Effect of 0.025% FK-506 Eyedrops on Botulinum Toxin B–Induced Mouse Dry Eye

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Purposes. To investigate the effect of FK-506 eye drops on Botulinum toxin B (BTX-B)-induced mouse dry eye.

Methods. Forty-five CBA/J mice were followed up for 4 weeks after treatment with 0.025% FK-506, vehicle or 0.9% saline eye drops 3 days after intralacrimal glands injection with 20 milliunits BTX-B. Tear production, corneal fluorescein staining, the mRNA, and protein expression of cytokines were measured. The activation of nuclear factor–κB (NF-κB) was detected by Western blotting. The infiltration of inflammatory cells was examined by immunohistochemistry.

Results. After treated with FK-506 eye drops, aqueous tear production in the mice began to recover at week 1, and then increased to the levels of pre–BTX-B injection at week 4 (2.21 ± 0.43 vs. 2.52 ± 0.71 mm, $t = 0.84$, $P > 0.05$). The severity of corneal epithelial defects was alleviated at week 2 and further improved at week 4 when compared with those in the vehicle- and saline-treated groups. The gene expression of IL-1β and TNF-α in the FK-506 and vehicle-treated groups were 47.01% and 45.56%, 85.91% and 115.83% of that in the saline-treated group in the ocular surface, while in the lacrimal glands 49.16% and 67.60%, 94.91% and 95.77% of that in the saline-treated group, respectively. The ratio of phosphorylated IκB-α to total IκB-α in the keratoconjunctival tissues was lower in the FK-506–treated group than in the vehicle- and saline-treated groups (both $P < 0.05$). No inflammatory cells were detected in all groups.

Conclusions. Topical application of FK-506 can inhibit NF-κB activation and related inflammatory response and alleviate the signs of dry eye.

Keywords: dry eye, inflammation, NF-κB, FK-506

Dry eye is a multifactorial disease of lacrimal functional unit characterized by symptoms of discomfort, visual disturbance, and tear-film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.[1] Although the definitive mechanism of dry eye is not exactly clarified, there is now increasing evidence indicating that inflammation plays a prominent role in the pathological process of this disease.[2] Inflammatory mediators, such as IL-1, IL-6, TNF-α, IL-8/CXCL8, and MIP-1α/CCL3 are increased in both the tear film and the ocular surface epithelia in patients with dry eye.[3–8] Much clinical evidence also demonstrated that the signs and symptoms of dry eye were relieved after treatment with anti-inflammatory drugs such as cyclosporine A (CsA), corticosteroids, and doxycycline.[9–11]

Cyclosporine A is now a therapeutic option for treating dry eye. Topical application of 0.05% and 0.1% CsA in dry eye patients has been shown to significantly improve in corneal fluorescein staining and tear production.[10,12] FK-506 is another immunosuppressant with similar a mechanism to CsA, but with 50 to 100 times more potency.[13,14] It forms complexes with an intracellular protein, FK-506-binding protein 12 (FKBP12), to block the activity of calcineurin and inhibits the activation of JNK, p38, and p65/nuclear factor–κB (NF-κB) through calineurin-independent pathways in T cells.[15–18] Systemic application of FK-506 has improved tear production in patients with dry eye associated with chronic graft versus host disease.[19,20]

Besides their character of immunosuppression, both CsA and FK506 showed inhibitory effects of noninfectious inflammation on nonimmune cells in a recent research.[21] Cyclosporine A eyedrops (0.05%) was demonstrated to effective for treating Botox-B (BTX-B)-induced dry eye, in the absence of T-cell infiltration.[22] However, there was no report on whether topical FK-506 could treat non-Sjögren's dry eye in the literature. The purpose of this study was to investigate the effects of topical application of FK-506 eye drops in an animal model of non-Sjögren's dry eye, BTX-B-induced mouse dry eye.[23]

Materials and Methods

Botulinum Toxin B–Induced Murine Dry Eye Model

Sixty female CBA/J mice of 6 to 8 weeks old (Jackson Labs, Bar Harbor, ME, USA) were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision

Research. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center at Sun Yat-sen University (Guangzhou, China).

The mouse model was created according to a previously reported method.[23] In brief, all mice were anesthetized with 45 mg/kg ketamine and 4.5 mg/kg xylazine. Then 0.05 mL 0.9% saline or 20 mU BTX-B in 0.05 mL 0.9% saline was injected into the right lacrimal gland through the conjunctiva with modified 33-G needles (Hamilton, Reno, NV, USA) under an operating microscope. All mice were maintained under relatively constant temperature (21°C–24°C) and humidity conditions (<20%).

Treatment of Dry Eye

Forty-five BTX-B–injected mice were randomized into three groups to receive topical medication four times daily with 0.025% tacrolimus eye drops (FK-506 group, this concentration was determined based on our previous experiments), vehicle drops (a menstruum used for dissolving FK-506 powder, vehicle group), or 0.9% saline sodium (saline group) 3 days after lacrimal gland injection for a 4-week trial period. Fifteen saline-injected mice were set to be blank controls (control group) without any treatment. FK-506 powder was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA), and dissolved in the mixture of normal saline, hydrogenated castor oil (20%, wt/vol), Tween-80 (5.5.%, vol/vol), hydroxymethyl cellulose (0.3%, wt/vol), glycerol (8%, vol/vol), and thiomersalate (0.002%, wt/vol; vehicle). The detailed preparation was described in a patent application by Zhongshan Ophthalmic Center (Patient application number: 200910038228.9). Tear production and ocular surface changes were evaluated in all the above four groups. At the end of 4 weeks following topical medication, all the mice were euthanized for tissue harvest.

