Time Polymerase Chain Reaction Analysis Corneal tissues from three rats (6 eyes) per group were used for RNA extraction, which was reverse-transcribed into cDNA using a kit [Seven Innovation (Beijing) Biotechnology Co., Beijing, China]. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was conducted in triplicate using gene-specific primers (Table 1) and the $2^{-\Delta\Delta CT}$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control.

Western Blot Corneal tissues from four rats (8 eyes) per group were used for protein extraction, protein samples (30 μg each) were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% skim milk for 1h, incubated overnight at 4℃ with primary antibodies (Proteintech Group, Inc., Wuhan, China), and then incubated with secondary antibodies (Proteintech Group, Inc., Wuhan, China) for 2h. Chemiluminescence imaging analysis was used to detect protein bands, which were quantified with Image J software.

Statistical Analysis Data were analyzed using SPSS 25.0 software and reported as mean±standard deviation. One-way ANOVA was used for normally distributed data with homogenous variances, followed by Tukey's test for multiple comparisons. Nonparametric tests were employed for non-normal distributions or heterogeneous variances. Statistical significance was defined as $P<0.05$.

Table 1 qRT-PCR gene primer sequences

Name	Sequences
TNF-α F	CTCAAGCCCTGGTATGAGCC
TNF-α R	GGCTGGGTAGAGAACGGATG
IL-1β F	GAGTCTGCACAGTTCCCCAA
IL-1β R	TCCTGGGGAAGGCATTAGGA
IL-6 F	CCTACCCCAACTTCCAATGCT
IL-6 R	CATAGCACACTAGGTTTGCCG
MMP9 F	GCTCCTCTTTTGCTTCAGCG
MMP9 R	CAAGAAAGGACAGCGTGCAG

qRT-PCR: Quantitative reverse transcription polymerase chain reaction; TNF: Tumor necrosis factor; IL: Interleukin; MMP9: Matrix metalloproteinase 9.

RESULTS

QRY on Dry Eye Symptom Indicators in Rats Baseline measurements revealed no significant differences in tear secretion among the experimental groups. Fourteen days after BAC induction, tear secretion significantly decreased in all modeled groups compared to the control group ($P<0.01$), confirming successful establishment of the dry eye model. Following 14d of treatment, tear secretion significantly improved in the QRY medium- and high-dose groups, as well as in the SB203580 group, compared to the model group ($P<0.01$; Figure 1A).

Similarly, initial CFS scores showed no significant differences among the groups. After 14d of BAC exposure, CFS scores were significantly elevated in all modeled groups compared to the control group ($P<0.01$), further validating successful model establishment. Treatment with QRY and SB203580 for 14d reduced CFS scores in the QRY low-dose group ($P<0.05$) and significantly decreased scores in the medium- and high-dose QRY groups, as well as in the SB203580 group ($P<0.01$; Figure 1B and 1C).

Histopathologic Changes in the Cornea and Conjunctiva and Goblet Cell Counts Histological analysis showed that the control group exhibited normal corneal morphology with a smooth epithelial surface, organized squamous cells, and a uniform stromal layer. In contrast, the model group demonstrated severe epithelial loss, stromal edema, and disorganized squamous cells. QRY treatment (low, medium, and high doses) and SB203580 partially restored corneal epithelial structure and reduced stromal edema, with the high-dose QRY group showing the most significant improvement (Figure 2A).

In the conjunctiva, the control group displayed tightly packed epithelial cells and a smooth surface, whereas the model group exhibited disorganized cells, prominent edema, and abnormal morphology. QRY and SB203580 significantly improved

