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Original Article

Vitamin A palmitate eye gel improves the density of conjunctival goblet cells and the production of mucin-5 subtype AC in rabbits with dry eye syndrome

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Abstract: Purposes: This study is to examine the efficacy of vitamin A palmitate eye gel in improving the density of conjunctival goblet cells and mucin-5 subtype AC (MUC5AC) production. Methods: Dry eye model was established in 60 rabbits. The rabbits were then randomly divided into 3 groups: group A - untreated; group B - treated with carboxymethylcellulose sodium eye drops three times a day for 28 days; group C - treated with vitamin A palmitate eye gel three times a day for 28 days. Break-up time, fluorescein and rose bengal staining, and Schirmer test were performed. Conjunctival impression cytology was used to detect conjunctival goblet cell density. Immunofluorescence and Western blotting was performed to detect MUC5AC. Results: On day 3, break-up time and the wet length for group C were lower than those for group B ($P < 0.01$). Fluorescein score of group C was higher than that of group A and B ($P < 0.01$). On day 14, fluorescein scores were decreased in groups B and C compared with that in group A ($P < 0.01$). On day 28, break-up time was significantly increased in group C ($P < 0.05$). On days 14 and 28, rose bengal scores were decreased in group C. Goblet cell density and MUC5AC production was significantly increased in group C on day 28, higher than that in group A and B. Conclusions: Vitamin A palmitate eye gel is effective in improving conjunctival goblet cell density and mucin production in dry eye syndrome.

Keywords: Dry eye, vitamin A, conjunctival goblet cells, mucin-5 subtype AC

Introduction

Dry eye syndrome is a complicated condition caused by different etiologies [1]. Various types of dry eye syndrome are accompanied by the loss of goblet cells [2]. Goblet cells, the main source of mucoproteins at the ocular surface, play a key role in tear film stability and goblet cell protection [3]. Goblet cell regression is a consequence of eye dryness [4, 5], and is also considered a pathological factor contributing to the development of dry eye.

Epithelial mucins are major protein components of tear fluid [6], among which goblet cell-specific mucin-5 subtype AC (MUC5AC) is a major secretory mucin in the tear film [7]. Mucin deficiency is originally proposed as a leading cause of dry eye [8]. The levels of MUC5AC protein are decreased significantly in the tears of dry eye patients [9].

In addition to tear substitutes, cyclosporine A 0.05%, tacrolimus 0.03% eye drops, and autologous serum eye drops are available pharmacological treatments for dry eye. Topical diquafosol is another drug under evaluation. Vitamin A palmitate eye gel is made up of Vitamin A Palmitate and Carbopol 980. Research suggests that vitamin A is released from the lacrimal gland and exists in retinol in the tears [10], maintaining a healthy ocular surface by regulating the proliferation and differentiation of keratoconjunctival epithelium on the ocular surface and preserving conjunctival goblet cells [11]. Previous studies show that retinol palmitate promotes the healing of keratoconjunctival damage that is particularly strong in the mucin layer, recovery of conjunctival goblet cells [12, 13], and mucin production [14]. In addition, ophthalmic solutions containing retinol palmitate are effective and cause few side-effects [15]. Carbopol 980 is the most efficient thick-

ener of all carbopolpolymers on the market. Furthermore, it is already incorporated in commercial ophthalmic gels to treat dry eye syndrome [16]. Some previous studies have only revealed that vitamin A has important effects on ocular surface repair, or only reported that conjunctival goblet cells and mucin have important effect on dry eye. In the present study, we use rabbit model with dry eye to clarify the efficacy and mechanism of action of vitamin A palmitate eye gel in topical treatment.