Clinical Measurements

Measurements of aqueous tear production and corneal fluorescein staining were performed when the mice were unanesthetized as previously described.[23] In brief, phenol red-impregnated cotton threads (Zone-Quick; Oasis, Glendora, CA, USA) were applied in the lateral canthus of unanesthetized mouse for 15 seconds. The wet threads were measured under a microscope with a micron-scale digital ruler. Corneal epithelial defects were shown by fluorescein staining with 5 μL 1% sodium fluorescein solution (Sigma-Aldrich Corp.) and photographed with a digital camera (Nikon, Tokyo, Japan) adapted onto a slit-lamp microscope under cobalt blue light. The whole cornea of front view was divided into five equal sections for scoring.[1] Each section was scored separately. The total score was the sum of all the scores of the five sections. It was designated as score 0 when there was no staining, score 1 when sporadic spot staining, score 2 when diffuse spot staining, and score 3 when dense spot or mass staining. All measurements were performed 1 day before injection and at day 3, weeks 1, 2, and 4 after injection. The corneal staining was graded by one of the author (SXF), who did not know the identities of the mice, with cobalt blue lighting under slit-lamp biomicroscopy to prevent bias.

Histopathological and Immunohistochemical Examination

Four weeks after topical medication, five mice in each group were randomly killed and their eyeballs and lacrimal glands were collected. The eyeballs and lacrimal glands were fixed in a 10% neutral buffered formaldehyde solution and embedded in paraffin. The paraffin blocks of samples were cut until the first slide with corneal tissue or lacrimal glands was seen. Then the next three slides of 5-μm thick were collected for immunostaining and another three for hematoxylin-eosin (H&E) staining. These paraffin-embedded sections were dewaxed in xylene twice for 5 minutes and rehydrated in a series alcohol gradients. Antigen retrieval was performed by incubation in 10 mM of sodium citrate at 100°C for 30 minutes. The elimination of endogenous peroxidase activity was performed with 3% H_2O_2 solution in methanol. The sections were incubated with normal goat serum for blocking nonspecific staining and then with anti-CD4 antibody (1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD8 antibody (1:100; Santa Cruz Biotechnology), and anti-F4/80 antibody (1:150; Novus Company, Littleton, CO, USA) at 37°C for 1 hour, subsequently with horseradish peroxidase labeled anti-mouse goat IgG (Dako, Inc., Glostrup, Denmark) for 30 minutes at room temperature. Finally, sections were detected with 3,3′-diaminobenzidine tetrahydrochloride (Dako) and counterstained with hematoxylin for 3 seconds. Negative controls were performed by replacing primary antibodies with PBS. For histopathological study, the sections were deparaffinized and stained with H&E. The slides were visualized and photographed using an Olympus BX3 microscope (Olympus Corp., Tokyo, Japan).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Real-time qPCR was performed to detect the mRNA expression levels of gene IL-1β and TNF-α in mouse ocular surface tissues and lacimral glands. Mouse keratoconjunctival tissues and lacrimal glands ($n = 5$/group) were harvested on day 28 after treatment. Total RNA was extracted (RNeasy Mini Kit, Qiagen, Inc., Shanghai, China) from keratoconjunctival tissues and lacrimal glands according to the manufacturer's instructions. After quantification of the RNA concentration, total RNA was treated with DNase I (Sigma-Aldrich Corp.) to remove any contaminated genomic DNA. The first-strand cDNA was synthesized from 1 μg of total RNA using a cDNA synthesis kit (Fermentas International, Inc., Burlington, ON, Canada). Real-Time qPCR was performed using a SYBR Green I Master Kit (Invitrogen, Inc., Carlsbad, CA, US) on an ABI7000 Real-Time PCR Detection System (Applied Biosystems, Inc., Foster City, CA, US) in 20 μL reaction containing 2 μL cDNA. The sequences of the PCR primer pairs are listed in the Table. Thermal cycling consisted of denaturation for 3 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 30 seconds at 60°C. The PCR amplification efficiency of the primer sets has been determined to be essentially 100% before qPCR. Data were analyzed according to the comparative Ct (ΔΔCT) method and were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. Data are representative of three independent experiments.

