Therapeutic Effects of Topical Doxycycline in a Benzalkonium Chloride–Induced Mouse Dry Eye Model

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Purpose. We investigated the therapeutic effects and underlying mechanisms of topical doxycycline in a benzalkonium chloride (BAC)–induced mouse dry eye model.

Methods. Eye drops containing 0.025%, 0.1% doxycycline or solvent were administered to a BAC-induced dry eye model four times daily. The clinical evaluations, including tear break-up time (BUT), fluorescein staining, inflammatory index, and tear volume, were performed on days 0, 1, 4, 7, and 10. Global specimens were collected on day 10 and processed for immunofluorescent staining, TUNEL, and periodic acid–Schiff assay. The levels of inflammatory mediators in the corneas were determined by real-time PCR. The total and phosphorylated nuclear factor-kB (NF-kB) were detected by Western blot.

Results. Both 0.025% and 0.1% doxycycline treatments resulted in increased BUT, lower fluorescein staining scores, and inflammatory index on days 4, 7, and 10, while no significant change in tear volume was observed. The 0.1% doxycycline-treated group showed more improvements in decreasing fluorescein staining scores, increasing Ki67–positive cells, and decreasing TUNEL- and keratin-10–positive cells than other groups. The mucin-filled goblet cells in conjunctivas were increased, and the expression of CD11b and levels of matrix metalloproteinase-9, IL-1β, IL6, TNF-α, macrophage inflammatory protein-2, and cytokine-induced neutrophil chemoattractant in corneas were decreased in both doxycycline-treated groups. In addition, doxycycline significantly reduced the phosphorylation of NF-kB activated in the BAC-treated corneas.

Conclusions. Topical doxycycline showed clinical improvements and alleviated ocular surface inflammation on BAC-induced mouse dry eye, suggesting a potential as an anti-inflammatory agent in the clinical treatment of dry eye.

Keywords: dry eye, topical doxycycline, benzalkonium chloride, anti-inflammation

Dry eye, a common and multifactorial ocular surface disease, affects millions of population worldwide, and results in symptoms of ocular discomfort, visual disturbance, and the instability of tear film.¹⁻³ It is well recognized that ocular surface inflammation may have a critical role in the pathogenesis of dry eye. Disease or dysfunction of the tear secretory glands leads to changes in tear composition, such as hyperosmolarity, which stimulate the production of inflammatory mediators on the ocular surface.³⁻⁵ Inflammation may, in turn, cause dysfunction or loss of cells responsible for tear secretion or retention.⁶ Regardless of the initiating cause, a vicious circle of inflammation can develop on the ocular surface in dry eye. Therefore, the anti-inflammatory therapy has become a common therapeutic strategy for dry eye disease. However, very limited anti-inflammatory eyedrops are available for the dry eye treatment, such as topical corticosteroids and cyclosporine A (CsA), and the efficacy and long-term safety of these agents are not ideal.⁷⁻⁸ Hence, it is desirable to identify and evaluate suitable ophthalmic anti-inflammatory agents.

Doxycycline, a semisynthetic long-acting tetracycline derivative, exhibits potent anti-inflammatory effects with subantibacterial dose and is used to treat several inflammatory diseases, such as periodontitis,⁹ chronic inflammatory lung diseases,¹⁰,¹¹ atherosclerotic cardiovascular diseases,¹²,¹³ rheumatoid arthritis,¹⁴ and so forth. Oral doxycycline has been proved effective in ocular surface inflammatory diseases, such as chronic blepharitis,¹⁵ meibomian gland dysfunction,¹⁶,¹⁷ ocular rosacea,¹⁸,¹⁹ and recurrent corneal erosions.²⁰,²¹ Doxycycline has been shown to inhibit pathologically excessive matrix metalloproteinases (MMPs), proinflammatory cytokines and activation of p38 MAPK signaling in human corneal epithelial cells.²²⁻²⁵ In an experimental mouse dry eye model, topical doxycycline was found to preserve corneal epithelial smoothness and barrier function.²⁶,²⁷ In addition to the known effects, the reported mechanisms of doxycycline in recent studies include regulation of mucin secretion,²⁸ and inhibitions of inducible nitric oxide synthase (iNOS), p38 MAPK, and nuclear factor-kB (NF-kB)²⁹,³⁰ in various inflammatory models. However, the therapeutic effects of topical doxycycline on dry eye–associated pathologic changes have not yet been fully investigated so far. Based on the potent anti-inflammatory effects, the aim of this study was to investigate the effects of topical doxycycline on benzalkonium chloride (BAC)–induced dry eye and the underlying mechanisms.
Topical Doxycycline and Dry Eye