Materials and methods

Animals

White New Zealand rabbits weighing between 2 and 2.5 kg were used for this study. All rabbits were purchased from the Department of Laboratory Animal Science at Peking University. All of the animals had no ocular or systemic diseases. The experiments performed were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Declaration of Helsinki. All animal protocols were approved by the Animal Research Committee of the Peking University Health Science Center. The establishment of dry eye model briefly followed the methods reported by Xiong et al. [1]. Sixty New Zealand rabbits were treated by topical administration of 0.1% benzalkonium chloride (BAC) (Sigma-Aldrich, St. Louis, MO, USA) drops in one eye of each rabbit three times a day for 14 days (day 0). On day 0, the rabbits were then randomly divided into 3 groups. Group A had preconditioning with BAK but no further treatment; group B was treated with carboxymethylcellulose sodium eye drops (Allergen Pharmaceutical Co., Ltd., Irvine, CA, USA) three times a day for 28 days; group C was treated with vitamin A palmitate eye gel (Shenyang Sinqi Pharmaceutical Co., Ltd., Shenyang, China) three times a day for 28 days. Normal control group was used for differential expression for the immunohistochemistry and Western blotting analyses, and was not treated with BAK or used in the comparisons for Schirmer's, break-up time test and fluorescein staining. Intramuscular injection of a mixture of 50 mg ketamine and 25 mg chlorpromazine was administered to keep the animals immobile. On day 28, all animals were sacrificed with overdoses of phenobarbital sodium.

Schirmer tear test

Tear production was measured with Schirmer test using 5 mm × 35 mm filter paper strips on days 0, 3, 7, 14 and 28. The paper strip was inserted into the junction of the middle and outer thirds of the lower lid of the conjunctival sac. The wet length (mm) of the paper strip was read after 5 minutes.

Break-up time test

Corneal fluorescein sodium staining was performed on days 0, 3, 7, 14 and 28. Fluorescein sodium (2 μl, 1%) was dropped into the conjunctival sac. The animal was allowed to blink several times to distribute the fluorescein in the cornea, and then break-up time from opening of the eyes to the appearance of the first dry spot in the central cornea was measured three times and the mean value was recorded [17].

Fluorescein staining

Fluorescein sodium staining was examined and graded under a slit lamp microscope with a cobalt blue filter at a magnification of 16 (Topcon, Tokyo, Japan). According to the grading standards of the corneal fluorescein staining described by Li et al. [18], the cornea was divided into four quadrants. The staining intensity in each quadrant was scored on a scale of 0-3 (for a maximum score of 12). Scoring was achieved using the following guidelines: no punctate staining on the cornea was assigned 0 point; punctate staining of 1-10 was assigned 1 point; punctate staining of 11-30 was assigned 2 points; and either punctate staining > 30 or clumped staining was assigned 3 points. After summing up the scores of the four quadrants, scores ranging from 1 to 4 were defined as mild, scores from 5 to 8 were moderate, and scores from 9 to 12 were considered severe.

Rose bengal staining

Rose bengal staining was performed on days 0, 3, 7, 14, and 28. After dropping 2 μl of 1% rose bengal into the conjunctival sac for 15 seconds, the intensity of staining of the medial and lateral bulbar conjunctiva and the cornea was recorded on a cornea diagram and scored using a standardized grading system of 0 to 3, for a total maximum of 9 per eye [19].