Enzyme-Linked Immunosorbent Assay (ELISA)

The protein levels of cytokine IL-1β and TNF-α in the keratoconjunctival tissues and lacrimal glands ($n = 5$/group) were determined using ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). The keratoconjunctival tissues and lacrimal glands were homogenized with 0.5 mL lysis buffer (150 mM Tris-HCl at pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF) containing Complete

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Table. Primer Sequences for qRT-PCR in the Mice

Gene		Sequence	PCR Product Sizes (bp)
IL-1β (NM_008361.3)	Forward	CTCCATGAGCTTTGTACAAGG	247
	Reverse	TGCTGATGTACCAGTTGGGG	
TNF-α (NM_013693.2)	Forward	CAGCCTCTTCTCATTCCTGCTTG	133
	Reverse	GGGTCTGGGCCATAGAACTGA	
GAPDH (XM_003085774.1)	Forward	CACATTGGGGGTAGGAACAC	201
	Reverse	CTCATGACCACAGTCCATGC	

Protease Inhibitor Mixture (Roche Diagnostics, Basel, Switzerland). All samples were centrifuged at 12,000*g* for 5 minutes at 4°C, and each supernatant was assayed in duplicate for IL-1β and TNF-α proteins detection in accordance with the manufacturer's instructions. The reported sensitivity of these assays were 3.0 pg/mL for IL-1β and 3.0 pg/mL for TNF-α. The data were expressed as the target molecule (pg) per minigram total proteins for each sample. This experiment was repeated once.

Western Blot Analysis

Western blot analysis of the activation of NF-κB signaling pathway in the mouse keratoconjunctival tissues and lacrimal glands (n = 5/group) was performed by the ECL system. Briefly, tissues were individually homogenized in the ice-cold lysis buffer (150 mM Tris-HCl at pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, 1 mM Na_3VO_4, 1 mM NaF) containing Complete Protease Inhibitor Mixture (Roche Diagnostics). All samples were centrifuged at 13,000*g* for 25 minutes at 4°C. The supernatant was collected and stored at −80°C until assay. Total protein concentrations were measured by bicinchoninic acid (BCA) protein assay (Pierce Chemical Company, Rockford, IL, USA). For detection of total IκB-α and phosphorylated IκB-α, 40 μg total protein lysate was separated on a 12% SDS-PAGE. Then proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline supplemented with Tween 20 (TBST), and incubated overnight at 4°C with antibodies against total IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or phosphorylated IκB-α (Santa Cruz Biotechnology) and subsequent incubation with HRP peroxidase conjugated secondary antibody (Santa Cruz Biotechnology). The reaction was further detected by an ECL kit (Pierce Chemical Company) and exposed to high performance film (Eastman Kodak Company, New York, NY, USA). The films were scanned with Quantity One systems (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The images were analyzed with ImageJ software (V. 1.62; http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) to obtain integral optical density (IOD) readings of the target bands. Duplicate immunoblots were run for each sample. Data are the average of these two experiments.

Statistical Analysis

Statistical analysis was performed using the SPSS software for Microsoft Windows XP (V. 16.0; SPSS, Inc., Chicago, IL, USA). The Kruskal-Walis H test was used for comparing the scores of corneal fluorescein staining among the four groups, and the Mann-Whitney U test was used for comparing between any two groups. One-way ANOVA method was used for comparing the difference in tear production among the four groups, and Least-significant difference (LSD) analysis was used for comparison between any two groups. The data of real-time PCR, ELISA, and Western blot assay were first analyzed with a one way ANOVA and then Student's t-test. All data were shown as mean ± SD. A P value of less than 0.05 was considered statistically significant.

Results

All mice kept normal eyelid blinking after BTX-B injection. Before injection, there were no statistically significant differences in aqueous tear production and corneal fluorescein staining between all groups. The aqueous tear production was significantly decreased (Fig. 1), and corneal staining score increased (Fig. 2), in the mice at day 3 after intralacrimal gland injection of BTX-B compared with preinjection values (tears, 1.83 ± 0.42 vs. 2.66 ± 0.54mm, $t = 5.94$, $P < 0.001$; corneal staining score, 8.53 ± 1.64 vs. 0.00 ± 0.00, $Z = -20.13$, $P < 0.0001$). There were no statistically significant differences in aqueous tear production and corneal fluorescein staining among the three treatment groups at day 3 after BTX-B intralacrimal gland injection. It did not change at any time point in the mice of the blank control group (with saline injection). The signs of eye irritation, such as skin scratching

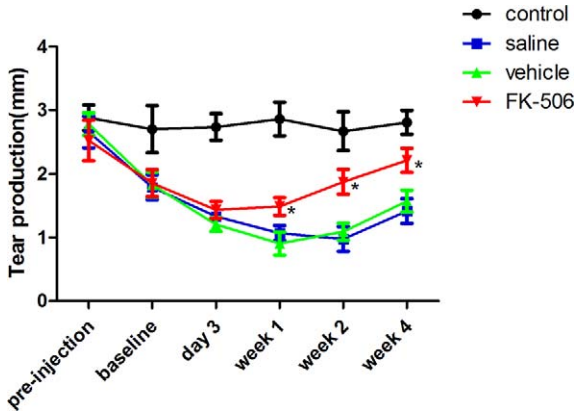

Figure 1. FK-506 promoted aqueous tear production in BTX-B induced dry eye model (n = 5/group). At day 3 after BTX-B injection, the tear production in all three groups was significantly decreased from 2.66 ± 0.54 to 1.83 ± 0.42 mm. The tear production increased at week 2 and then recovered to the pre–BTX-B injection level at week 4 after treatment with FK-506 eye drops. In comparison, the tear production in the vehicle and saline groups reduced further with time, but without statistical differences between them. (The "pre-injection" means the measurement 1 day before BTX-B injection. The "baseline" means the measurement 3 days after intra-acrimal gland injection without any treatment. Day 3 and week xx refers to the checkpoint time after treatment. *$P < 0.05$).

