• Basic Research •

Obtusifolin ameliorates dry eye model in rats by reducing inflammation and blocking MAPK/NF-κB pathways

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Abstract

● **AIM:** To investigate the functions and potential mechanisms of obtusifolin in dry eye disease (DED) in a rat model.

● **METHODS:** A rat DED model was established *via* topical administration of benzalkonium chloride (BAC), followed by administration of obtusifolin. Conjunctival irritation score and tear production were measured to evaluate DED symptoms. Enzyme-linked immunosorbent assay (ELISA) was employed for determining inflammatory cytokine levels in rat conjunctiva. Periodic acid-Schiff staining and corneal fluorescein staining were implemented for assessing goblet cell numbers and corneal epithelial defects, respectively. Western blotting showed zonula occludens-1 (ZO-1), matrix metalloproteinase-9 (MMP-9), and mitogen-activated protein kinase (MAPK)/nuclear factor kappa B (NF-κB) signaling-related protein levels in the conjunctiva.

● **RESULTS:** Topical application of obtusifolin alleviated conjunctival irritation and enhanced tear production in BAC-induced DED rats. Obtusifolin attenuated conjunctival inflammatory response and goblet cell loss as well as corneal epithelial barrier disruption in DED rats. Obtusifolin suppressed extracellular signal-regulated kinase (ERK), p38, and NF-κB phosphorylation in the conjunctiva of DED rats.

● **CONCLUSION:** Obtusifolin ameliorates DED in rats possibly by alleviating inflammation *via* the inactivation of MAPK/NF-κB signaling.

● **KEYWORDS:** dry eye disease; obtusifolin; inflammation; tear production; mitogen-activated protein kinase/nuclear factor kappa B; rats
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INTRODUCTION

Dry eye disease (DED) is a common ocular surface disease caused by insufficient tear production or excessive tear evaporation[1]. It is characterized by the disrupted homeostasis of the tear film, causing tear film instability and/or ocular surface damage and inflammation, leading to eye discomfort and visual dysfunction[2]. The disease is clinically manifested by irritation, itching, photophobia, and foreign body sensation, which impair patients' quality of life[3]. It has been reported that DED affects approximately 5%-50% of the population worldwide, and the increasing use of visual displays and contact lenses is contributing to the growing prevalence of the disease[4]. Despite advances in DED treatment, there is still a lack of safe and effective drugs to cure this disease[5]. Hence, exploring new medications for DED is pressingly needed.

The pathogenesis of DED is thought to be a vicious cycle involving a hyperosmolarity-triggered inflammatory cascade. A state of hyperosmolarity triggers a nonspecific innate immune response, which causes chronic and self-sustaining ocular surface inflammation, leading to goblet cell loss as well as conjunctival and corneal epithelial damage[6]. The damage induces a more prolonged adaptive immune response, consequently perpetuating inflammation and leading to the vicious cycle of DED[7]. Thus, targeting inflammation is a promising therapeutic approach for DED treatment[8]. Multiple signal transduction pathways can modulate inflammation, including mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) signaling pathways[9]. Ling *et al*[10] proposed that Dendrobium extracts ameliorate DED in a rat model partially by modulating MAPKs/NF-κB signaling.

Obtusifolin is an anthraquinone extracted from Cassiae Semen, the mature seeds of *Cassia obtusifolia* L. and *Cassia tora* L. which

have been used as a traditional herbal medicine in many Asian regions to eliminate liver fire, improve eyesight, and mitigate photophobia[11]. Previous reports have indicated that obtusifolin possesses multiple pharmacological properties, such as neuroprotective, anti-diabetic, antioxidant, and antifungal[12]. Moreover, Wang *et al*[13] demonstrated that obtusifolin inhibits the growth of retinal pigment epithelial cells with exposure to hypoxia, indicating the potential effect on eye diseases. Intriguingly, a recent report illuminated that obtusifolin can relieve inflammation and repress NF-κB signaling in a mouse osteoarthritis model[14]. Nonetheless, obtusifolin's function and potential mechanism in DED remain unillustrated.

