

Rebamipide ophthalmic solution modulates the ratio of T helper cell 17/regulatory T cells in dry eye disease mice

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Abstract. The aim of the present study was to confirm the effect of 2% rebamipide ophthalmic solution on a scopolamine-induced dry eye (DE) mouse model, and to investigate its effect on the ratio of T helper cell 17 (Th17)/regulatory T cell (Treg) numbers. C57BL/6 mice received subcutaneous injections of scopolamine and were exposed to a low-humidity environment in order to establish a DE model. Rebamipide eye drops (2%) administered four times daily for 2 weeks, significantly reduced the corneal staining scores and increased the tear film breakup time and tear production in the DE mice. Pathologically, the rebamipide restored the histological changes induced by DE in the cornea, conjunctiva and lacrimal gland. At a molecular level, it downregulated pro-inflammatory and upregulated anti-inflammatory cytokines in the conjunctiva and lacrimal gland. Furthermore, the increased Th17 and Treg levels were restored following treatment with rebamipide. In conclusion, the anti-inflammatory and Th17/Treg balance-preserving effects of rebamipide may contribute to the treatment of DE in mice.

Introduction

Dry eye (DE), a common ophthalmic disorder, is defined as a multifactorial disease of the tears and ocular surface that results in 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). DE may be induced by a number of causes, including aging, excessive computer use, contact lenses usage, androgen deficiency, medication and air pollution (2). Worldwide, 5-30% of the population suffer

from DE (3). Although vision may not be adversely affected, DE causes various problems, including tearing, dryness and photophobia, leading to a measurable adverse impact on visual function and daily life (4,5). If untreated, DE does not heal naturally and its symptoms persist. However, treatments for the improvement of the symptoms and signs may not cure the condition completely (6). Current DE treatments aim to attenuate the clinical symptoms and objective signs, and improve the life quality of patients.

Rebamipide ophthalmic solution was developed by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan), and launched in Japan for the treatment of DE (Mucosta[®] ophthalmic suspension, unit dose 2%). Rebamipide has been used as a therapeutic agent for gastritis and gastric ulcers due to its anti-inflammatory, antioxidant and gastric epithelial mucin-promoting effects (7-11). The use of rebamipide has been extended to DE treatment due to the discovery of its ocular surface mucin-increasing action. The compound was revealed to increase mucin-like substances on the cornea and conjunctiva of N-acetylcysteine-treated rabbits (12). This outcome has been confirmed *in vitro* in cultured conjunctival goblet cells and corneal epithelial cells (13-16). In addition, rebamipide has been demonstrated to exert an anti-inflammatory response in ocular autoimmune lesions in a Sjogren's syndrome mouse model (17). In clinical studies, rebamipide has been demonstrated to be effective in improving the symptoms and signs of DE (18-20). It has also been demonstrated to be well tolerated in a 52-week trial (21). Studies over the past five years have further evaluated the action of rebamipide on various types of DE. Their results revealed that the regimen is effective in treating keratoconjunctivitis sicca in patients with Sjögren syndrome (22), chronic ocular graft-versus-host disease-associated DE and ocular cicatricial pemphigoid-like disease (23), and postoperative DE (24).

Regarding the pharmacological mechanisms of rebamipide in DE, the majority of studies have focused on mucin production. One previous study indicated that a T helper cell 17 (Th17)/T regulatory cell (Treg) imbalance occurs in DE, and interleukin (IL)-17 blockage reduces the severity of the disease, indicating that the measurement of the Th17/Treg ratio may have a diagnostic use in the treatment of DE (25). In the present study, the effects of rebamipide on the modulation of the Th17/Treg balance were evaluated, and possible novel mechanisms of action on rebamipide in DE treatment were investigated.

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Materials and methods

Animals. For the present study, a total of 40 female C57BL/6 mice (8-week-old, 20–22 g) obtained from the Experimental Animal Center of China Medical University (Shenyang, China) were housed in 50–60% humidity and kept on a 12-h light/dark cycle at 22±2°C with free access to food and water. The experimental protocol was approved by the Animal Ethics Committee of The Fourth Affiliated Hospital of China Medical University.