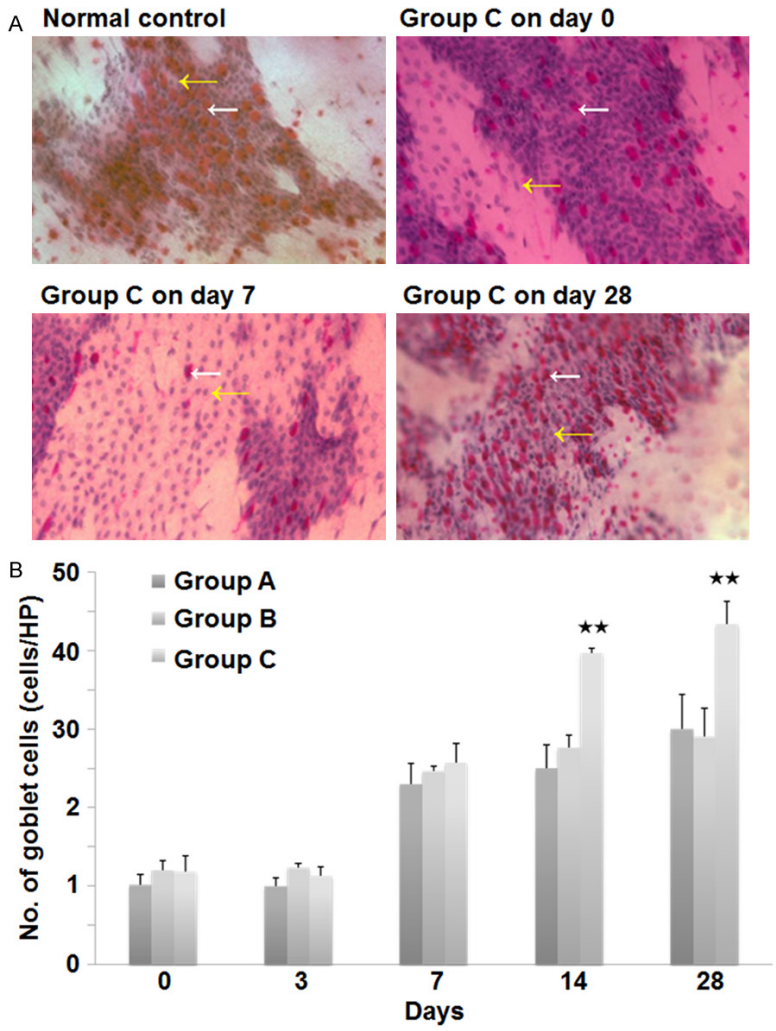

Figure 1. Morphology and amount of goblet cells. A. Images of goblet cells after PAS staining. White arrows show the goblet cells, while yellow arrows show epithelial cells. B. Comparison of goblet cell number among three groups. Data are means ± standard deviations. Double stars indicate $P < 0.01$ compared with group A or B on the same day.

Conjunctival impression cytology

Samples were collected on day 28. Intramuscular injection of 50 mg ketamine and 25 mg chlorpromazine was given to immobilize the rabbits. Two 3.5 mm × 3.5 mm circular disks of nitrocellulose filter paper (Pall Corporation, New York, NY, USA) were placed separately on the nasal and temporal bulbar conjunctiva with the filter paper dull-side down. After 20 seconds, the filter paper was fixed with 95% alcohol. After staining with hematoxylin and periodic acid-Schiff (PAS) reagents, the morphology of the conjunctival epithelium was graded according to the Nelson system. Three different sections of each sample were selected randomly and an average was calculated (cells/high-power [HP] visual field, 400 ×).

Immunofluorescence staining

Immunofluorescence staining of MUC5AC was performed on cryosections on day 28. Normal corneal samples were used as negative controls. Sections were fixed in acetone at 4°C for 10 minutes. After being washed 3 times of 5 min with phosphate-buffer saline, the sections were blocked with 1% goat serum for 30 minutes at room temperature and incubated overnight at 4°C with a 1:150 dilution of mouse anti-rabbit MUC5AC antibody (Abcam, Cambridge, UK). The sections were incubated with DyLight594-conjugated AffiniPure goat anti-mouse IgG (ZF-0413, ZSGB-BIO, Beijing, China) for 45 minutes at room temperature, and then the nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA). The sections were observed under a confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany).

Western blotting

To determine MUC5AC expression on day 28, proteins were prepared by the following procedures. Tissues were re-suspended in 250 μl radioimmunoprecipitation assay buffer (1 mM) and incubated at 4°C. After lysis, the samples were centrifuged at 12,000 × g for 5 min. Protein concentration was determined by bicinchoninic acid protein assay kit (P0010, Beyotime Institute of Biotechnology, Nantong, China). The 150 μg protein was loaded on 8% SDS-PAGE gel at 110 V for 70 min. After separation by electrophoresis, the proteins were transferred to polyvinylidene fluoride membrane. The mem-