This study aims to probe the function of obtusifolin and its mechanism of action in DED using a benzalkonium chloride (BAC)-induced rat model. We speculated that obtusifolin could ameliorate DED by alleviating inflammation and mediating MAPK and NF-κB signaling.

MATERIALS AND METHODS

Ethical Approval All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals. The approval was obtained from the Animal Ethics Committee of Wuhan Myhalic Biotechnology Co., Ltd (approval number: HLK-202309366).

Preparation of Obtusifolin and Benzalkonium Chloride Obtusifolin (99.8% purity, HY-N2098) and BAC solution (≥98.0% purity, HY-B2232) were obtained from MedChemExpress (Shanghai, China). Obtusifolin's chemical structure is shown in Figure 1A.

Animals Female Sprague-Dawley rats (6-7wk, 180-220 g) purchased from Cavens (Changzhou, China) were housed under 12-h light/dark cycles in a temperature- and humidity-controlled (23℃±1℃, 55%±5%) with free access to food and water.

DED Model and Drug Administration After one week of acclimatization, a rat DED model was established *via* topical administration of BAC according to the previous description[15]. In brief, thirty-two rats were randomly grouped as: 1) control+dimethyl sulfoxide (DMSO); 2) control+obtusifolin; 3) DED+DMSO; 4) DED+obtusifolin (n=8/group). To induce DED, rats were topically administered with 0.5% BAC (5 μL/eye) twice daily on the ocular surface for 5d. After successful modelling, rats were treated topically with 0.5% obtusifolin (5 μL/eye, dissolved in 2% DMSO) or 2% DMSO (vehicle) alone four times daily (3h intervals) for 10d. All rats were euthanized, and the entire eye tissue was collected at the end of experiments.

Conjunctival Irritation Score Conjunctival edema, hyperemia, and secretion were assessed under a slit-lamp microscope (Haag-Streit Diagnostics, Wedel, Germany) to evaluate conjunctival irritation as previously described[15]. The scores for each aspect were summed (maximum, 10 points). The conjunctival irritation evaluation was performed by two experienced ophthalmologists blinded to the group treatments.

Tear Production Phenol red cotton thread testing (Tianjin Jingming Technological Development Co., Ltd., Tianjin, China) was performed for quantifying tear production. Threads were placed at the lateral 1/3 of the lower eyelid margin for 15s, with wetting length measured using precision calipers. Calipers were employed to measure the length of the moistened red thread. Both eyes were measured to obtain an average. Bilateral measurements were obtained and averaged.

Corneal Fluorescein Staining Corneal fluorescein staining was conducted for corneal epithelial damage evaluation. In brief, 1% sodium fluorescein (1 μL; Sigma-Aldrich, St. Louis, MO, USA) was dropped into the rat inferior conjunctival sac using a micropipette. Following three blinks, a slit-lamp microscope was employed for observation under cobalt blue light. Each cornea was divided into four quadrants and the degree of corneal fluorescein staining in each quadrant was scored as: no staining, 0; slight punctate staining with <30 spots, 1; evident puncture staining with >30 spots, but without diffuse staining, 2; severe diffuse staining, but without positive plaque, 3; positive fluorescein plaque, 4. The scores of each quadrant were summed (maximum, 16 points). Corneal fluorescein staining was conducted by two experts unaware of animal grouping.