Experimental procedure. DE was induced as described previously (26). Briefly, the mice were randomly divided into four groups: Control (con); DE model (DE); DE with balanced salt solution treatment (DE+vehicle); and DE with 2% rebamipide treatment (DE+Reb) (n=10 mice/group). The mice in the three DE groups were exposed to an environment of <20% relative humidity, 21–23°C and airflow of 15 l/min for 16 h/day, and received subcutaneous injections of scopolamine hydrobromide (0.5 mg/0.2 ml resolved in saline; Dalian Meilun Biotech Co., Ltd., Dalian, China) at 8:00 a.m., 11:00 a.m., 2:00 p.m. and 5:00 p.m. daily for 2 weeks. The mice in the control group were placed in a normal environment without airflow and received subcutaneous injections of an equal volume of saline at the same time points. Following the establishment of the model, the mice in the DE+Reb group were treated with 2% rebamipide eye drops (Mucosta®; Otsuka Pharmaceutical Co., Ltd.) four times daily for 2 weeks. The mice in the DE+vehicle group were treated with salt solution at the same time points.

Tear secretion and stability of tear film examinations. Tear secretion was evaluated using the phenol red thread test. The mice were fixed in a 50-ml tube with the bottom cut off and phenol red-impregnated cotton threads (Tianjin Jingming New Technological Development Co., Ltd., Tianjin, China) were applied to the lateral canthus for 15 sec. The length of wetting of the thread, which turned red, was measured (mm).

The stability of the tear film was examined using a tear film breakup time (BUT) assay and corneal fluorescein staining. The mice were anesthetized with 50 mg/kg pentobarbital sodium (intraperitoneal) and 1 µl 1% fluorescein sodium (Alcon Laboratories, Inc., Duluth, GA, USA) were applied to the conjunctival sac. The BUT was recorded as the duration between the natural blink response of the mice to the appearance of a dark area, representing tear film breakup, using slit-lamp biomicroscopy and a cobalt blue light (BX900; Haag-Streit AG, Koeniz, Switzerland). The BUT was recorded three times and the average time was used. Then excess fluorescein sodium was rinsed using PBS and the corneal epithelium deficit was evaluated using fluorescein punctate staining the duration of which depended on the natural blink response time of each mouse. The staining was scored according to previously established standards (27).

Histological examination. From each group, six mice were randomly selected for histological examination. Following rebamipide administration, the mice were sacrificed by deep anesthesia and the cornea and lacrimal gland were collected and fixed in 10% neutral formalin at 4°C for 24 h. The fixed samples were embedded in optimal cutting temperature (OCT)

medium and cut into 8-µm-thick cryosections. The sections were stained with hematoxylin for 5 min and eosin for 3 min at room temperature. The histological alterations in the cornea and lacrimal gland were observed under a light microscope at x400 or x200 magnification (DP73; Olympus Corporation, Tokyo, Japan).

ELISA. ELISA was used to measure the levels of IL-1β, IL-6, tumor necrosis factor-α (TNF-α), IL-10, IL-17, and IL-23 in the conjunctiva and lacrimal gland. The tissues were homogenized in PBS at 4°C and subjected to three freeze-thaw cycles. The homogenates were centrifugated at 10,000 x g at 4°C for 20 min. The supernatant was collected for measurement. The measurements were performed using commercial ELISA kits [MultiSciences (Lianke) Biotech Co., Ltd., Hangzhou, China], according to the manufacturer's protocols. The catalog number of each kit was: IL-1β: EK201B2/2; IL-6: EK2062/2; TNF-α: EK2822/2; IL-10: EK2102/2; IL-17: EK2172/2; IL-23: EK2232.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the conjunctiva and lacrimal gland samples using the RNAPure total RNA Fast Isolation kit (BioTeke Corporation, Beijing, China). The isolated RNA was quantified and reverse transcribed in a 20 µl reaction system containing 1 µg RNA sample, 1 µl random primer, 2 µl dNTP, 1 µl oligo(dT)₁₅, 4 µl 5XBuffer, 0.5 µl RNasin and 1 µl Moloney murine leukemia virus reverse transcriptase. Reverse transcription reactions were carried out at 42°C for 50 min and 95°C for 5 min. RT-qPCR was performed using 2X Power Taq PCR Master Mix (BioTeke) and SYBR Green (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) on an Exicycler 96 real-time PCR system (Bioneer Corporation, Daejeon, Korea). All reactions were incubated at 94°C for 10 min, 60°C for 20 sec, and 72°C for 30 sec, followed by 40 cycles of 72°C for 2 min 30 sec, 40°C for 5 min 30 sec, 60°C for 30 sec, and 25°C for 1 min. The primers used are listed in Table I. The relative expression of the genes was measured against β-actin and was calculated using the 2^{-ΔΔC_q} method (28).