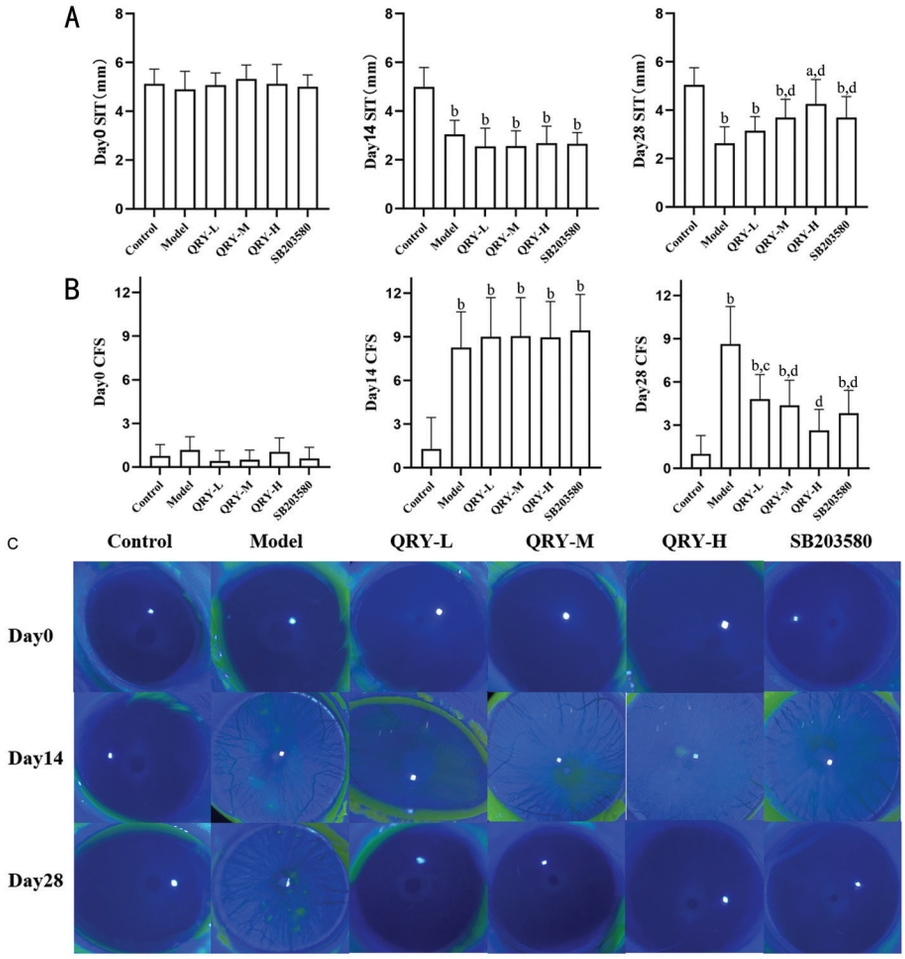

Figure 1 Changes on dry eye symptom indicators in rats A: Tear secretion test (SIT) results on days 0, 14, and 28; B: Changes in CFS scores on days 0, 14, and 28; C: Representative images of fluorescein staining from each group. [a]*P*<0.05, [b]*P*<0.01 *vs* control group; [c]*P*<0.05, [d]*P*<0.01 *vs* model group. QRY: Qingxuan Runmu Yin; QRY-L: QRY low-dose group; QRY-M: QRY medium-dose group; QRY-H: QRY high-dose group; CFS: Corneal fluorescein staining.

conjunctival structure and reduced edema, with the high-dose QRY group exhibiting the most pronounced recovery (Figure 2B). Additionally, goblet cell counts, which were markedly reduced in the model group, increased significantly following QRY and SB203580 treatment, particularly in the medium- and high-dose QRY groups (*P*<0.01; Figure 2C).

Immunohistochemistry The control group exhibited minimal TLR4 staining in corneal and conjunctival tissues, whereas the model group showed markedly increased positive staining (*P*<0.01). QRY and SB203580 treatment significantly reduced TLR4 expression, with the high-dose QRY group displaying the most substantial reduction (*P*<0.05; Figure 3).

Enzyme-Linked Immunosorbent Assay Results ELISA analysis revealed that BAC-induced DED significantly elevated IL-1β, IL-6, TNF-α, and MMP9 protein levels in corneal tissues compared to the control group (*P*<0.01). QRY treatment significantly reduced these markers, with the most pronounced effects observed in the medium- and high-dose groups (*P*<0.01; Figure 4).