Western Blotting The rats' conjunctival and corneal tissues were excised and lysed using radioimmunoprecipitation buffer (Solarbio, Beijing, China), followed by quantifying protein concentration using a bicinchoninic acid assay kit (Beyotime). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) was employed to separate protein samples, which were then blotted on polyvinylidene difluoride membranes (Beyotime), and blocked with 5% defatted milk. The membranes were incubated overnight at 4℃ with the following primary antibodies: anti-zonula occludens-1 (ZO-1; 1:5000, 21773-1-AP, Proteintech, Wuhan, China), anti-matrix metalloproteinase-9 (MMP-9; 1:1000, ab228402, Abcam, Shanghai, China), anti-extracellular signal-regulated kinase (ERK; 1:10000, ab184699, Abcam), anti-p-ERK (1:1000, ab201015, Abcam), anti-p38 (1:1000, ab170099, Abcam), anti-p-p38 (1:1000, ab4822, Abcam), anti-NF-κB (1:1000, 10745-1-AP, Proteintech), anti-p-NF-κB (1:2000, 82335-1-RR, Proteintech), and β-actin (1:200, ab115777, Abcam). After washing three times with Tris-buffered saline in Tween, the horseradish peroxidase-conjugated secondary antibody (1:2000, ab288151, Abcam) was used for further incubation for 1h. Lastly, the blot signals were detected with an enhanced chemiluminescence kit (Solarbio) and quantified with Image J software.

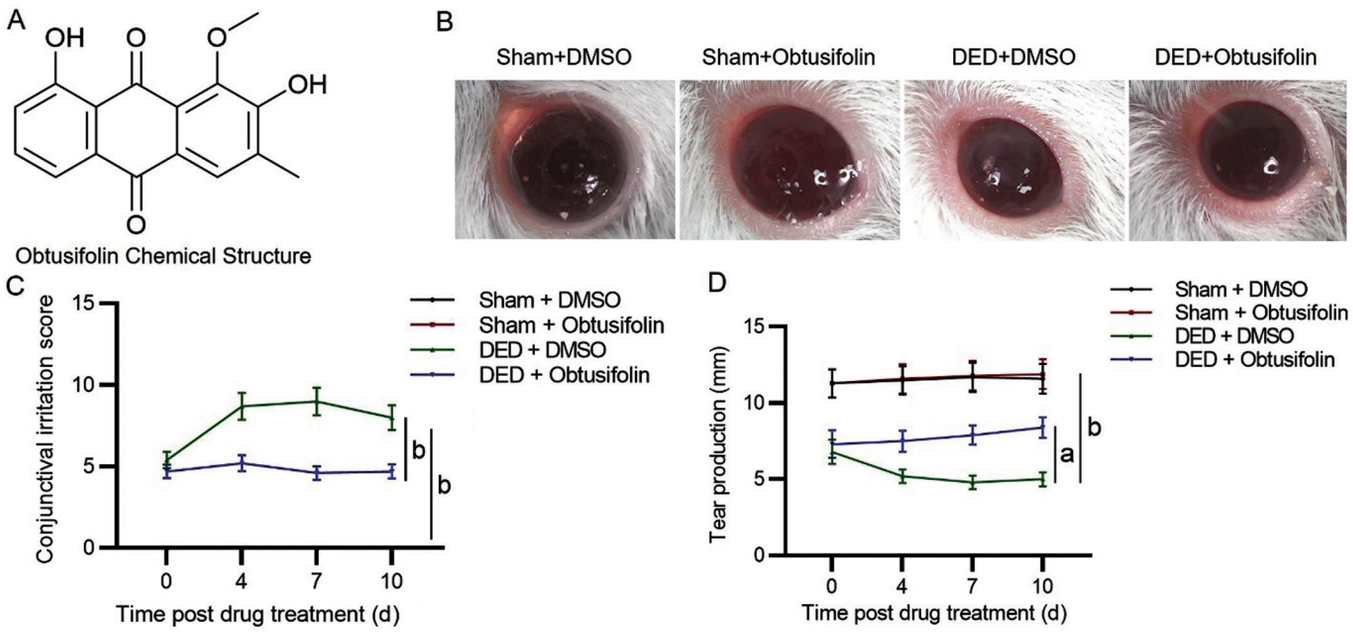

Figure 1 Obtusifolin alleviates DED symptoms in rats A: Obtusifolins chemical structure; B: Representative rat eye images after topic administration of obtusifolin for 4d; C: Conjunctival irritation score in each group (Sham+DMSO and Sham+Obtusifolin groups had scores of 0, overlapping with the x-axis); D: Evaluation of tear secretion in each group (n=8). ^{a}P<0.05, ^{b}P<0.01. DED: Dry eye disease; DMSO: Dimethyl sulfoxide.