Immunofluorescence staining. The fixed samples were embedded in OCT medium and cut into 10-µm-thick cryosections. The sections were boiled in a citrate buffer in a microwave oven for 10 min for antigen retrieval. Following blocking in 10% goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at room temperature, the sections were incubated with anti-keratin 10 (K10; 1:50; cat. no. sc-53252), or anti-IL-17 (1:50; cat. no. sc-374218) (both Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-cluster of differentiation (CD)4 (1:100; cat. no. 19068-1-AP; Proteintech Group, Inc., Chicago, IL, USA) antibodies or with anti-forkhead box P3 (Foxp3; 1:50; cat. no. sc-53876; Santa Cruz Biotechnology, Inc.) and anti-CD4 (1:100) antibodies at 4°C overnight. Following washing, the sections were incubated with fluorescein isothiocyanate-conjugated goat/anti-mouse (1:200; A0568; Beyotime Institute of Biotechnology, Haimen, China) and Cy3-conjugated goat/anti-rabbit secondary antibodies (1:200; A0516; Beyotime Institute of Biotechnology) for

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction analysis.

Genes	Upstream	Downstream
IL-1 β	5'-TTGGGCCTCAAAGGAAAGAAT-3'	5'-TGCTTGTGAGGTGCTGATGTA-3'
IL-6	5'-TGTATGAACAACGATGATGCAC-3'	5'-CTGGCTTTGTCTTTCTTGTT-3'
TNF- α	5'-GAGTCCGGGCAGGTCTACTT-3'	5'-CCAGGTCAGTGTCCAGCATC-3'
IL-17	5'-TGTGTCTCTGATGCTGTTGCT-3'	5'-TTCATTGCGGTGGAGAGTC-3'
IL-23	5'-GCCTGCTCTACTCCCTGAT-3'	5'-GTTGGCACTAAGGGCTCA-3'
IL-10	5'-GAAGACAATAACTGCACCCACT-3'	5'-ACCCAAGTAACCCTTAAAGTCC-3'
β -actin	5'-CTGTGCCCATCTACGAGGGCTAT-3'	5'-TTTGATGTACGCACGATTTC-3'

IL, interleukin; TNF, tumor necrosis factor.

60 min at room temperature. The staining was observed under a BX50 fluorescence microscope (Olympus Corporation) at $\times 400$ magnification.

Statistical analysis. The data are expressed as the mean \pm standard deviation. Experiments were repeated 6 or 10 times as described above. Groups were compared using one-way analysis of variance followed by Tukey's post hoc test for data with a normal distribution, and by Games-Howell analysis with Dunn's multiple comparisons test for skewed data using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Rebamipide attenuates the symptoms of DE. The effects of rebamipide on the symptoms of DE were evaluated by the examination of corneal fluorescein staining, BUT and tear production. A total of 4 weeks after the model was established, the mean corneal fluorescein staining score was significantly increased in the DE group (8.10 ± 1.37) compared with the control group (1.30 ± 0.48) ($P < 0.01$). Vehicle treatment for 2 weeks did not affect the score (7.90 ± 1.37 ; $P > 0.05$ vs. the control group). However, following 2 weeks of treatment with 2% rebamipide, the corneal fluorescein staining score decreased to 3.5 ± 0.71 , which was significantly lower compared with that in the DE+vehicle group ($P < 0.01$) (Fig. 1A and B).

Regarding the measurement of tear film BUT, the results revealed that the values taken from the DE group were significantly lower compared with those from the control group (2.80 ± 1.22 vs. 12.70 ± 1.42 ; $P < 0.01$), and rebamipide treatment partly restored this change (5.70 ± 1.16 ; $P < 0.05$ vs. the DE+vehicle group) (Fig. 1C). In accordance with the results from the BUT measurement, the tear volume (represented as the wetting length of the phenol red thread) in the DE group was markedly reduced compared with the control group (0.51 ± 0.17 vs. 4.50 ± 0.29 ; $P < 0.01$), and rebamipide treatment slightly but significantly increased the tear production in DE mice (0.93 ± 0.17 ; $P < 0.01$ vs. the DE+vehicle group). The tear production in the vehicle group demonstrated no statistically significant difference when compared with that in the DE group (0.54 ± 0.16 ; $P > 0.05$) (Fig. 1D).

Rebamipide attenuates the histological changes in the cornea and lacrimal glands in DE mice. Hematoxylin and eosin staining revealed that superficial epithelial cells in the corneas of the control mice were well arranged and tightly attached, and the surface of the cornea was smooth (Fig. 2A). However, the epithelial layers of the cornea were disrupted, and the epithelial cells were desquamated in the DE mice. In addition, the lacrimal glands in the control group exhibited a unbroken structure and uniform size of the acinus (Fig. 2B). In the DE mice, the lacrimal glands exhibited notable vacuolization and a disordered cellular arrangement. The vehicle did not affect the histological alterations in the lacrimal glands, whereas 2% rebamipide administration markedly attenuated these pathological alterations.