MATERIALS AND METHODS

Mouse Dry Eye Model

A total of 35 male BALB/c mice (18–20 g; purchased from Shanghai SLAC Laboratory Animal Center, Shanghai, China) were used in this study. They were kept in the facility with standard environment: temperature 25° ± 1°C, relative humidity 60% ± 5%, and 12 hours light-dark cycles (9 AM to 9 PM). All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Experimental Animal Ethics Committee of Xiamen University.

The mouse dry eye model was induced as described previously. Both eyes of 28 mice received 5 μL of 0.2% BAC (Sigma-Aldrich, St. Louis, MO, USA) administered topically twice daily (9 AM, 9 PM) for 14 days. Based on the clinical evaluations (described below), the mice with dry eye condition then were divided randomly into four groups (seven mice per group): blank control group, solvent-treated group, 0.025% doxycycline (Anyu, Inc., Zhengzhou, China)-treated group, and 0.1% doxycycline-treated group. Seven other mice were used as the normal control.

Experimental Procedure

After grouping, the blank control group was left untreated for baseline comparison. The other three groups received topically administered 0.025% and 0.1% doxycycline, and solvent (physiologic saline solution), respectively, with the dosage of 5 μL, four times daily (9 AM, 1 PM, 5 PM, 9 PM). The clinical evaluations, including tear break-up time (BUT), corneal fluorescein staining, inflammatory index, and tear volume were performed on days 0, 1, 4, 7, and 10. The ocular and orbit tissues were harvested carefully on day 10 for histologic examination, reverse transcriptase-polymerase chain reaction (RT-PCR), and Western blot analysis following the methods described below.

BUT and Fluorescein Staining

Three blinks after 1 μL of 0.1% liquid sodium fluorescein was dropped into the conjunctival sac, BUT was recorded in seconds. At 90 seconds later, the corneal epithelial integrity was graded with a cobalt blue filter under a slit-lamp microscope (BQ990; Haag-Streit AG, Koeniz, Switzerland). The cornea was divided into four quadrants, which were scored respectively. The grade was assessed as described previously briefly as follows: absent, 0; slightly punctuate staining less than 30 spots, 1; punctuate staining more than 30 spots, but not diffuse, 2; severe diffuse staining but no positive plaque, 3; and positive fluorescein plaque, 4. The scores from four quadrants were summed to a final grade (total, 16 points).

Evaluation of Inflammatory Index

The inflammatory index was assessed as described previously, and based on three parameters: ciliary hyperemia (absent, 0; present but less than 1 mm, 1; present and between 1 and 2 mm, 2; present and more than 2 mm, 3), central corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present without visible pupil, 3), and peripheral corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present with no visible iris, 3). The final inflammatory index was the sum of three parameters divided by a factor of 9.

Measurement of Tear Volume

Tear volume was measured with phenol red-impregnated cotton threads (Zone-Quick, Yokota, Tokyo, Japan) at a similar time point (7 PM) in the standard environment. The lower eyelid was pulled down slightly, and a 1 mm portion of the thread was placed on the palpebral conjunctiva for 15 seconds, at a specified point approximately one third of the distance from the lateral canthus of the lower eyelid. The length of the wet red thread was measured in millimeters.

Immunofluorescent Staining

Immunofluorescent staining was performed in cryosections (6 μm thick) of the eyeballs. Sections were fixed in acetone at −20°C, blocked, and then incubated at 4°C overnight with rabbit anti-Ki-67 antibody (1:200; Abcam, San Francisco, CA, USA), rabbit anti-keratin-10 (K10) antibody (1:500; Covance, Harrogate, UK), and rat anti-CD11b antibody (1:100; eBioscience, San Diego, CA, USA). After incubation with Alexa Fluor 594 donkey anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA, USA), or Alexa Fluor 488 donkey anti-rat IgG (1:500; Invitrogen), sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA), mounted, and photographed using the Leica upright microscope (DM2500; Leica Microsystems, Wetzlar, Germany).