Periodic Acid-Schiff Staining The eye tissue was fixed in 10% neutral buffered formalin, paraffin-embedded, and sectioned (5-μm-thick). Each section was stained with periodic Acid-Schiff (PAS) reagent (Solarbio) as per the manufacturer's guidelines. The conjunctival morphology was observed under a microscope (Leica Microsystems, Shanghai, China). For each section, goblet cells were counted in three randomly chosen visual fields, and the average was determined.

Enzyme-Linked Immunosorbent Assay Inflammatory factor concentrations in rat conjunctival tissues were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Abcam): interleukin-6 (IL-6, ab234570), IL-10 (ab214566), tumor necrosis factor-alpha (TNF-α, ab236712), monocyte chemoattractant protein-1 (MCP-1, ab100778), as per the manufacturer's guidelines.

Statistical Analysis Data were presented as mean±standard deviation. Each experiment was implemented in triplicate. Difference comparisons between groups were evaluated by one-way or two-way analysis of variance followed by Tukey's post hoc analysis using GraphPad Prism 8.0.2 (GraphPad, San Diego, CA, USA). P<0.05 indicated statistical significance.

RESULTS

Effect of Obtusifolin on DED Symptoms in Rats To investigate obtusifolin's effect on DED, we established a rat DED model by topical administration of BAC followed by obtusifolin's administration. As shown in Figure 1B, relative to the control rats, the BAC-treated rats exhibited significant conjunctival damage, as indicated by hyperemia, edema, and secretion, while administration of obtusifolin for 4d significantly attenuated the above symptoms. Consistently, the conjunctival irritation score was markedly higher in BAC-stimulated rats than in controls and was prominently decreased after obtusifolin administration (Figure 1C). Furthermore, we estimated tear production of rats in each group. Notably, tear secretion was markedly repressed after BAC induction, whereas obtusifolin treatment restored tear secretion in DED rats (Figure 1D). Collectively, topical administration of obtusifolin could improve DED symptoms in rats.

Obtusifolin's Effect on BAC-induced Conjunctival Inflammation and Goblet Cell Loss Ocular surface inflammation is widely considered a pivotal regulator in DED progression[16]. To elucidate obtusifolin's effect on BAC-triggered ocular inflammation, we estimated the concentrations of inflammatory mediators in rat conjunctival tissues. As expected, DED rats exhibited prominently higher levels of TNF-α, IL-6, and MCP-1 than the controls (Figure 2A-2C). In addition, BAC induction markedly decreased the anti-inflammatory cytokine IL-10 concentration in rat conjunctival tissues (Figure 2D). However, these alterations of inflammatory mediator levels in DED rats were restored after obtusifolin administration (Figure 2A-2D), suggesting that obtusifolin could mitigate BAC-triggered conjunctival inflammatory response in DED rats. Furthermore, the loss of goblet cells is an important hallmark of DED. As depicted by PAS staining, topical application of BAC resulted in a reduced number of goblet cells in the conjunctiva of rats, while obtusifolin treatment prominently abated BAC-evoked conjunctival goblet cell loss (Figure 2E-2F).