Rebamipide increases the density of goblet cells and decreases squamous metaplasia. In the conjunctival sections, the number of goblet cells was decreased in the DE mice compared with that in the control mice (Fig. 3). Rebamipide treatment significantly increased the number of goblet cells. Furthermore, epidermis-specific K10 expression was examined in the corneal epithelia. In the control group, few K10-positive epithelial cells were observed in the cornea, whereas DE induced a marked increase in the expression of K10, which is a hallmark of squamous metaplasia. Rebamipide treatment significantly weakened the intensity of K10 expression compared with that observed in the vehicle-treated group (Fig. 3).

Rebamipide modulates the production of inflammatory-associated factors. To further investigate the alterations in inflammatory-associated factors following rebamipide treatment, the levels of known pro- and anti-inflammatory cytokines in the lacrimal gland and corneal tissues were determined. As demonstrated in Fig. 4, the mRNA expression levels and protein content of the pro-inflammatory cytokines IL-1 β , IL-6, TNF- α , IL-17 and IL-23 were significantly enhanced, whereas the anti-inflammatory cytokine IL-10 was significantly decreased, in the samples from DE mice. Treatment with 2% rebamipide for 2 weeks markedly reversed these effects.

Rebamipide modulates Th17 differentiation in the ocular microenvironment of DE mice. To evaluate the impact of

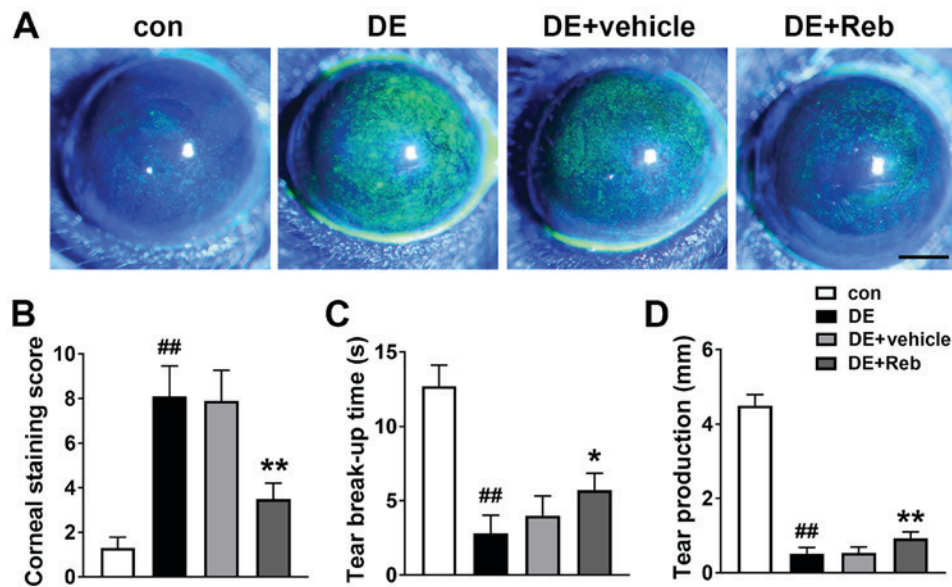


Figure 1. Rebamipide (2%) improves the clinical symptoms of DE in mice. (A) Corneal fluorescein staining in the various groups of mice. Scale bar=1 mm. (B) Quantification of corneal fluorescein staining. (C) Tear film breakup time. (D) Tear production expressed as the length of wetting of the thread. The data are expressed as the mean \pm standard deviation (n=10). ^{##}P<0.01 vs. the control group; ^{*}P<0.05 vs. the DE+vehicle group; ^{**}P<0.01 vs. the DE+vehicle group. con, control; DE, dry eye; DE+vehicle, dry eye group treated with vehicle; DE+Reb, dry eye group treated with 2% rebamipide.