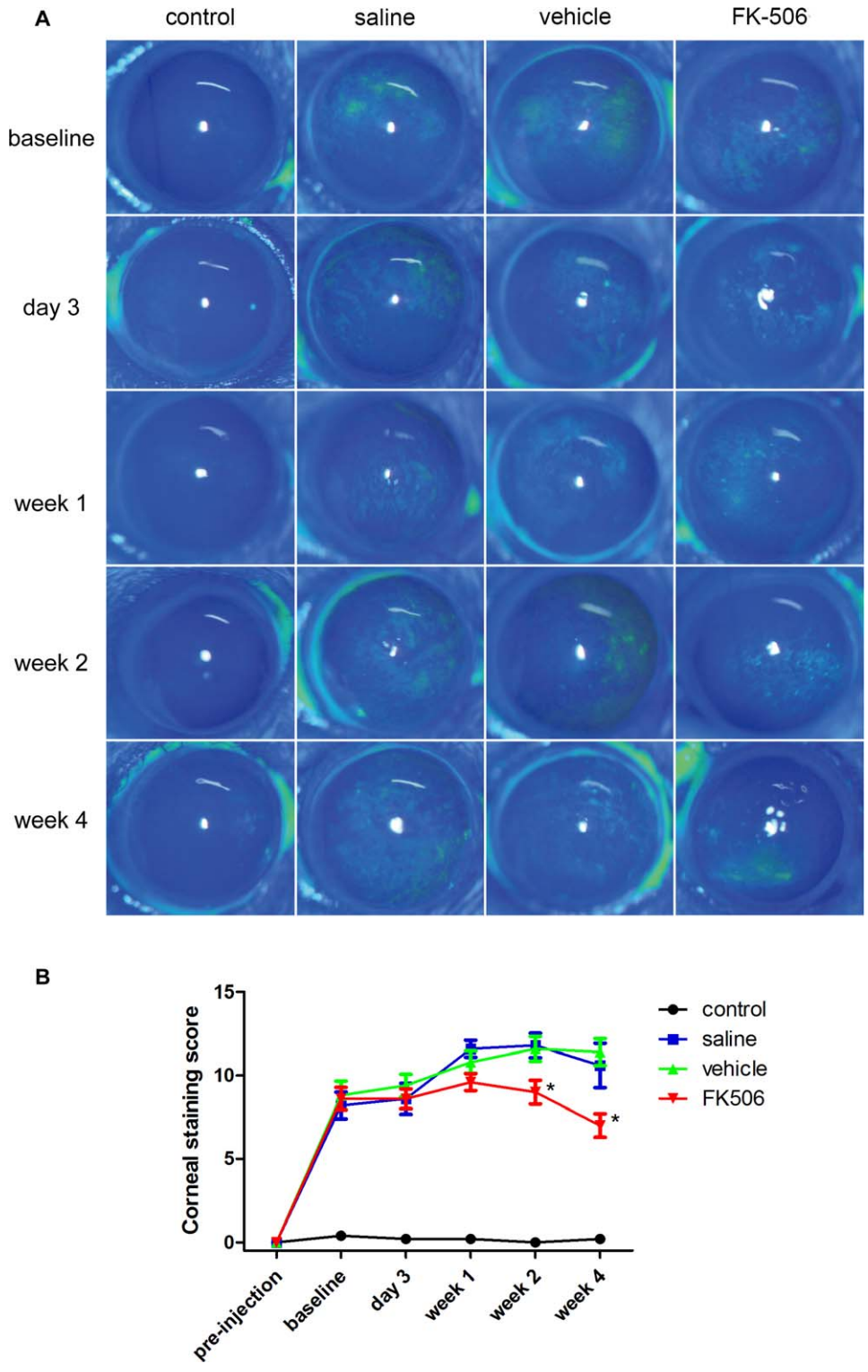

Figure 2. The severity of corneal staining was alleviated after use of FK-506 eye drops ($n = 5$/group). (**A**) Representative images of the mice corneas with fluorescein staining treated with saline, vehicle, and FK-506 after BTX-B injection. (**B**) The corneal staining scores were increased at day 3 after BTX-B injection. There were significant differences in the score of corneal staining at week 2 and 4 after treatment between the FK-506 group (9.00 ± 1.00 and 7.00 ± 1.58) and the vehicle group (11.60 ± 1.67 and 11.40 ± 1.82), as well as the saline group (11.80 ± 1.64 and 10.60 ± 2.97). There was no corneal staining in the control group at any time point (Baseline means the value at day 3 after intralacrimal gland injection without any treatment, $^*P < 0.05$.).