Western Blot Results Western blot analysis showed significantly increased TLR4, p-TAK1/TAK1, and p-p38MAPK/p38MAPK expression in the model group compared to the control group (*P*<0.01). QRY and SB203580 treatment significantly reduced these protein levels, with the most notable reductions observed in the high-dose QRY group (*P*<0.01; Figure 5).

QRY on TLR4/TAK1/p38MAPK Pathway-Associated mRNAs qRT-PCR analysis revealed that mRNA expression levels of IL-1β, IL-6, TNF-α, and MMP9 were significantly upregulated in the model group compared to the control group (*P*<0.01). QRY treatment significantly reduced these mRNA levels in a dose-dependent manner, with the high-dose group showing the greatest reduction (*P*<0.01; Figure 6).

DISCUSSION

DED currently lacks a definitive cure, and existing treatments, such as artificial tears, antibiotics, and corticosteroids[17], are often limited by significant side effects and the potential for adverse reactions[18-19]. Consequently, many patients are turning to TCM as an alternative therapy[20]. TCM has consistently demonstrated advantages in DED management, including fewer side effects and enhanced safety profiles[21],

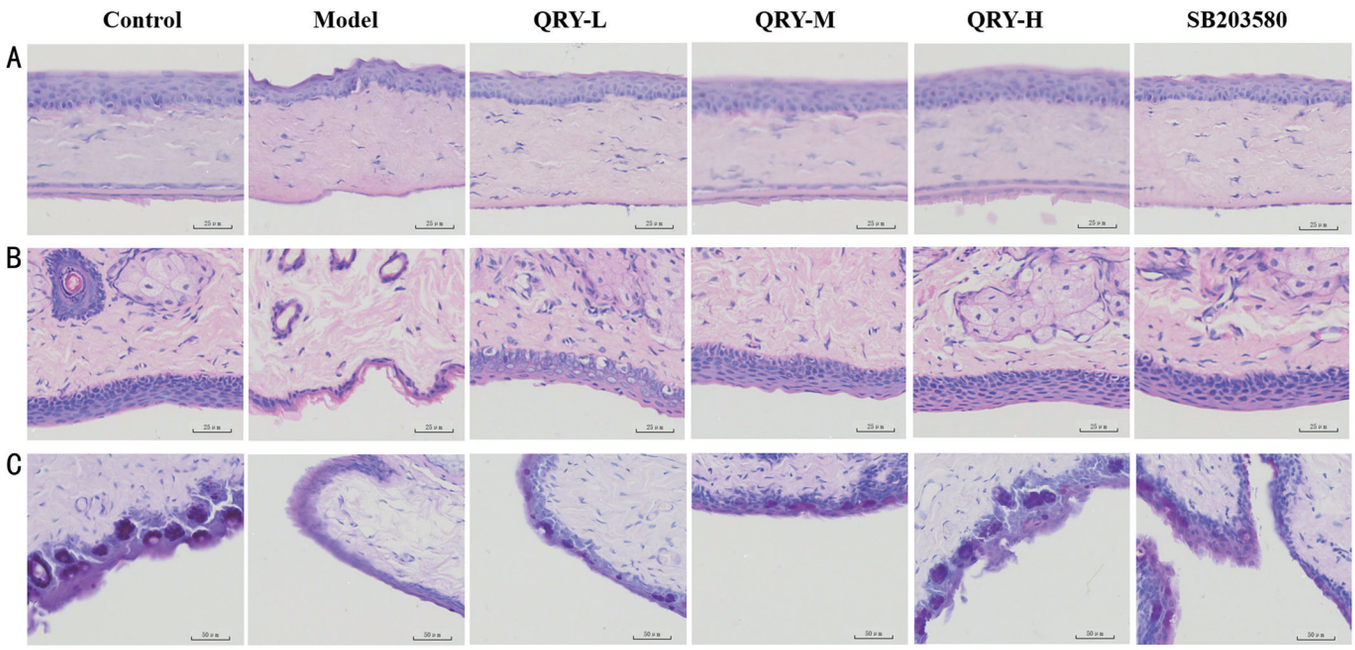

Figure 2 Effect of QRY on histology in dry eye rats A: Hematoxylin and eosin staining of corneal tissue (×400, scale bars=25 μm); B: Hematoxylin and eosin staining of conjunctival tissue (×400, scale bars=25 μm); C: PAS staining showing goblet cell counts (×200, scale bars=50 μm). QRY: Qingxuan Runmu Yin; QRY-L: QRY low-dose group; QRY-M: QRY medium-dose group; QRY-H: QRY high-dose group; PAS: Periodic Acid-Schiff stain.