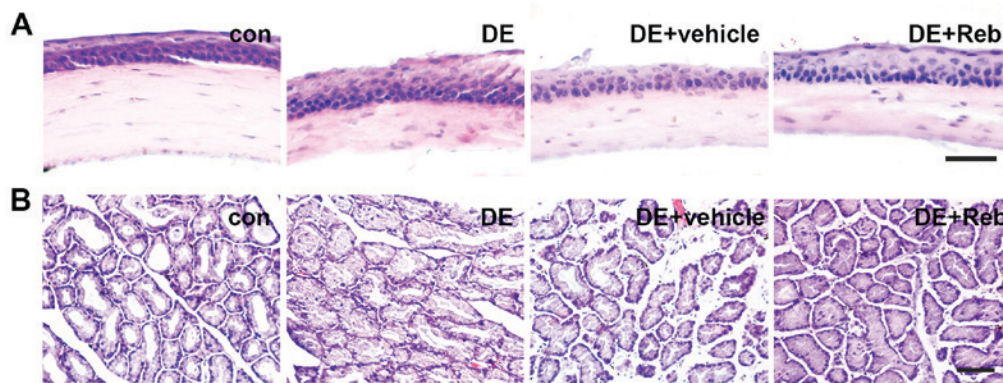


Figure 2. Rebamipide (2%) attenuates the histological changes in the cornea and lacrimal glands in DE mice. Hematoxylin and eosin staining of (A) the cornea and (B) the lacrimal glands. Scale bars, 50 and 100 μ m, respectively. con, control; DE, dry eye; DE+vehicle, dry eye group treated with vehicle; DE+Reb, dry eye group treated with 2% rebamipide.

rebamipide on the ratio of Th17 and Tregs in the ocular micro-environment, double-immunofluorescence was performed to locate CD4⁺/IL-17⁺ and CD4⁺/Foxp3⁺ double-positive cells in the cornea and lacrimal gland. As demonstrated in Fig. 5, more CD4⁺/IL-17⁺ and CD4⁺/Foxp3⁺ cells were observed in the cornea and lacrimal gland specimens from the DE mice compared with those of the control, indicating that DE altered the ratio of Th17/Tregs in the ocular microenvironment. Treatment with rebamipide (2%) led to decreases in the numbers of CD4⁺/IL-17⁺ cells and CD4⁺/Foxp3⁺ cells.

Discussion

In the present study, the effect of 2% rebamipide on scopolamine-induced DE mice was confirmed. Rebamipide eye drops led to the restoration of corneal, conjunctival and lacrimal gland DE-induced alterations, and the regulation of the secretion of inflammatory cytokines. Furthermore, it was

revealed that rebamipide modulated the Th17/Treg imbalance by suppressing Th17 cells and increasing the differentiation of Treg cells.

The tissue components that comprise the ocular surface are responsible for maintaining its integrity and function. The cornea is one of the most important tissues in the maintenance of visual function and microenvironment of ocular surface. The cornea consists of epithelial cells and keratocytes. The epithelial layer is directly exposed to the external environment, and the tightly arranged epithelial cells provide protection from exposure to pathogens. The corneal keratocytes are distributed in the stroma and maintain the extracellular matrix by synthesizing glycosaminoglycans and collagen. In DE, desiccation and excessive inflammation lead to corneal thinning and the reduction of superficial corneal epithelial microvilli (29,30). In the present study, a relatively thin cornea and disordered epithelial and stromal layers were observed in DE mice. In addition, the epithelial cells were detached from