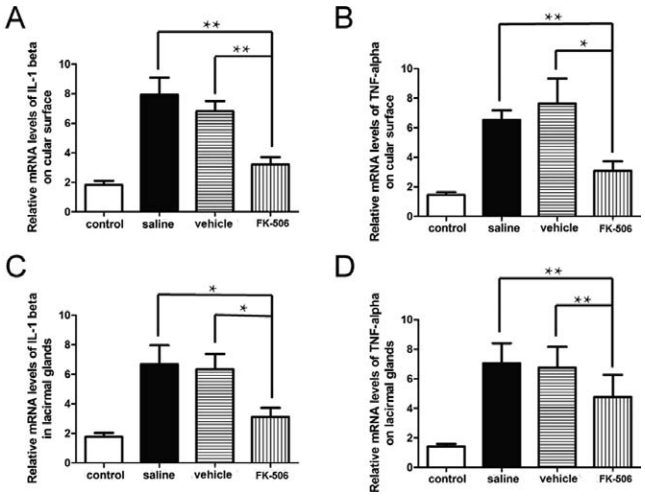

FIGURE 3. The expression of IL-1β and TNF-α mRNA was measured by qRT-PCR 4 weeks after treatment. All the data represented the relative fold change of mRNA expression of the genes of interest. The gene expression of IL-1β in the vehicle and FK-506 groups were 85.91% and 47.01% of those in the saline group in the ocular surface (**A**), while 94.91% and 49.16% in the lacrimal glands (**C**), respectively. The gene expression of TNF-α in the vehicle and FK-506 groups were 115.83% and 45.56% of that in the saline group in the ocular surface (**B**), while in the lacrimal glands 95.77% and 67.60% of that in the saline group (**D**), respectively. ($^{*}P < 0.05$, $^{**}P < 0.01$). The data are means + SD of five samples in each group, each were determined in triplicates.

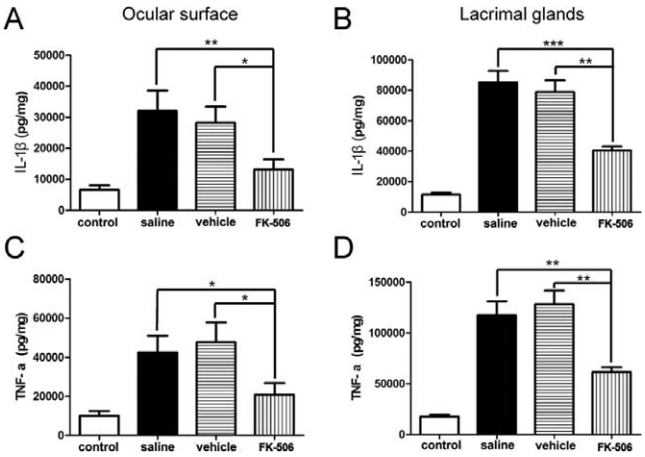

FIGURE 4. The expression of IL-1β and TNF-α protein were detected by ELISA 4 weeks after treatment. The levels of IL-1β protein in the ocular surfaces (**A**) and lacrimal glands (**B**) were 6580.9 ± 3371 pg/mg and 11496 ± 2915 pg/mg, 32040 ± 6545 pg/mg and 85190 ± 7515 pg/mg, 28244 ± 11671 pg/mg and 78822 ± 17236 pg/mg, and 13114 ± 7335.3 pg/mg and 40419 ± 5721 pg/mg, in the control, saline, vehicle, and FK-506 groups, respectively. The levels of TNF-α protein in the ocular surface (**C**) and lacrimal glands (**D**) were 10009 ± 5457 pg/mg and 17624 ± 4447 pg/mg, 42432 ± 19259 pg/mg and 117560 ± 30556 pg/mg, 47706 ± 22648 pg/mg and 128310 ± 29600 pg/mg, and 20294 ± 13101 pg/mg and 61566 ± 10311 pg/mg in the control, saline, vehicle, and FK-506 groups, respectively. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.01$). The data are means ± SD of five samples in each group, each were determined in duplicates.

and eye rubbing, were not observed after instillation of eye drops in all groups.

Effects of Topical FK-506 on Aqueous Tear Production

After treatment with FK-506 eye drops, the production of aqueous tears in the mice was greater than that of the vehicle group at week 1 and week 2. (FK-506 versus vehicle, week 1, 1.48 ± 0.31 vs. 0.90 ± 0.40 mm, $t = 2.55$, $P < 0.05$, week 2, 1.88 ± 0.43 vs. 1.09 ± 0.30 mm, $t = -3.33$, $P < 0.05$). Then the aqueous tear production recovered to the pre–BTX-B–injection level after 4-weeks treatment in FK-506 group (2.21 ± 0.43 vs. 2.52 ± 0.71 mm, $t = 0.84$, $P > 0.05$). Meanwhile, tear production was significantly decreased compared with the BTX-B–injection level at all points in the vehicle and saline groups (Fig. 1).