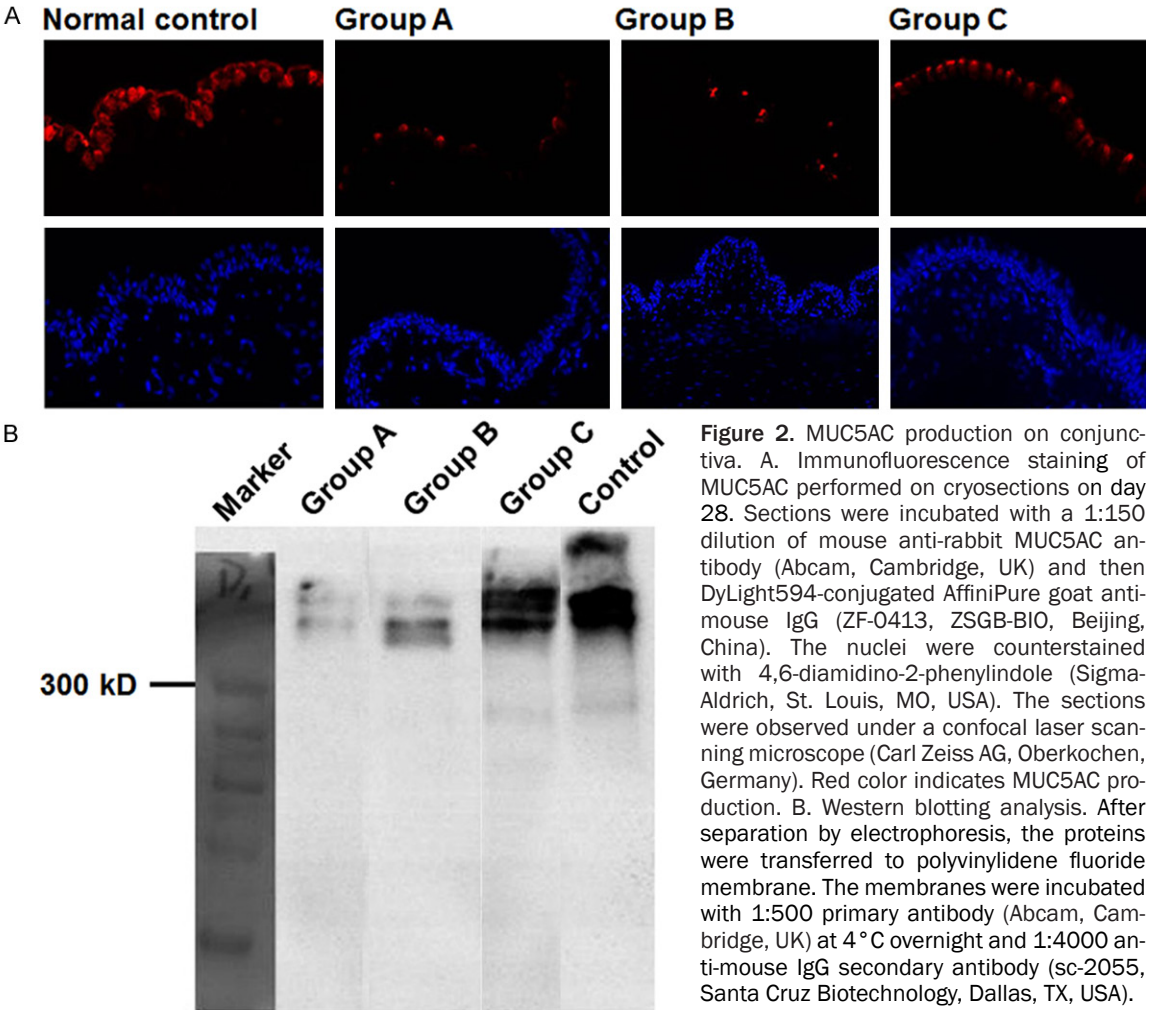

Figure 2. MUC5AC production on conjunctiva. A. Immunofluorescence staining of MUC5AC performed on cryosections on day 28. Sections were incubated with a 1:150 dilution of mouse anti-rabbit MUC5AC antibody (Abcam, Cambridge, UK) and then DyLight594-conjugated AffiniPure goat anti-mouse IgG (ZF-0413, ZSGB-BIO, Beijing, China). The nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA). The sections were observed under a confocal laser scanning microscope (Carl Zeiss AG, Oberkochen, Germany). Red color indicates MUC5AC production. B. Western blotting analysis. After separation by electrophoresis, the proteins were transferred to polyvinylidene fluoride membrane. The membranes were incubated with 1:500 primary antibody (Abcam, Cambridge, UK) at 4°C overnight and 1:4000 anti-mouse IgG secondary antibody (sc-2055, Santa Cruz Biotechnology, Dallas, TX, USA).

brane was then blocked with 5% bovine serum albumin in Tris-buffered saline with Tween 20 at room temperature for 2 h. The membranes were incubated with 1:500 primary antibody (Abcam, Cambridge, UK) at 4°C overnight and 1:4000 anti-mouse IgG secondary antibody (sc-2055, Santa Cruz Biotechnology, Dallas, TX, USA) for 2 h at room temperature with shaking. The protein side of the membrane was exposed to X-ray film for imaging.

Statistical analysis

Statistical analyses were performed using SPSS16.0 statistical software (IBM, Armonk, NY, USA). One-way ANOVA (LSD) was used to evaluate test data. Experimental data were shown by means ± standard deviations. *P* value less than 0.05 was considered statistically significant.

Results

Dry eye model is successfully constructed