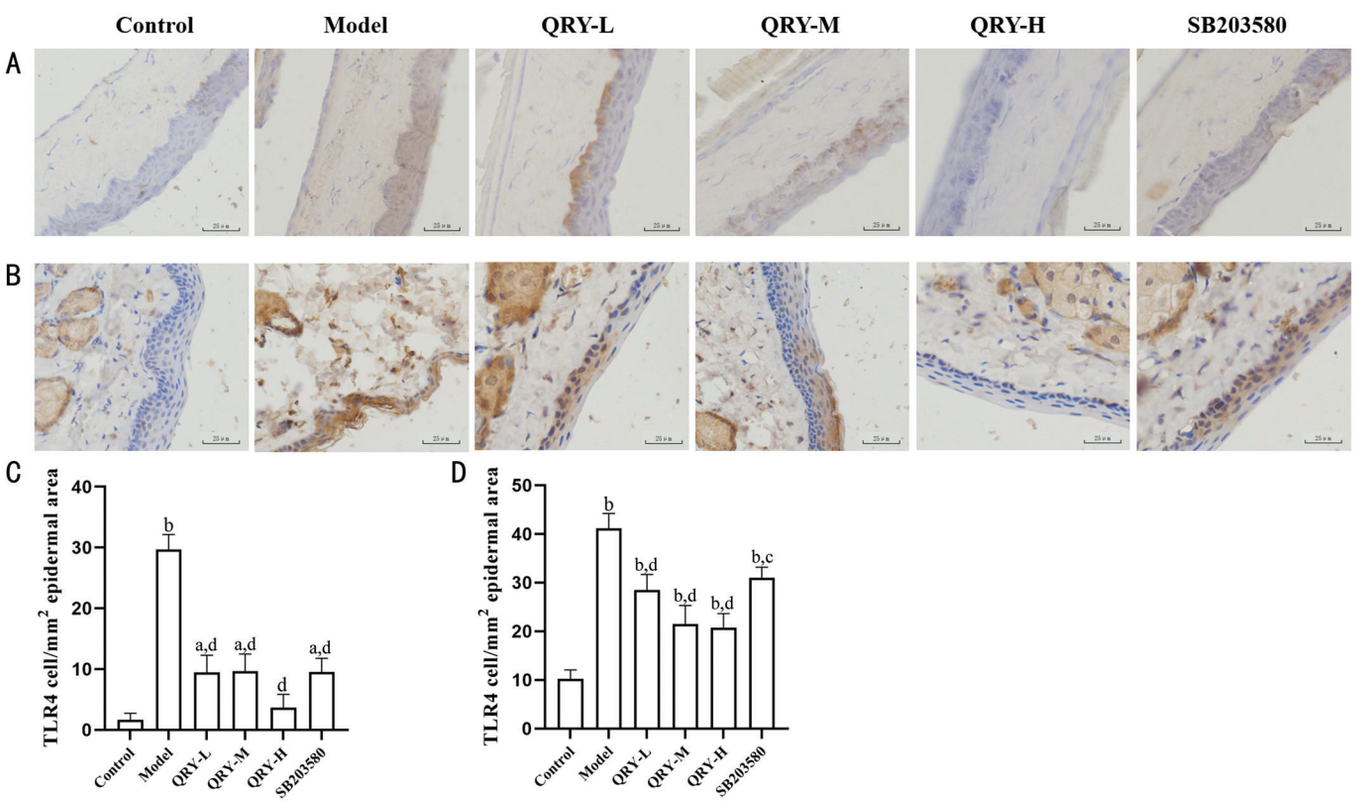

Figure 3 TLR4 protein expression in rat ocular surface tissues in each group A: Immunohistochemistry showing TLR4 expression in corneal tissues; B: Immunohistochemistry showing TLR4 expression in conjunctival tissues; C: Quantification of TLR4-positive areas in corneal tissues; D: Quantification of TLR4-positive areas in conjunctival tissues. [a]*P*<0.05, [b]*P*<0.01 *vs* control group; [c]*P*<0.05, [d]*P*<0.01 *vs* model group. QRY: Qingxuan Runmu Yin; QRY-L: QRY low-dose group; QRY-M: QRY medium-dose group; QRY-H: QRY high-dose group; TLR-4: Toll-like receptor 4.