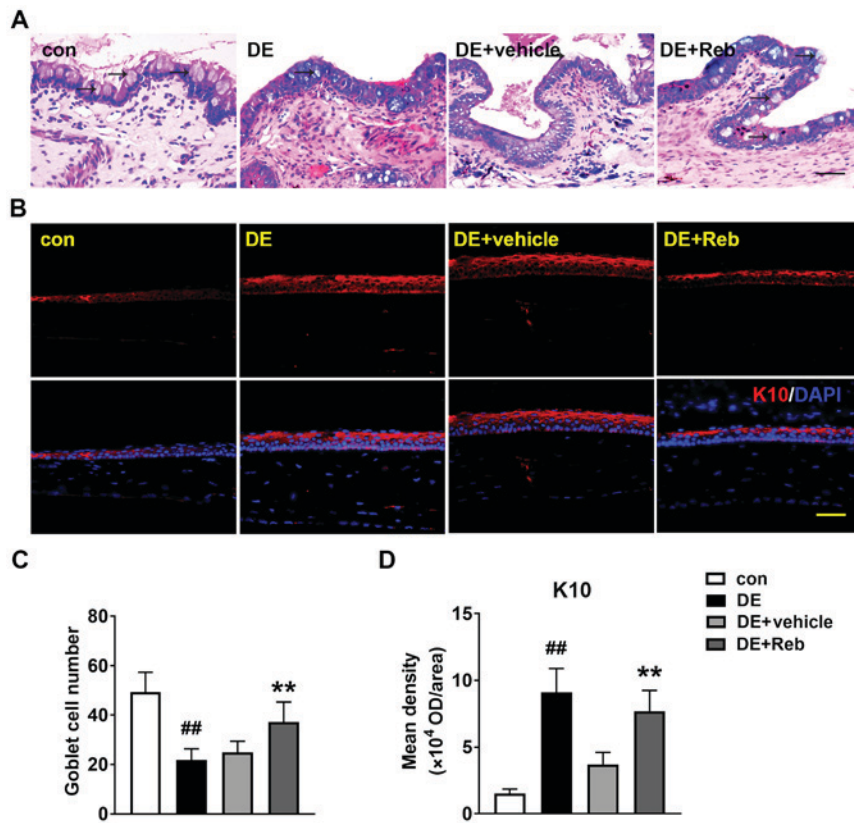


Figure 3. Rebamipide (2%) increases the conjunctival goblet cell number and ameliorated the corneal epithelium squamous metaplasia. (A) Hematoxylin and eosin staining of the conjunctiva. (B) Immunofluorescence staining of K10 co-stained with DAPI. Scale bar, 50 μ m. (C) Conjunctival goblet cell count based on the hematoxylin and eosin staining. (D) Quantification of the mean fluorescence density of the K10-positive area. n=6. ^{##}P<0.01 vs. the control group; ^{**}P<0.01 vs. the DE+vehicle group. K10, keratin 10; con, control; DE, dry eye; DE+vehicle, dry eye group treated with vehicle; DE+Reb, dry eye group treated with 2% rebamipide.

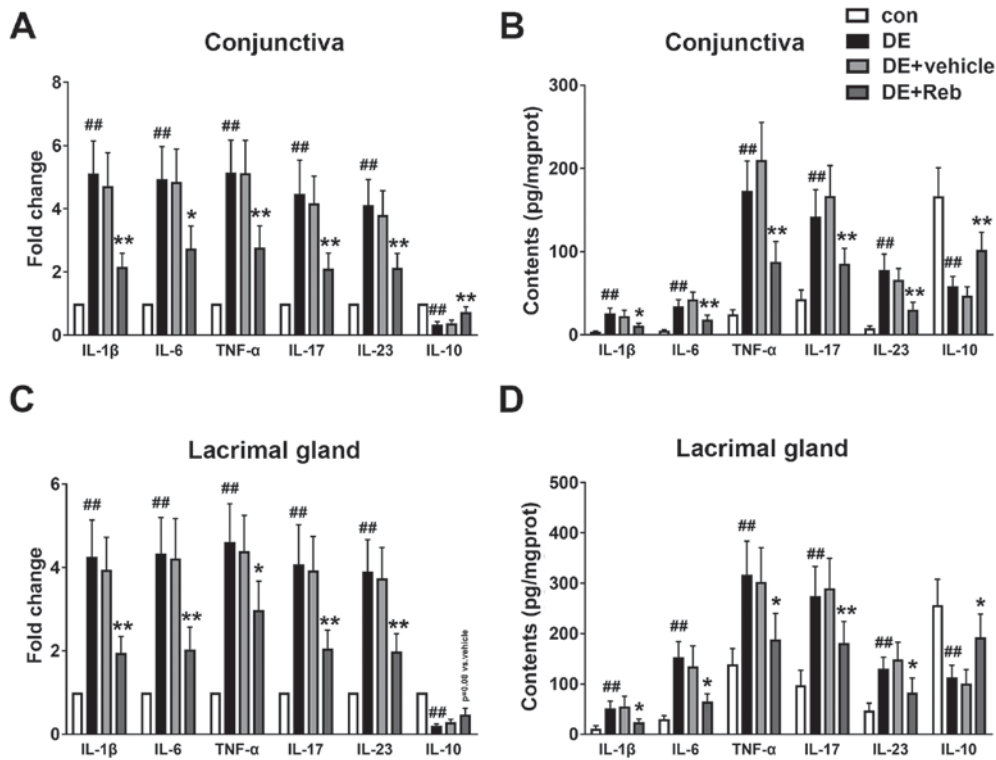


Figure 4. Anti-inflammatory effects of 2% rebamipide in the lacrimal gland and conjunctiva of DE mice. (A) mRNA and (B) protein expression levels of inflammatory cytokines in the conjunctiva. (C) mRNA and (D) protein expression levels of inflammatory cytokines in the lacrimal gland. The data are expressed as the mean \pm standard deviation (n=6). ^{##}P<0.01 vs. the control group; ^{*}P<0.05 vs. the DE+vehicle group; ^{**}P<0.01 vs. the DE+vehicle group. con, control group; DE, dry eye; DE+vehicle, dry eye group treated with vehicle; DE+Reb, dry eye group treated with 2% rebamipide.