To examine whether the dry eye model was successfully built, we performed break-up time test, Schirmer tear test, fluorescein staining and rose bengal staining. Before being treated by 0.1% BAC, break-up time of normal control group was 15.67 ± 1.53 s. The wet length in Schirmer tear test was 7.33 ± 0.58 mm. Fluorescein and rose bengal scores were 0.00 ± 0.00 and 0.33 ± 0.58, respectively. The number of conjunctival goblet cells was 88.33 ± 8.02 cells/HP. After being treated by 0.1% BAC for 14 days (day 0), the mean break-up time in all the rabbits was 0.90 ± 1.20 s. The wet length in Schirmer tear test was 3.55 ± 0.89 mm. Fluorescein and rose bengal scores were 3.65 ± 0.67 and 4.35 ± 1.14, respectively. The

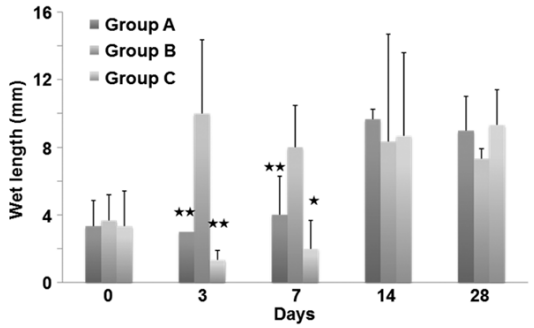

Figure 3. Schirmer's test showing tear production status among groups A-C. Data are means ± standard deviations. Single star indicates $P < 0.05$ and double stars indicate $P < 0.01$ compared with group B on the same day.

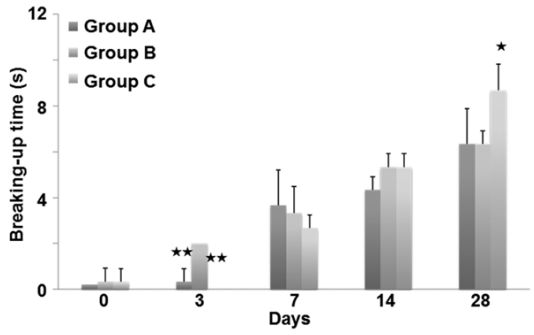

Figure 4. Break-up time comparison among groups A-C. Data are means ± standard deviations. Single star indicates $P < 0.05$ compared with group A or B on the same day. Double stars indicate $P < 0.01$ compared with group B on the same day.

number of conjunctival goblet cells was 10.85 ± 1.63 cells/HP. There were significant differences compared with those before being treated by 0.1% BAC ($P < 0.01$). All the rabbits were randomly divided into three groups on day 0. There were no significant differences among the three groups in Schirmer tear test, break-up time, fluorescein staining or rose bengal staining ($P > 0.05$). The results suggest that dry eye model is successfully constructed.