Effects of Topical FK-506 on Ocular Surface Status

In all mice injected with BTX-B, the cornea showed diffuse punctuate defects. The score of corneal fluorescein staining reached its first peak from the score of 0 (pre–BTX-B injection) to 8.5 ± 1.6 at day 3 after injection. The corneal epithelial defects became more severe later on in the vehicle and saline groups. After treatment with FK-506, the scores of corneal epithelial staining decreased from 9.60 ± 1.14 (baseline) to 9.00 ± 1.58 at week 2 ($Z = -0.64$, $P > 0.05$), and to 7.00 ± 1.58 at week 4 ($Z = -2.27$, $P < 0.05$). The severity of corneal epithelial defects was significantly alleviated in 2 weeks (9.00 ± 1.00 vs. 11.60 ± 1.67, $Z = -2.12$, $P < 0.05$) and further improved at week 4 (7.00 ± 1.00 vs. 11.40 ± 1.81, $Z = -2.63$, $P < 0.05$) when compared with that of vehicle group (Fig. 2).

Effects of Topical FK-506 on Inflammatory Cytokines and Its Associated Signaling Pathway Expressed in Keratoconjunctival Tissues and Lacrimal Glands

The mRNA and protein levels of TNF-α and IL-1β were increased after injection of BTX-B in lacrimal glands in both keratoconjunctival tissues and lacrimal glands. The gene expression of TNF-α and IL-1β in saline group were 446% and 435% of that in the control group in the ocular surface, while 49% and 358% of that in the control group in the lacrimal glands. The protein levels of TNF-α and IL-1β expressed in the ocular surface increased from 10009 ± 5457 and 6580.9 ± 3371 pg/mg in the saline group to 42432 ± 19259 and 32 040 ± 6545 pg/mg compared with control group, while in the lacrimal glands from 17624 ± 4447 and 11496 ± 2.915 pg/mg to 117560 ± 30556 and 85190 ± 7515 pg/mg. The results also showed that the mRNA and protein levels of TNF-α and IL-1β, which expressed in both keratoconjunctival tissues and lacrimal glands, were significantly lower in the FK-506 group those in the saline and vehicle groups (Figs. 3, 4). Meanwhile, the levels of activated NF-κB signaling (phosphorylated IκB-α/total IκB-α) in the ocular surface tissues (Fig. 5A) were upregulated after injection of BTX-B in lacrimal glands as compared with the control group (0.17 ± 0.03 vs. 0.74 ± 0.16, $t = -5.30$, $P < 0.05$). There was no significant increase in the lacrimal glands (Fig. 5B, 0.34 ± 0.10 vs. 0.36 ± 0.07, $t = -0.18$, $P > 0.05$). The percentage of phosphorylated IκB-α in the keratoconjunctival tissues of mice was lower in the FK-506 group (0.38 ± 0.05) than in the saline (0.56 ± 0.07, $t = 3.50$) and vehicle groups (0.55 ± 0.08, $t = 3.00$; both $P < 0.05$; Fig. 5C).

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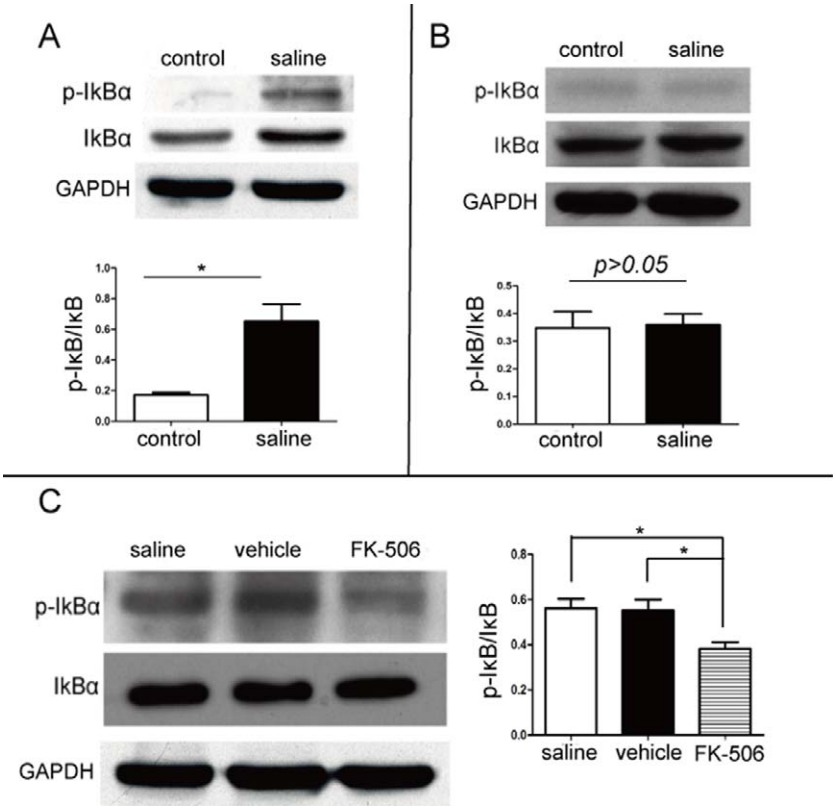