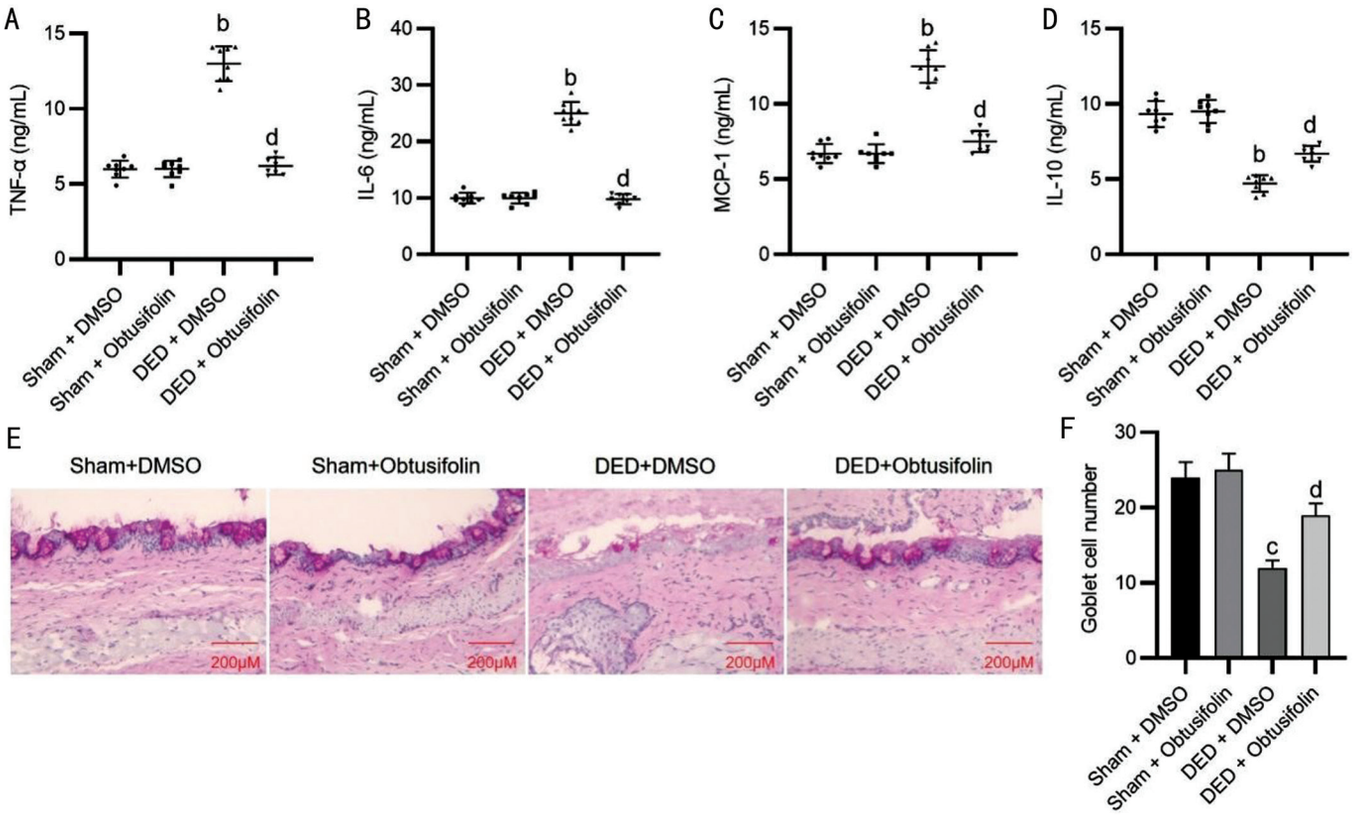

Figure 2 Obtusifolin attenuates BAC-induced conjunctival inflammation and goblet cell loss A-D: Measurement of the concentrations of inflammatory mediators in the conjunctiva of rats using ELISA kits (n=8); E: Representative conjunctival PAS staining images in each group; F: Quantification of goblet cell numbers in the conjunctiva (n=4). ^{b}P<0.01, ^{c}P<0.001 *vs* Sham+DMSO group; ^{d}P<0.01 *vs* DED+DMSO group. BAC: Benzalkonium chloride; ELISA: Enzyme-linked immunosorbent assay; PAS: Periodic acid-Schiff; DED: Dry eye disease; DMSO: Dimethyl sulfoxide.