Vitamin A palmitate eye gel is efficient in enhancing goblet cell density

To determine goblet cell density, conjunctival impression cytology was employed. For PAS staining, conjunctival impression cytology was grade 0 in normal control group. The nucleocytoplasmic ratio of epithelial cells was 1:2. Goblet cell density was 500 cells/mm^2. Conjunctival impression cytology was grade 3 in group C on day 0. The nucleocytoplasmic ratio of epithelial cells was 1:5. Goblet cell density was 100 cells/mm^2. Conjunctival impression cytology was grade 2 in group C on day 7. The nucleocytoplasmic ratio of epithelial was about 1:3. Goblet cell density was 100-350 cells/mm^2. Conjunctival impression cytology was grade 1 in group C on day 28. The nucleocytoplasmic ratio of epithelial cells was about 1:2. Goblet cell density was 300-500 cells/mm^2. Goblet cell density kept increasing in groups A and B, but was lower than that in group C on days 14 and 28 ($P < 0.01$). There were no apparent differences between groups A and B. (**Figure 1**). The results suggest that vitamin A palmitate eye gel is efficient in enhancing goblet cell density.

MUC5AC production is enhanced in group C

To measure MUC5AC production on conjunctiva, immunofluorescence staining and Western blotting were performed. MUC5AC production in all three groups was lower than that of normal control group according to immunofluorescence staining and Western blotting analysis. MUC5AC production in groups A and B had no significant increase on day 28 compared with control. MUC5AC secretion in group C had a mild increase compared with that in group A or B (**Figure 2A**). Western blotting revealed that MUC5AC production in group B had mild increase compared with that in group A. The increase of MUC5AC production in group C was the most significant among all three groups (**Figure 2B**). These results indicate that MUC5AC production is enhanced in group C.

Vitamin A palmitate eye gel does not influence tear production

To study tear production, Schirmer tear test was used. The wet length of the paper strip in group B was significantly increased compared with that in group A or C on day 3 ($P < 0.01$). On day 7, the wet length of the paper strip in group A ($P < 0.05$) and group C ($P < 0.01$) were still shorter than that in group B. On day 14, the wet length in groups A and C was dramatically increased. On days 14 and 28, the length remained stable. There were no significant differences among the three groups on days 14 and 28 (**Figure 3**). The results indicate that vita-