In Situ TUNEL

To measure end-stage apoptosis, TUNEL assay was performed in cryosections of the corneas using the DeadEnd Fluorometric TUNEL System (G3250; Promega, Madison, WI, USA) according to the manufacturer’s instructions. Sections were counterstained with DAPI (Vector), mounted, and photographed using the Leica upright microscope (DM2500; Leica Microsystems).

PAS Staining

The mucin-filled goblet cells in the conjunctival fornices were stained by the PAS Staining System (395B-1KT; Sigma-Aldrich) in paraffin sections of the whole orbit tissues, and the counterstaining with hematoxylin was performed. The mucin-filled goblet cells were counted in six representative slices of homologous positions from each sample (three samples for each group).

RNA Isolation and Real-Time PCR

Total RNA of the corneas was extracted using the TRIzol reagent (Invitrogen), and cDNA was synthesized using a reverse transcription kit (RR047A; TaKaRa, Shiga, Japan). Real-time PCR was performed on a StepOne Real-Time PCR System (Applied Biosystems, Alameda, CA, USA) using a SYBR Premix Ex Taq Kit (RR420A; TaKaRa), and the primer sequences are summarized in the Table. The amplification program included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, and 60°C for 30 seconds, after which a melt curve analysis was conducted to check amplification specificity. The results of quantitative PCR were analyzed by the comparative threshold cycle (Ct) method, normalized with β-actin as an endogenous reference, and calibrated against the normal control group.

Western Blot

The corneal proteins were extracted with cold RIPA buffer, and total protein concentration of the cell extract was measured.
with a protein assay kit (MicroBCA; Pierce Biotechnology, Rockford, IL, USA). Equal amounts of proteins were subjected to electrophoresis and then transferred electronically to polyvinylidene fluoride (PVDF) membranes. After 1 hour of blocking in 2% BSA, the membranes were incubated overnight at 4°C with rabbit anti–NF-κB p65 antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-NF-κB p65 (Ser536) antibody (1:1000; Cell Signaling Technology), and horseradish peroxidase (HRP)–conjugated anti-β-actin antibody (1:20,000; Sigma-Aldrich) as a loading control. After three washes, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000; Sigma-Aldrich) as a loading control. Western blot, followed by the Bonferroni post test. Differences with P value < 0.05 were considered statistically significant.

**Statistical Analysis**

Two-way ANOVA was applied to make clinical comparisons between groups at different time points, and 1-way ANOVA was conducted to analyze the data from real-time PCR and Western blot, followed by the Bonferroni post test. Differences with P value < 0.05 were considered statistically significant.

**RESULTS**

**Doxycycline Ameliorated Clinical Evaluations of Dry Eye**

Clinical evaluations of dry eye were first performed before and during the treatment. There was a general trend of increased fluorescein staining scores and inflammatory index, shortened BUT, and decreased tear volume after topical administration of 0.2% BAC for 14 days. At baseline, no statistically significant differences in clinical evaluations were found between groups (D0; Figs. 1A–D).

The 0.1% doxycycline treatment significantly decreased fluorescein staining scores compared to the other groups on days 4, 7, and 10 (Figs. 1A, 1E–I). There was also a decrease in fluorescein staining scores in the 0.025% doxycycline-treated group, though no statistically significant difference was found compared to those in the solvent-treated group on day 10 (Fig. 1A). The BUT was increased significantly in both doxycycline-treated groups on days 4, 7, and 10, while 0.1% doxycycline resulted in longer BUT than 0.025% doxycycline (Fig. 1B). In addition, both doxycycline-treated groups significantly decreased inflammatory index on days 4, 7, and 10, compared to the blank control and solvent-treated groups, and no significant difference between the doxycycline-treated groups was observed (Fig. 1C). However, phenol red thread tests revealed no significant change in tear volume among the four groups at any time points (Fig. 1D).