Amelioration of Corneal Epithelial Barrier Disruption by Obtusifolin in DED Rats The degree of corneal epithelial damage was determined by measuring the fluorescein staining. As depicted in Figure 3A-3B, the DED+DMSO group had a significantly higher fluorescein staining score than the control+DMSO group, further confirming the successful induction of the rat DED model. Notably, this score in DED rats was reduced after treatment with obtusifolin (Figure 3A-3B), suggesting that obtusifolin mitigated BAC-induced corneal epithelial damage in rats. Moreover, we measured the protein levels of ZO-1, a corneal epithelial tight junction protein, and MMP-9, a key factor in corneal epithelial barrier disruption. Notably, ZO-1 protein expression was decreased, and MMP-9 protein expression was elevated in the DED+DMSO group (Figure 3C-3E), further elucidating that topical application led to corneal epithelial barrier disruption. Nonetheless, after topical administration of obtusifolin, ZO-1 expression was remarkably enhanced, and MMP-9 expression was reduced in the cornea of DED rats (Figure 3C-3E). Taken together, the above results revealed the ameliorative effect of obtusifolin on BAC-evoked corneal epithelial barrier disruption.

Obtusifolin-Mediated Inhibition of MAPK/NF-κB Signaling in DED Rats To investigate the potential mechanism underlying obtusifolin's therapeutic effect on DED, we tested obtusifolin's effects on MPAKs and NF-κB signaling in the rat conjunctiva. As depicted by Western blotting, the DED+DMSO group showed much higher levels of p-ERK, p-p38, and p-NF-κB than the control+DMSO group, whereas the DED+obtusifolin group showed decreased levels of p-ERK, p-p38, and p-NF-κB as compared with the DED+DMSO group (Figure 4A-4B). Collectively, MAPK/NF-κB inactivation might be involved in obtusifolin-mediated ameliorative effect on DED in rats.

DISCUSSION

DED is a multifactorial disorder of the ocular surface that seriously impairs patients' quality of life[17]. BAC is a quaternary ammonium compound that is a common preservative in ophthalmic formulations for ocular surface diseases, with concentrations ranging from 0.005% to 0.02%[18]. Topical administration of BAC has been widely employed in the induction of DED animal models due to its detergent-like properties, which lead to excessive tear evaporation and tear film disruption[19]. Thus, we established a rat DED model by topical application of 0.5% BAC to investigate the effect of obtusifolin. This study demonstrated that topical administration of obtusifolin restored BAC-induced conjunctival irritation and tear evaporation in rats.