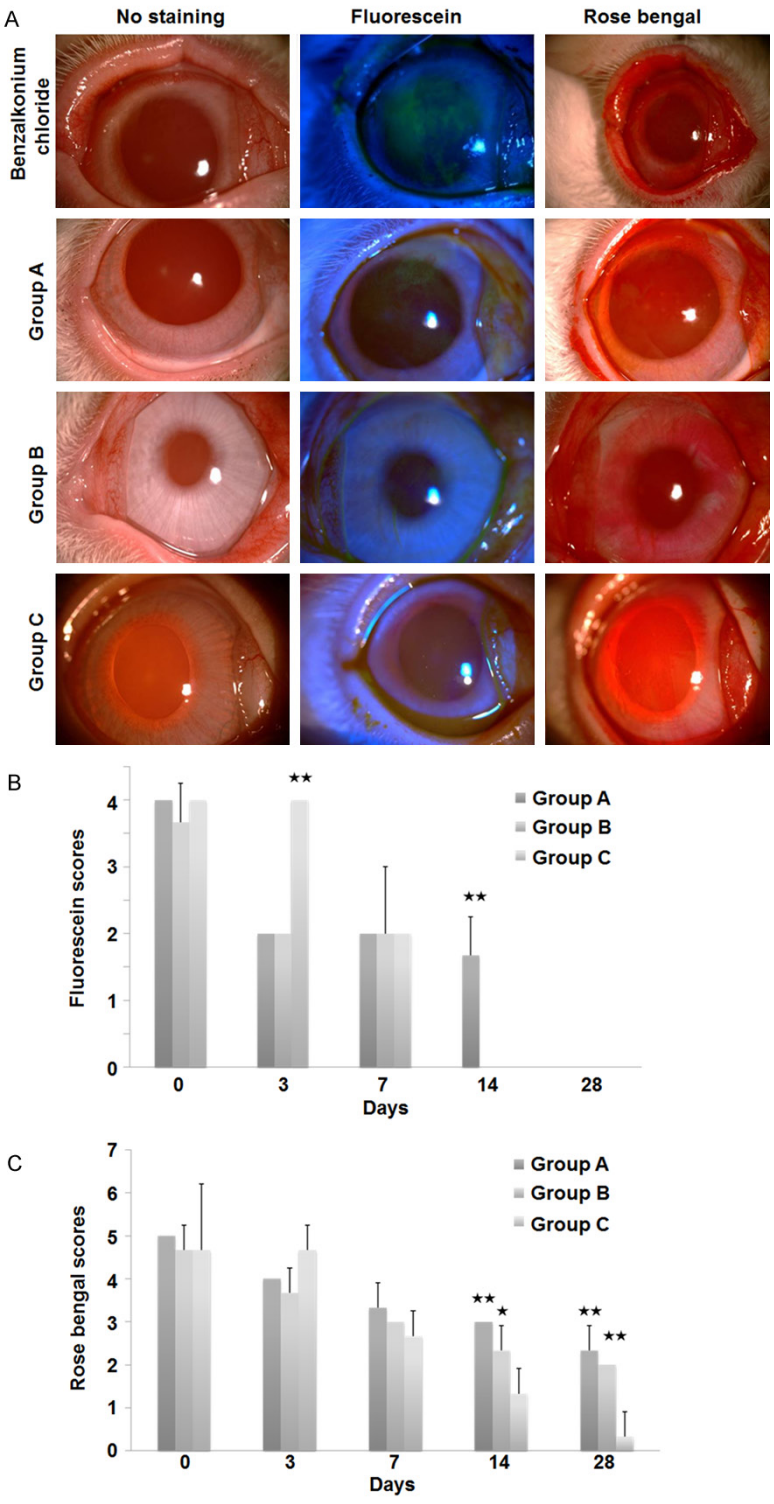

Figure 5. Study of corneal status. A. Images of corneal fluorescein and rose bengal staining on day 14 in groups A-C. B. Fluorescein staining scores in groups A, B and C. Data are means ± standard deviations. Double stars indicate $P < 0.01$ compared with all other groups on the same day. C. Rose bengal scores in groups A, B and C. Data are means ± standard deviations. Single star indicates $P < 0.05$ and double stars indicate $P < 0.01$ compared with group C on the same day.

min A palmitate eye gel does not influence tear production.

Vitamin A palmitate eye gel prolongs break-up time

To evaluate the stability of lacrimal film, break-up time was measured. On day 3, break-up time in group B showed a mild increase compared with that in group A and C ($P < 0.01$). Break-up time in group C was the lowest among three groups on day 3. After day 3, break-up time was continuously increased in all three groups. On day 28, break-up time of group C was significantly higher than that in group A or B ($P < 0.05$) (**Figure 4**). The results suggest that vitamin A palmitate eye gel prolongs break-up time.