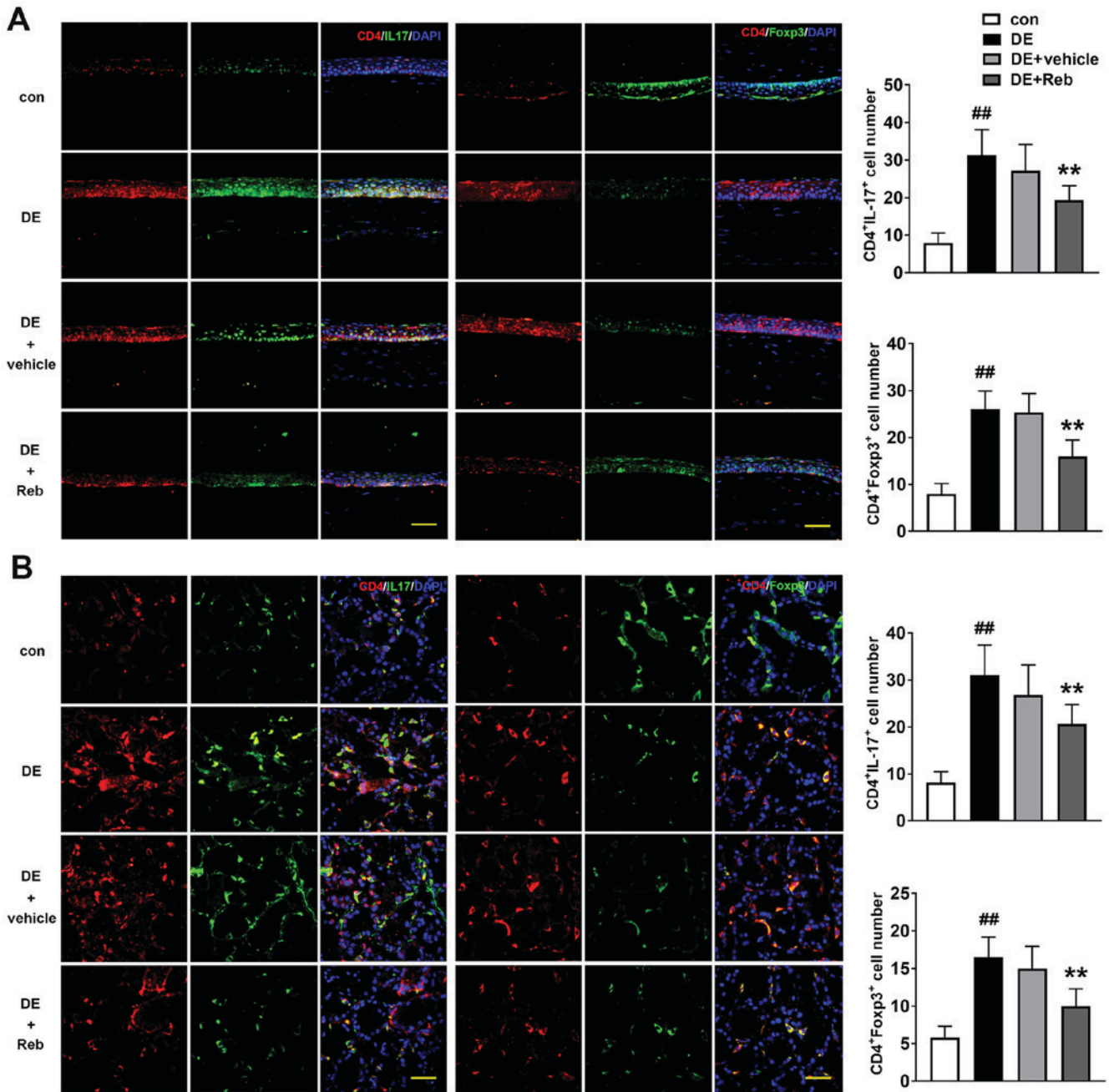


Figure 5. Regulatory effects of 2% rebamipide on the Th17/Treg ratio in DE mice. The presence of CD4⁺IL-17⁺ cells and CD4⁺Treg⁺ cells in (A) the cornea and (B) lacrimal gland was detected with double immunofluorescence staining. The red staining represents CD4⁺ and the green staining represents IL-17⁺ or Treg⁺. Nuclei were counterstained with DAPI (blue). Scale bar=50 μm. Data are expressed as the mean ± standard deviation (n=6). ^{##}P<0.01 vs. the control group; ^{**}P<0.01 vs. the DE+vehicle group. Th17, T helper 17 cell; Treg, regulatory T cell; CD, cluster of differentiation; IL, interleukin; Foxp3, forkhead box P3; con, control; DE, dry eye; DE+vehicle, dry eye group treated with vehicle; DE+Reb, dry eye group treated with 2% rebamipide.