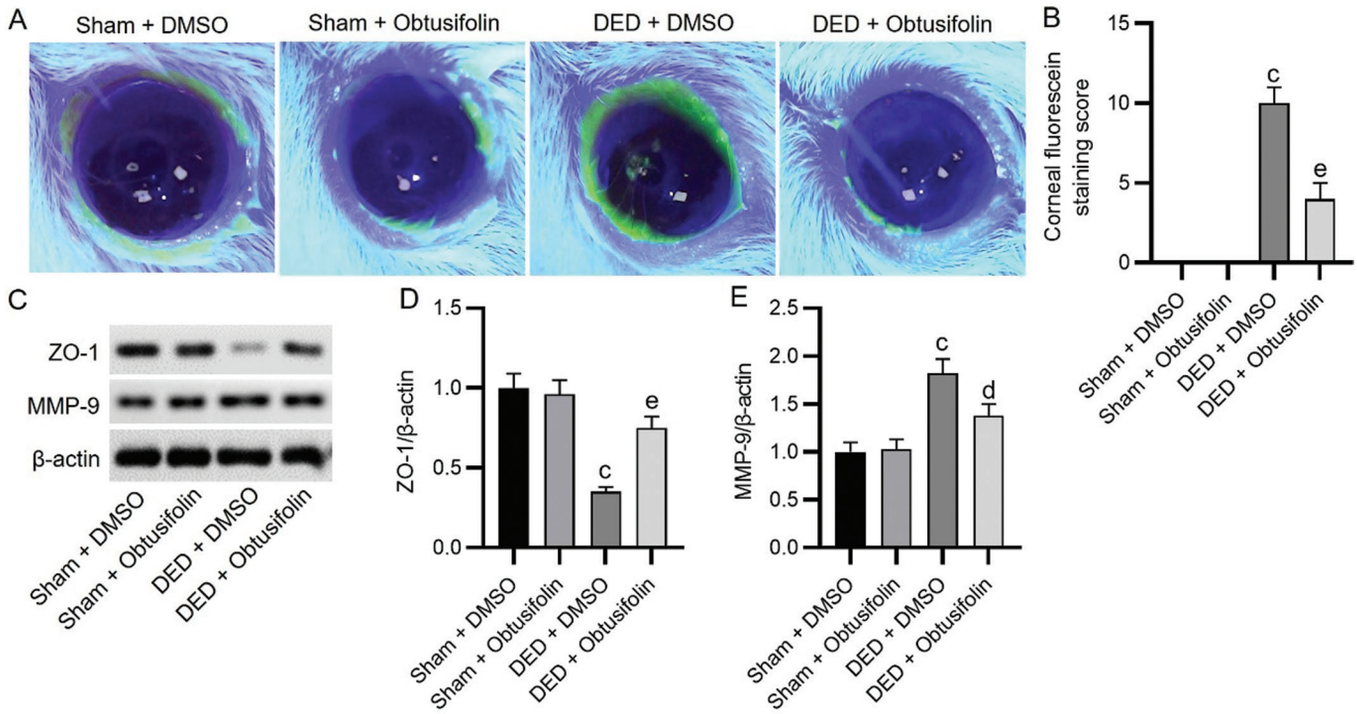

Figure 3 Obtusifolin ameliorates ocular corneal epithelial barrier disruption in DED rats A: Representative images of corneal fluorescein staining for estimating corneal epithelial damage of rats after treatment with obtusifolin for 4d; B: Assessment of corneal fluorescein staining scores in each group (n=8); C: Western blotting for evaluating ZO-1 and MMP-9 protein expression in the cornea of rats; D-E: Quantification of protein levels (n=4). ^{c}P<0.001 *vs* Sham+DMSO group; ^{d}P<0.01, ^{e}P<0.001 *vs* DED+DMSO group. DED: Dry eye disease; ZO-1: Zonula occludens-1; MMP-9: Matrix metalloproteinase-9; DMSO: Dimethyl sulfoxide.