**Effect of Doxycycline on Corneal Epithelial Cell Proliferation, Apoptosis, and Squamous Metaplasia**

To investigate further whether the effect of doxycycline on dry eye is associated with cell proliferation, the level of Ki-67, a marker of cell proliferation that is known to be present during active phases of the cell cycle (G1, S, G2, and mitosis) but absent from resting cells (G0),35 was detected to determine the growth fraction of the corneal epithelial cells. It was shown that fewer cells were labeled by Ki-67 in the blank control group (Figs. 2C, 2D) than those in the normal control group (Figs. 2A, 2B), and there was no statistically significant difference between the solvent- and 0.025% doxycycline-treated groups (Figs. 2E–H). However, the 0.1% doxycycline treatment induced more Ki-67–positive cells than the other groups (Figs. 2I, 2J).

The TUNEL assay was used to measure cell apoptosis. It was revealed that the dry eye condition resulted in more apoptotic cells in the superficial corneal epithelium of the blank control and solvent-treated groups than those in the normal control group (Figs. 3A–F). Meanwhile, the 0.025% doxycycline-treated group had a few apoptotic cells (Figs. 3G, 3H), but less than the solvent-treated group. There were hardly any apoptotic cells after the 0.1% doxycycline treatment (Figs. 3I, 3J).

Squamous metaplasia is a hallmark of various severe ocular surface inflammatory disorders, including dry eye.36 The epidermis-specific K10 expression was assessed to determine the alteration of squamous metaplasia. The normal corneal epithelium was K10-negative (Figs. 4A, 4B), while the superficial corneal epithelium of the blank control and solvent-treated groups were K10-positive (Figs. 4C–F). The doxycycline treatment significantly decreased K10-positive cells, though there still were some K10-positive cells in the 0.025% doxycycline-treated group (Figs. 4G, 4H). In addition, the 0.1% doxycycline treatment made the superficial corneal epithelium return to normal (Figs. 4I, 4J).

**Effect of Doxycycline on Goblet Cell Density**

The number of goblet cells is associated closely with MUC5AC in the tear film and reduced by the inflammatory conditions, such as dry eye.37 As such, PAS staining was performed and the PAS-stained mucin-filled goblet cells were counted in the conjunctival sections to determine whether doxycycline had an effect on MUC5AC. As shown in Figure 5, the PAS-stained cells in the conjunctiva were decreased in dry eye condition (Figs. 5A–C) and the doxycycline treatment significantly increased the number of mucin-filled goblet cells (Figs. 5D, 5E), to a level close to, but less than the normal level.

**Effect of Doxycycline on Corneal Inflammation**

To evaluate the effect of doxycycline on the corneal inflammation caused by dry eye, immunofluorescence staining for CD11b (a macrophage marker) and real-time PCR for the

### Table: Mouse Primer Sequences Used for Real-Time PCR

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<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>PCR Product, bp</th>
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**Effect of Doxycycline on Corneal Inflammation**

To evaluate the effect of doxycycline on the corneal inflammation caused by dry eye, immunofluorescence staining for CD11b (a macrophage marker) and real-time PCR for the
levels of MMP-9, proinflammatory cytokines, and chemokines in the corneas were performed. No macrophages were infiltrated in the normal corneal stroma, while CD11b-positive cells were increased significantly in the blank control and solvent-treated groups (Figs. 6A–C). However, there were hardly any CD11b-positive cells in both doxycycline-treated groups (Figs. 6D, 6E). Meanwhile, the data of real-time PCR revealed the increases of mRNA expression of MMP-9, IL-1b, IL-6, TNF-a, MIP-2, and KC in the BAC-induced corneas, which were decreased after the doxycycline treatment (Figs. 6F–K).

**Effect of Doxycycline on NF-κB Activation**

To elucidate further the mechanisms underlying the above effects of doxycycline on BAC-induced dry eye, we focused on the activation of NF-κB by Western blot analysis. It was demonstrated that BAC induction significantly increased the ratios of phosphorylated NF-κB p65 to total NF-κB p65 in the blank control and solvent-treated groups, compared to those in the normal control group. However, the activation of NF-κB was inhibited significantly in both doxycycline-treated groups (Fig. 7).