Figure 5. The level of phosphorylated IκB-α protein (p-IκB-α) was detected by Western blotting analysis 4 weeks after treatment. The percentage of p-IκB-α to total IκB-α (ratio of integral optical density) was calculated as the level of phosphorylated IκB-α. In the keroconjunctival tissues (**A**) and lacrimal glands (**B**), the density of phosphorylated IκB-α/total IκB-α in keroconjunctival tissues, and lacrimal glands were 303.99 ± 61.74/469.53 ± 144.35 and 405.08 ± 86.85/1112.6 ± 115, respectively, in the saline group. While density of phosphorylated IκB-α/total IκB-α in keroconjunctival tissues, and lacrimal glands were 69.20 ± 17.45/403.07 ± 36.63 and 370.11 ± 110.92/1064.3 ± 63.69, respectively in the control group. In the keroconjunctival tissues after treatment (**C**), the density of phosphorylated IκB-α/total IκB-α in keroconjunctival tissue was 629.77 ± 117.97/1646.9 ± 79.21 in the FK-506 group, 738.12 ± 151.19/1329.70 ± 95.57 in vehicle group and 838.15 ± 69.94/1511.00 ± 280.74 in the saline group. (*$P <$ 0.05). The figure is a representative of five samples in each group and each sample were determined by duplicate immunoblots.

Infiltrated Inflammatory Cells in the Ocular Surface and the Lacrimal Glands After Application of Topical FK-506

Histopathological results showed that there were no obvious inflammatory cells in the ocular surface and the lacrimal glands of the mice in all groups (Fig. 6). Immunohistochemical results confirmed that there were no CD4-, CD8-, and F4/80-positive cells in the ocular surface and the lacrimal glands of the mice in all groups.

Discussion

Dry eye is an ocular disease with multiple causes. Inflammation is one of the key mechanisms in the development of dry eye.[24] Anti-inflammatory therapy is required for patients, particularly if inflammatory signs and irritation symptoms are observed.[25] Cyclosporine A eye drops 0.05% is the only commercial and nonsteroid topical drug for treating dry eye in clinic.[10,12] In this study, we demonstrated that topical application of another immunosuppressant agent, 0.025% FK-506 eye drops, could increase aqueous tear production, reduce corneal epithelial defects, and alleviate inflammatory cytokines secretion and activation of its signaling pathway in both keratoconjunctival tissues and lacrimal glands of the mice with BTX-B–induced dry eye. This indicates that 0.025% FK-506 eye drops could effectively improve the signs of dry eye through its anti-inflammatory role.

Several animal models of dry eye have been established to investigate the pathogenesis of dry eye. Among these models, the mouse model of BTX-B–induced dry eye was reported to manifest non-Sjögren's dry eye.[23] In this model, the state of dry eye can keep more than 1 month, which is more consistent with the chronic condition of human dry eye than other animal models.[23,26–28] In this study, we observed similar changes in tear production, corneal fluorescein staining, and inflammatory cytokines after BTX-B injection as in previous reports.[23,29,30] After topical application of 0.025% FK-506 eye drops, the tear production and the state of ocular surface began to recover within 2 weeks and reached the preinjection normal state in 4 weeks.

It has demonstrated that FK-506 is 50 to 100 times potency than CsA for immunosuppression. However, FK-506 was compared with CsA for treating dry eye. The effective concentration of FK-506 for treatment of dry eye was 0.025%, whereas the effective concentration of CsA for treatment of dry eye was 0.05%.[22] It appears that FK506 was 2-fold more potent than CsA in treatment of dry, much lower than comparative potency in their immunosuppressive effects. This may be due to the differences in drug accumulation, distribution, and clearance. The molecular weight of FK-506 and CsA are 822 and 1202 daltons, and the partition coefficient (log PC) of FK-506 and CsA was 2.74 and 3.14 in an octanol/water system, which indicates that FK-506 is less lipophilic