Vitamin A palmitate eye gel improves corneal status

To detect corneal status, ocular surface was stained with fluorescein and rose bengal. Fluorescein scores had no significant change in group C on day 3 compared with that on day 0. On day 14, fluorescein scores in groups B and C decreased to their lowest level (**Figure 5A** and **5B**); Fluorescein score in group A was the lowest on day 28 (**Figure 5B**). For rose bengal staining, the rose bengal score in group C was higher than that in group B on day 3 ($P < 0.05$). On day 14, there was a significant decrease in group C compared with that in group A ($P < 0.01$) or group B ($P < 0.05$). On day 28, the rose bengal score in group C was significantly lower than those in groups A and B ($P < 0.01$)

(**Figure 5A** and **5C**). In all these tests, there were significant changes in all three groups on day 28 compared with those on day 0 (**Figure 5A-C**). The results indicate that vitamin A palmitate eye gel improves corneal status.

Discussion

BAC, a surface-active preservative commonly used in eye drop preparations, has been shown to hasten the drying of the tear film [20, 21]. Previous studies show that BAC-induced dry eye is in accordance with the natural etiology and pathophysiology of the tear film, and that BAC-induced dry eye model is both satisfactory and applicable [1, 22]. Our study shows that treatment of rabbits' ocular surface with 0.1% BAC for 14 days leads to significant decrease in length in Schirmer tear test and break-up time test, significant increase in fluorescein and rose bengal staining scores in cornea and conjunctiva, and decrease of goblet cell density and MUC5AC production. Our study demonstrates that 0.1% BAC effectively induces dry eye.

On day 28, we found that MUC5AC production and goblet cell density were significantly increased by treatment with vitamin A palmitate eye gel. Corresponding to this situation, break-up time was increased and fluorescein and rose bengal scores in cornea and conjunctiva were decreased on day 28. This suggests that vitamin A palmitate eye gel increases MUC5AC secretion and the number of goblet cells in dry eye, and improves tear film restoration. Meanwhile, we found that on days 14 and 28, the fluorescein score disappeared in vitamin A palmitate eye gel group, but rose bengal staining was still positive. Rose bengal can stain both live and dead cells if the cells are not protected by an intact mucin layer [23]. Our result shows that treatment with vitamin A palmitate eye gel for 28 days significantly improves the condition of the dry eye, but still doesn't completely recover its condition to normal level. Therefore, topical long-term application is needed.

On day 3, we found that the results of Schirmer tear test, break-up time, and fluorescein staining are improved in carboxymethylcellulose sodium group, being better than the results for vitamin A palmitate eye, especially in Schirmer test. We consider that carboxymethylcellulose sodium eye drops are only supplement of tear fluid that can increase the amount of the aqueous tear fluid, increase break-up time and wet length for Schirmer tear test temporarily. However, it cannot induce the regeneration of goblet cells and MUC5AC production.

It is well known that vitamin A deficiency has a close relationship with dry eye. Dry eye induces the damage of the ocular surface, resulting in a reduction of vitamin A absorption and production. Vitamin A deficiency leads to the decrease of goblet cells and mucin, accelerating the occurrence of dry eye and aggravating the process of keratoconjunctivitis sicca. However, the regulatory mechanisms of MUC5AC and goblet cells in dry eye by vitamin A have not been characterized. Bossenbroek et al. [24] have reported the presence of all subtypes of retinoic acid receptors (RARs) and retinoid X receptors (RXRs) in rabbits' corneal epithelium, as well as corneal and conjunctival fibroblasts. Furthermore, Mamoru et al. [25] have speculated that vitamin A may have potential direct regulation at transcriptional levels on MUC5AC, and that one of the major action mechanisms for vitamin A modulation of conjunctival goblet cell differentiation is through the control of mucin expression [26]. Therefore, our data not only suggest that local vitamin A palmitate eye gel treatment is effective in dry eye, but also provide some evidences for the mechanisms of action of vitamin A palmitate eye gel treatment. In summary, vitamin A palmitate eye gel is a promising pharmacological approach for the treatment of dry eye by improving the number of conjunctival goblet cells and mucin secretion. It is also much better to use carboxymethylcellulose sodium eye drops at the early phase of application of vitamin A palmitate eye gel.

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Disclosure of conflict of interest

None.

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