these advantages have prompted extensive research on the therapeutic effects of herbal components on DED[22].

Active components such as quercetin, kaempferol, and luteolin—found in honeysuckle and forsythia[23]—have been

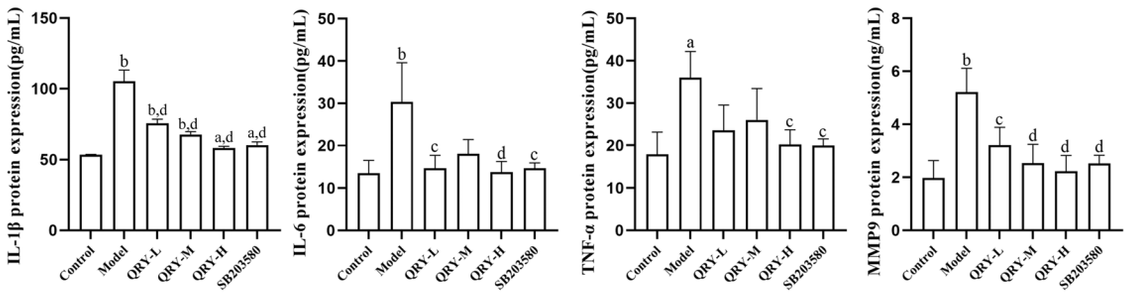

Figure 4 Protein expression of IL-1β, IL-6, TNF-α, and MMP9 in corneal tissues as determined by ELISA [a]*P*<0.05, [b]*P*<0.01 *vs* control group; [c]*P*<0.05, [d]*P*<0.01 *vs* model group. QRY: Qingxuan Runmu Yin; QRY-L: QRY low-dose group; QRY-M: QRY medium-dose group; QRY-H: QRY high-dose group; IL: Interleukin; TNF: Tumor necrosis factor; MMP9: Matrix metalloproteinase 9; ELISA: Enzyme-linked immunosorbent assay.