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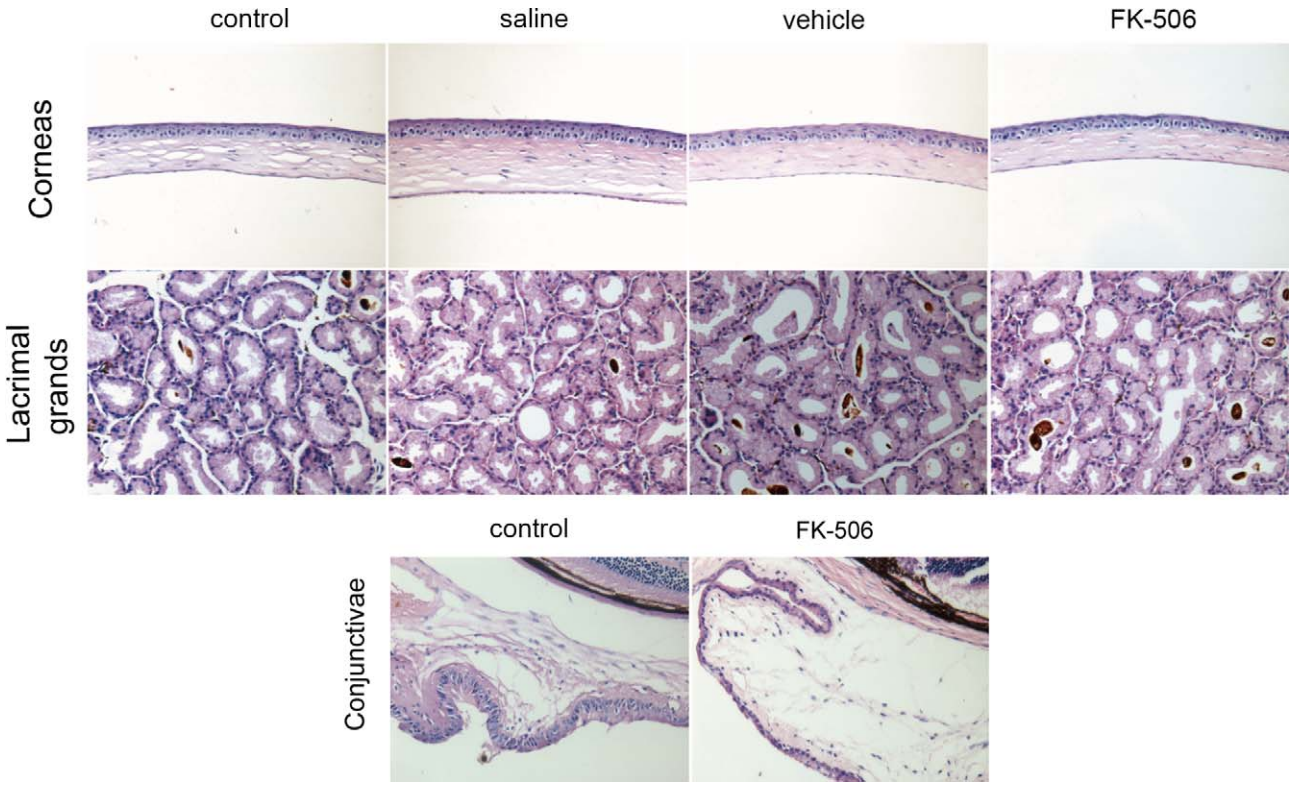

Figure 6. Histopathologic examination of the corneal and lacrimal glands after 4 weeks of topical medication ($n = 5$/group). Hematoxylin and eosin staining of the mice corneas and lacrimal glands (magnification, ×400). There were no lymphocytic and inflammatory cells infiltrated into the ocular surface and the lacrimal glands in all groups.

than CsA, and therefore enters the cornea less.[31,32] Previous reports showed that the accumulation of FK-506 in the corneal epithelium was less than that of CsA, and the distribution and clearance of FK-506 in the cornea were faster than those of CsA.[33,34] Another possible reason is their different anti-inflammatory mechanisms. In addition to inhibition of calcineurin, like FK-506 does, CsA could inhibit apoptosis by blocking the opening of mitochondrial permeability transition pore.[35]

As demonstrated in many previous reports, the tears are hyperosmotic in dry eye.[36,37] The state of tear hyperosmolarity will activate mitogen-activated protein kinase (MAPK) cascades in corneal and conjunctival epithelial cells.[38,39] The activated kinases initiate a cascade of protein phosphorylation involving multiple kinases and activate nuclear transcription factors such as NF-κB, activating protein 1, and activating transcription factor, and then stimulate expression of inflammatory cytokines (e.g., IL-1β and TNF-α).[40–42] This study also showed that the expression levels of IL-1β and TNF-α were downregulated in both lacrimal glands and keratoconjunctival tissues after use of FK-506. FK-506 was reported to be capable of inhibiting the activation of T lymphocytes, and then decreasing the production of inflammatory cytokines.[17] However, T cells and macrophages were not detected in either the ocular surface or the lacrimal gland of the BTX-B–injected mice in this study. So the decrease of TNF-α and IL-1β in the ocular surface and lacrimal glands should attribute to the inhibition of NF-κB activation in the epithelium of ocular surface and lacrimal gland after treatment with FK-506 eye drops. For those noninflammatory cells such as conjunctival and corneal epithelial cells immersed in the hyperosmotic tears, NF-κB signaling plays multiple roles as a broad pathway. In a rat renal tubular epithelial cell line (NRK-52E cells), FK-506 was reported to suppress hypertonic medium activated NF-κB signaling via unfolded protein response.[21] In this research, the phosphorylation of IκB-α was reduced along with a decrease of IL-1β and TNF-α in dry eye after treatment with FK-506. The IκB proteins retain NF-κB in the cytoplasm through masking of the nuclear localization sequences. Phosphorylation of IκB proteins (especially IκB-α) allows nuclear-localization sequences on NF- κB subunits be exposed from the complex of IκB-NF-κB, and then the inhibition of NF-κB will be released.[43] So the reduction of IκB-α phosphorylation would decrease the activation of NF- κB signaling and then the production of inflammatory cytokines in the ocular surface epithelium and lacrimal glands. The reduction of IL-1β and TNF-α production may decrease the activation and maturation of immature antigen-presenting cells and downregulate the expression of adhesion molecules, and thus alleviate the ocular surface epithelium from damage.[44,45]

In summary, topical administration of 0.025% FK-506 eye drops for 2 to 4 weeks could successfully ameliorate the signs of dye eye, including tear production and corneal epithelial defects. These effects may be due to the reduction in the production of inflammatory cytokines through suppressing the activation of NF-κB signaling by FK-506. FK-506 eye drops 0.025% is a promising agent for treating dry eye as a nonsteroid drug.

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