**DISCUSSION**

Oral doxycycline has been applied to treat some ocular surface inflammatory diseases, such as meibomian gland dysfunction, mainly attributed to the anti-inflammatory properties by inhibiting MMP-9 and IL-1 synthesis. The anti-inflammatory effects of topical doxycycline on dry eye have not yet been elucidated clearly. Our study systematically investigated the
FIGURE 2. Representative images for immunofluorescent staining of Ki-67 to determine corneal epithelial cell proliferation in the normal control (A, B), blank control (C, D), solvent-treated (E, F), 0.025% DOX-treated (G, H), and 0.1% DOX-treated (I, J) groups. *Scale bars*: 20 μm.
FIGURE 3. Representative images for TUNEL assay to measure corneal epithelial cell apoptosis in the normal control (A, B), blank control (C, D), solvent-treated (E, F), 0.025% DOX-treated (G, H), and 0.1% DOX-treated (I, J) groups. Scale bars: 20 μm.
FIGURE 4. Representative images for immunofluorescent staining of K10 to determine corneal epithelial squamous metaplasia in the normal control (A, B), blank control (C, D), solvent-treated (E, F), 0.025% DOX-treated (G, H), and 0.1% DOX-treated (I, J) groups. Scale bars: 20 µm.
Both 0.025% and 0.1% doxycycline have been used to treat dry eye.

The mucin-filled goblet cells in the conjunctival fornices observed by PAS staining in the normal control (A), blank control (B), solvent-treated (C), 0.025% DOX-treated (D), and 0.1% DOX-treated (E) groups, and mean number of PAS-positive cells in each group (F). Six representative slices from homologous positions of each sample were selected (three samples for each group). Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars: 20 μm.

Effects of topical doxycycline on BAC-induced dry eye for the first time, and suggested that topical doxycycline effectively improved clinical evaluations, including BUT, fluorescein staining scores, and inflammatory index, and was capable of maintaining the corneal epithelial integrity, stabilizing the tear film, and inhibiting the corneal inflammation. Hence, doxycycline shows a potential as a topical anti-inflammatory agent in the treatment of dry eye.

We have reported previously that BAC-induced dry eye resulted in corneal epithelial pathologic changes, including cell apoptosis and squamous metaplasia that were related closely to inflammatory progression.51 Previous studies showed that increased MMP-9 and IL-1β induced the loss of corneal epithelial barrier function associated with the ocular surface inflammation,38,39 and topical doxycycline was found to preserve corneal epithelial smoothness and barrier function in a desiccating stress-induced mouse dry eye model.26,27 In this study, we demonstrated that topical doxycycline was capable of maintaining the integrity and normal phenotype of the corneal epithelium, evidenced by lower fluorescein staining scores, increased Ki-67-positive cells, and decreased TUNEL- and K10-positive cells. The suppression of inflammation might contribute to the pathologic ameliorations, which were consistent with the improvement of clinical evaluations. Both 0.025% and 0.1% doxycycline have been used to treat ocular surface disorders in different animal models.26,40 In this study, we suggested more improvement in epithelial and goblet cell proliferation, suppressing epithelial cell apoptosis and squamous metaplasia, and lower expression of MMP-9, proinflammatory cytokines, such as IL-1β, and chemokines, such as KC in the 0.1% doxycycline-treated group than those in the 0.025% group, which provided the reference data for clinical application of topical doxycycline in the treatment of dry eye.