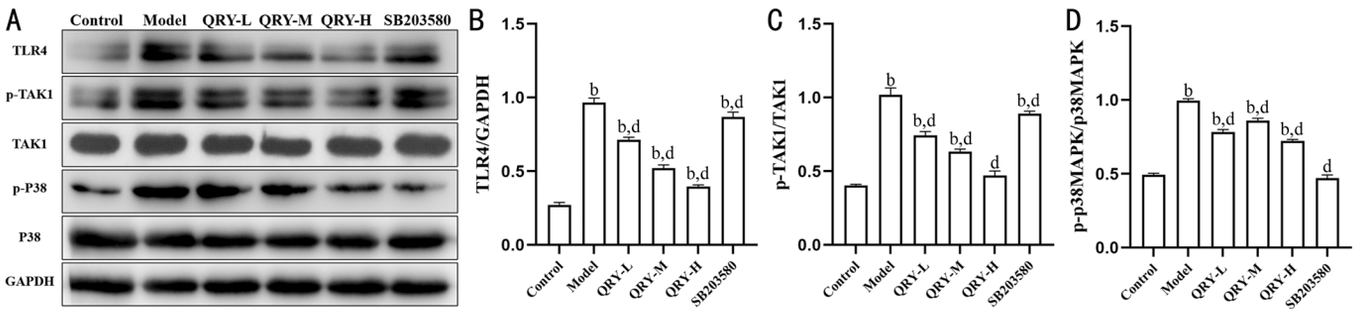

Figure 5 Western blot analysis of TLR4, TAK1, and p38MAPK expression in corneal tissues [a]*P*<0.05, [b]*P*<0.01 *vs* control group; [c]*P*<0.05, [d]*P*<0.01 *vs* model group. QRY: Qingxuan Runmu Yin; QRY-L: QRY low-dose group; QRY-M: QRY medium-dose group; QRY-H: QRY high-dose group; MAPK: Mitogen-activated protein kinase; TLR4: Toll-like receptor 4; TAK1: TGF-β-activated kinase 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

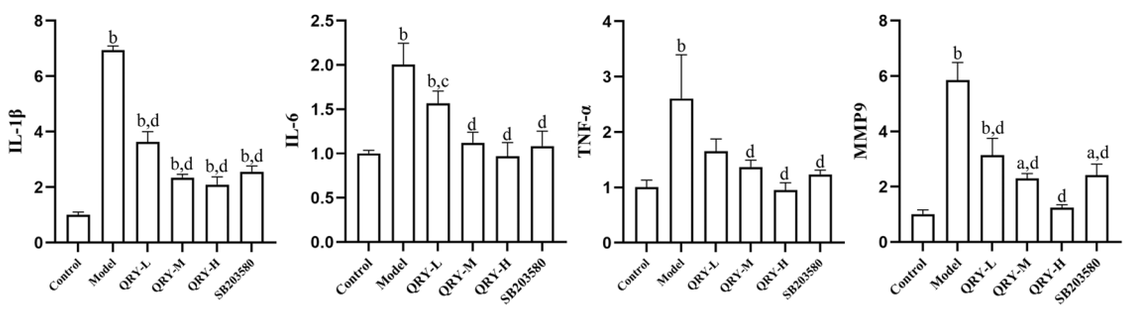

Figure 6 Relative mRNA expression levels of IL-1β, IL-6, TNF-α, and MMP9 in corneal tissues [a]*P*<0.05, [b]*P*<0.01 *vs* control group; [c]*P*<0.01, [d]*P*<0.01 *vs* model group. QRY: Qingxuan Runmu Yin; QRY-L: QRY low-dose group; QRY-M: QRY medium-dose group; QRY-H: QRY high-dose group; IL: Interleukin; TNF: Tumor necrosis factor; MMP9: Matrix metalloproteinase 9.

shown to increase tear secretion, support corneal repair, and alleviate DED symptoms through diverse mechanisms[24-26]. Moreover, *Dendrobium officinale* extract has demonstrated its ability to enhance tear production and reduce inflammatory responses, offering protection to the ocular surface[27]. The polysaccharide from *Atractylodes macrocephala Koidz* reduces oxidative stress and inflammation by regulating ferroptosis-related genes[28]. Steroidal saponins from *Ophiopogon japonicus* suppress pro-inflammatory cytokines (IL-6, TNF-α, IL-1β) and enhance antioxidant enzyme activity[29]. Additionally, 8-formylophiopogonanone B from this plant inhibits paraquat-induced reactive oxygen species (ROS) generation, mitigates lipid peroxidation, and preserves mitochondrial function[30]. Rehmannioside A alleviates cognitive deficits and hippocampal damage *via* suppression of oxidative stress, inflammation, and apoptosis[31]. Saponins such as platycodin D from *Platycodon grandiflorum* exhibit potent peroxyl radical scavenging and antioxidant effects[32]. Chromones in *Saposhnikovia divaricata* attenuate immune activation and inflammation by modulating TNF-α, IL-17, and chemokines[33]. Furthermore, Meng *et al*[34] demonstrated that *Glycyrrhiza glabra* counteracts strychnine-induced neuroinflammation and oxidative stress, demonstrating neuroprotective properties. These findings are consistent with the efficacy of QRY in suppressing ocular surface inflammation demonstrated in prior clinical and experimental studies, though

its precise mechanisms remained uncertain until now.