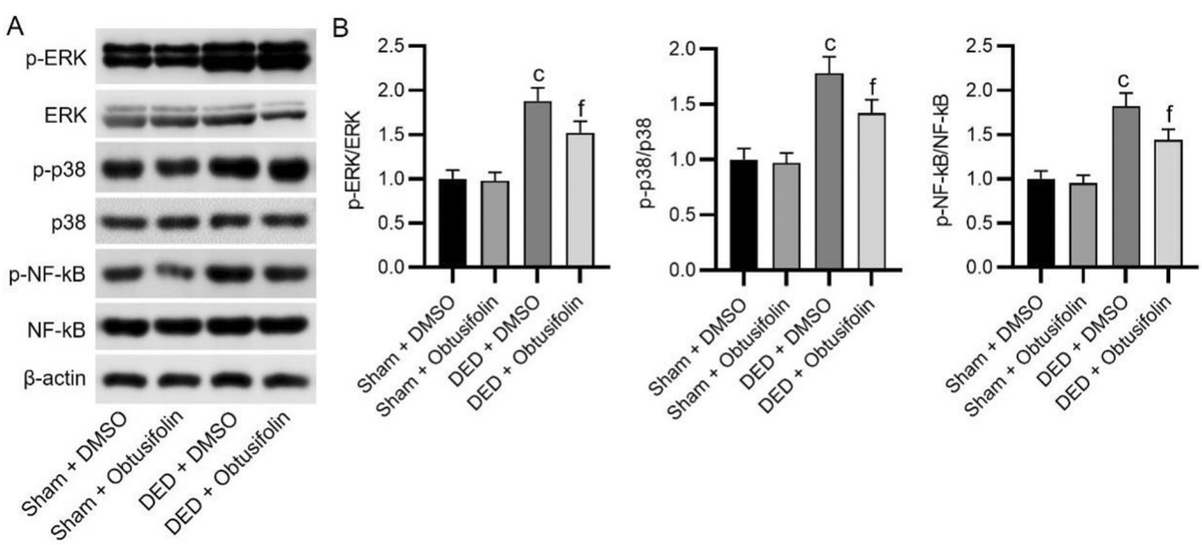

Figure 4 Obtusifolin blocks MAPK/NF-κB signaling pathway in DED rats A: Western blotting for assessing expression of MAPK/NF-κB signaling-related proteins in the conjunctiva of rats; B: Quantification of relative protein expression. n=4. ^{c}P<0.001 *vs* Sham+DMSO group; ^{f}P<0.05 *vs* DED+DMSO group. MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor kappa B; DED: Dry eye disease; DMSO: Dimethyl sulfoxide.

Conjunctival goblet cell loss is a well-characterized feature of DED[20]. Goblet cells in the conjunctiva produce gel-forming mucins which contribute to tear film stabilization, ocular surface lubrication, and protection[21]. Our study showed that obtusifolin protected against BAC-triggered goblet cell loss in the conjunctiva of rats. Moreover, corneal epithelial barrier disruption is also a key feature of DED. Evidence suggests that BAC induces corneal epithelial barrier disruption by downregulating corneal tight junction proteins (*e.g.* ZO-1) and increasing the production of MMPs, especially MMP-3 and -9[22-23]. Elevated MMP-9 levels contribute to corneal epithelial cell loss and extracellular matrix degradation[24]. Consistent with previous evidence, the present study depicted that BAC induced corneal epithelial barrier disruption in rats,

as evidenced by significantly elevated corneal fluorescein staining score and MMP-9 protein expression as well as reduced ZO-1 protein expression in the cornea. Nonetheless, topical application of obtusifolin prominently reversed the above effects in DED rats, indicating the protective effect of obtusifolin against BAC-evoked corneal epithelial damage. In addition, a previous report has demonstrated that obtusifolin represses MMP-3 and -13 protein expression in IL-1β-stimulated chondrocytes[14], which partially supports our findings.

Mounting evidence has indicated the significant role of inflammation in DED pathogenesis[25]. Obtusifolin has been revealed to inhibit inflammation in mice with osteoarthritis[14]. Similarly, our results displayed that obtusifolin elevated the anti-inflammatory cytokine IL-10 level and reduced the concentrations of proinflammatory factors (TNF-α, IL-6, MCP-1) in the conjunctiva of DED rats, confirming the anti-inflammatory activity of obtusifolin in DED. NF-κB signal transduction is a typical proinflammatory pathway and has been widely reported to participate in DED progression[26]. Here, our results depicted that obtusifolin administration repressed NF-κB phosphorylation in the conjunctiva of DED rats, which is consistent with previous evidence as mentioned above[14]. In addition to NF-κB signaling, the MAPK signaling pathway is also a classical inflammatory pathway. Activated MAPKs and NF-κB signaling mediate inflammatory response by enhancing the production of various proinflammatory cytokines including IL-6 and TNF-α[27]. Importantly, studies have shown that suppression of MAPKs and NF-κB signaling contributes to the alleviation of DED symptoms[10,28]. The present study revealed that obtusifolin blocked the ERK/p38 signaling pathway in the DED rat conjunctiva.

In conclusion, this study demonstrates for the first time that topical administration of obtusifolin can ameliorate DED in the rat model by increasing tear production, alleviating conjunctival irritation and goblet cell loss, and restoring corneal epithelial barrier function. Moreover, obtusifolin protects against BAC-triggered inflammation in rat conjunctiva possibly by blocking MAPKs and NF-κB signal transduction. Our findings indicate that obtusifolin may be a new candidate drug for treating DED.

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Conflicts of Interest: Zhu D, None; **Wu XY,** None; **Li LC,** None.

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