Goblet cells, located on the apical surface of the conjunctiva, are responsible for secreting the gel-forming MUC5AC as the mucous component of the tear film.57 We observed significantly fewer mucin-filled goblet cells in dry eye condition and an increase in the number of these goblet cells after the doxycycline treatments. The goblet cells secreted mucins contributing to the stabilized tear film evidenced by the improvement of BUT in this study. Goblet cells have been reported to be very sensitive to ocular surface inflammation or abnormalities, which leads to their depletion on the ocular surface epithelia. The goblet cell content of the conjunctiva was regarded as a sensitive indicator of ocular surface diseases.51,42 We speculated that the protective effect of doxycycline on goblet cells may benefit by the suppression of inflammation and epithelial repair of the ocular surface. Interestingly, doxycycline has been reported to have a therapeutic role in attenuating mucus production, such as MUC5AC, by inhibiting MMP-9 expression in the airway epithelial cells.43,44 Since the expression pattern and function of mucins in the ocular surface apparently are different with those in the airway epithelium,55,44 the effects of doxycycline on the regulation of different mucins might not be the same. Further studies will be needed to investigate the mechanisms of doxycycline on goblet cell protection and mucin production in dry eye.

It has been recognized widely that the ocular surface inflammation has a critical role in the pathogenesis of dry eye. Increased proinflammatory cytokines and inflammatory cell infiltration are found in the ocular surface and lacrimal tissues of dry eye patients.5–47 Therefore, the efficacy of a variety of anti-inflammatory agents for the treatment of dry eye disease has been evaluated. The CSA decreases the local inflammation through its immune-modulatory activity, thereby improving dry eye symptoms and signs.48–50 Corticosteroids have an effect on nearly every aspect of the inflammatory process, and have been proven to decrease the production of inflammatory cytokines, chemokines, and MMPs, and stimulate lymphocyte apoptosis.51–53 However, long-term use of corticosteroids has potential sight-threatening side effects,7 and the drawbacks of topical CSA include stinging sensations and a long-term use.
required to achieve a maximal therapeutic effect.\textsuperscript{8} The tetracyclines have anti-inflammatory effects by decreasing the activity of collagenase, phospholipase A2, and several MMPs in a wide range of tissues.\textsuperscript{9,10,16,17} In the BAC-induced dry eye model, the expression of MMP-9, proinflammatory cytokines IL-1\textbeta, IL-6, and TNF-\textalpha, chemokines MIP-2, and KC, as well as infiltration of CD11b-positive macrophages in the corneas were elevated, mimicking the inflammatory features of human dry eye. After the doxycycline treatments, the expression of MMP-9, proinflammatory cytokines, chemokines, and macrophage infiltration were decreased, consistent with previous reports that doxycycline suppressed pathologically excessive MMPs and proinflammatory cytokines in vivo and in vitro.\textsuperscript{22,24,25,52}

It has been reported that dry eye condition stimulates MAPK activation, which leads to increases in the activity of NF-kB and inflammatory mediators, such as MMPs and cytokines.\textsuperscript{5,54,55} Doxycycline was demonstrated previously to inhibit the activation of p38 MAPK signaling markedly in response to the dry eye-associated inflammation.\textsuperscript{52} Our data suggested that BAC induction stimulated NF-kB activation, which was reduced significantly by topical doxycycline, consistent with reduced expression of inflammatory mediators. The NLRP3 (NOD-like receptor family pyrin domain-containing protein 3) inflammasome is implicated in several human autoimmune and inflammatory diseases\textsuperscript{56,57} as well as some ocular diseases,\textsuperscript{58-62} and it is important by converting immature IL-1\textbeta into its biologically active form through the activation of caspase-1. NLRP3 is transcriptionally upregulated by the NF-kB signaling.\textsuperscript{64,65} We propose that the inhibition of NF-kB signaling by doxycycline downregulated the expression of inflammatory molecules, such as IL-1\textbeta, TNF-\textalpha, and MMP-9, via some underlying mechanisms. Further studies are needed to clarify the precise mechanisms of doxycycline.
Topical Doxycycline and Dry Eye

In summary, our study showed that topical doxycycline ameliorated clinical evaluations, and protected the ocular surface epithelium and goblet cells by suppressing the inflammation in BAC-induced dry eye, and proposed that the anti-inflammatory mechanisms involved the inhibition of NF-κB activation and inflammatory mediators. Moreover, topical doxycycline is a preferred treatment with less potentially systemic side-effects. Hence, we suggested a potential for doxycycline as a topical anti-inflammatory agent in the clinical treatment of dry eye. Further clinical investigations are needed to assess the efficacy and safety of topical doxycycline for the treatment of dry eye.

Acknowledgments

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