DED is characterized by chronic ocular surface inflammation triggered by increased tear osmolarity[4]. This condition initiates a cascade of inflammatory events that elevate pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and MMP9[35]. These cytokines damage the corneal and conjunctival epithelial cells, further destabilizing the tear film[36]. This vicious cycle—comprising inflammation, hyperosmolarity, apoptosis, and tear film instability—emphasizes the need for therapies that can disrupt these interconnected processes to restore homeostasis. Our study demonstrated that QRY significantly enhances tear secretion and restores tear film stability in BAC-induced DED rats. High-dose QRY treatments were particularly effective, achieving tear film homeostasis levels comparable to control groups. Additionally, QRY markedly reduced pro-inflammatory cytokine levels in ocular surface tissues, alleviating the inflammatory response. Histopathological analyses also revealed improvements in corneal and conjunctival tissue structure and goblet cell density, underscoring the formulation's therapeutic impact.

The TLR4/TAK1/p38MAPK signaling pathway has been extensively implicated in DED pathophysiology. Enhanced TLR4 activity in the ocular surface and lacrimal glands correlates with elevated cytokine expression and recruitment of inflammatory cells[37]. Moreover, phosphorylated p38MAPK levels rise before IL-1β and TNF-α elevation in DED models, underscoring its pivotal role in initiating inflammation[38]. Activation of this pathway also impairs neuromodulation, reducing tear secretion and exacerbating tear film instability[39]. TLR4 is a crucial pattern recognition receptor that connects innate and adaptive immunity[40]. Hyperosmolar tears activate TLR4 and downstream pathways involving TAK1 and p38MAPK[41-42]. The MAPK pathway regulates immune responses in DED by promoting leukocyte aggregation and amplifying inflammation, which plays a key role in the modulation of inflammatory responses[43-45].

In the present study, we established a BAC-induced DED rat model and administered low, medium, and high doses of QRY alongside the p38MAPK inhibitor SB203580 to investigate the specific mechanisms through which QRY modulates ocular surface inflammation. The results indicated that QRY significantly reduced the expression levels of TLR4, p-TAK1, and p-p38MAPK in ocular surface tissues, thereby demonstrating its ability to regulate ocular surface inflammation and stabilize the ocular environment by inhibiting the TLR4/TAK1/p38MAPK pathway. These findings highlight the potential of QRY as a novel therapeutic strategy for managing DED by targeting the TLR4/TAK1/p38MAPK pathway.

This study utilized the BAC-induced DED model to investigate disease mechanisms. While this established model effectively recapitulates critical pathological features of DED, including corneal epithelial barrier disruption, inflammatory cascade activation, and conjunctival goblet cell depletion, it inadequately replicates neurosensory abnormalities observed in human DED pathophysiology. Our findings demonstrate QRY's therapeutic potential in ocular surface repair and localized inflammation modulation under these experimental conditions. However, future studies should prioritize utilizing animal models that better approximate human disease characteristics to systematically evaluate QRY's effects on lacrimal secretion dysfunction, nociceptive neural sensitization, and other core pathophysiological mechanisms. The 14-day observation window was selected to align with the acute ocular surface injury progression in BAC-induced models, enabling effective assessment of QRY's early therapeutic actions on inflammatory resolution and epithelial regeneration. Given the chronic progressive nature of clinical DED, extended treatment durations will be implemented in subsequent investigations to delineate QRY's mechanistic dynamics within sustained inflammatory microenvironments.

In summary, QRY may alleviate ocular surface inflammation associated with dry eye by inhibiting the TLR4/TAK1/p38MAPK signaling pathway, highlighting its potential therapeutic efficacy for dry eye treatment.

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