the ocular surface and marked squamous metaplasia occurred. These results indicated that DE induces damage to the cornea. Following treatment with 2% rebamipide, these impairments were attenuated, and the structure of the cornea was repaired.

The lacrimal gland is another key tissue that contributes to the hemostasis of the ocular surface. In its healthy state, the lacrimal gland secretes tears that contain electrolytes, soluble proteins and mucus. Normal tear secretion maintains the moisture of the ocular surface and serves as a light refraction medium in the eye. Furthermore, the lacrimal gland secretes microbicidal proteins, which suppress pathogenic invasion onto the ocular surface (31). In DE, lacrimal gland secretion is

diminished due to the loss of acinar and ductal cells, induced by the neuroendocrine stimuli (32). In the scopolamine-induced DE mouse model used in the present study, markedly impaired acinar cells were observed and treatment with 2% rebamipide attenuated the damage. The rebamipide treatment also led to increased tear BUT and production, which may be associated with protection of the lacrimal gland. These results confirmed the beneficial effects of 2% rebamipide on DE and indicated that these actions may be associated with cornea and lacrimal gland protection.

CD4⁺ T cell-mediated inflammatory responses serve a key role in DE (33,34). In the disease condition, the lacrimal

functional unit, which is composed of the cornea, conjunctiva, lacrimal glands and meibomian glands (35), is vulnerable to pro-inflammatory cytokines (36). Various inflammatory markers have been discovered in the lacrimal gland and conjunctiva in different types of DE (37). These pro-inflammatory cytokines are able to inhibit neural activity and neurotransmitter release (38), and lead to goblet cell loss (39,40). In the present study, treatment with 2% rebamipide was demonstrated to exert effective anti-inflammatory functions, as evidenced by the significant decrease in the production of the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α , and the increased production of the anti-inflammatory cytokine IL-10, in the lacrimal gland and conjunctiva. These observations are consistent with previous findings in the tear fluid of superoxide dismutase-1(-/-) mice (41) and in the conjunctival tissues of NFS/sld mice (17). These results suggest that treatment with 2% rebamipide has an anti-inflammatory effect on the conjunctiva and lacrimal glands of DE mice.

Th17 are a subset of T helper cells that secrete the inflammatory cytokine IL-17 (42). Transforming growth factor- β 1 (TGF- β 1), IL-6, IL-21 and IL-23 are important for the differentiation of Th17 (43,44). Increased levels of IL-17 have been demonstrated in the lacrimal and submandibular glands of Sjögren syndrome model mice (45,46). IL-17 induces corneal epithelial barrier disruption in DE (47). The inhibition of IL-17 in various ways significantly decreases the severity of DE in animal models (48-50). Tregs, which are differentiated following TGF- β stimulation, regulate the formation of pathogenic T cells, particularly Th17. The balance between Th17 and Tregs is important for maintaining immune responses. However, in DE, the function of Tregs is impaired and the Treg-induced suppression of Th17 is overcome (25). In the present study, 2% rebamipide effectively restored this equilibrium by suppressing the differentiation of Th17 cells and upregulating that of Tregs in the cornea and lacrimal gland. The result was in accordance with the previous study on curdlan-induced spondyloarthritis mice (51). The present data indicated that the regulation of the balance between Th17 and Tregs may contribute to the remedial action of 2% rebamipide in DE mice.

In conclusion, 2% rebamipide acts on the cornea and lacrimal glands in DE mice in a protective manner, which may be associated with its anti-inflammatory and Th17/Treg regulatory functions. The present study revealed a novel pharmacological mechanism of action of rebamipide in DE therapy and highlighted a potential novel anti-DE drug.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RF, YJ and JZho performed the experiments. RF and JZho acquired and analyzed the data. RF and JZho interpreted the data. RF drafted the manuscript. JZha designed the experiment and revised the manuscript.

Ethics approval and consent to participate

The experimental protocol was approved by the Animal Ethics Committee of The Fourth Affiliated Hospital of China Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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