• Basic Research •

Rabbit models of dry eye disease: comparative analysis

Vytautas Baranauskas[1], Julija Daukantaitė[2], Saulius Galgauskas[2]

[1]Vilnius University Institute of Biochemistry, Life Science Centre, Sauletekio av 7, Vilnius, LT 10257, Lithuania
[2]Vilnius University Faculty of Medicine, M. K. Čiurlionio str. 21, Vilnius, LT 03101, Lithuania
Correspondence to: Vytautas Baranauskas. Vilnius University Institute of Biochemistry, Life Science Centre, Sauletekio av 7, Vilnius, LT 10257, Lithuania. vytautas.baranauskas@gmc.vu.lt
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Abstract

● **AIM:** To report ocular changes in rabbits after the implementation of three different induction methods to create dry eye (DE) conditions and provides evidence of DE-related disease evolution.

● **METHODS:** Experimental methods were divided into 3 models. The first model used involved triple injection of complete Freund's adjuvant, 50 μL each, also called the meibomian gland dysfunction (MGD) model. In the second model, DE conditions were created by the resection of nictitating membranes (NM), Harderian glands (HG), and main lacrimal glands (LG), also called the LGR model. The third model involved the topical administration of benzalkonium chloride (BAK) 0.1% solution. The Schirmer test, ocular surface staining with fluorescein, and tear break-up time tests were implemented before and after excision. After euthanasia, the ocular tissues were dissected. Cornea, conjunctiva, and meibomian glands were treated with periodic acid–Schiff (PAS) staining and haematoxylin–eosin staining.

● **RESULTS:** The MGD model triggered inflammation of meibomian glands. It detected changes in the lipid layer of the tear film. The bilateral resection of NM, HG, and LG reduced the watering layer of the tear film. The topical administration of BAK of 0.1% solution impacted the mucosal layer of the tear film.

● **CONCLUSION:** Different changes are observed with different DE syndrome models. The composition of the tear film differ depending on which part of the eye is targeted. More studies need to be done to confirm whether an increased thickness of the cornea has any impact on the DE disease.

● **KEYWORDS:** ocular surface; lid inflammation; keratitis; tear deficiency; rabbits; dry eye disease

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INTRODUCTION

The meibomian glands are vertically arranged sebaceous glands located in the upper and lower tarsal plates[1]. These glands synthesize and secrete meibomian, which is a secretion composed of phospholipids, cholesterol, wax esters, and cholesterol esters. Meibomian forms a layer on the eye called the tear film that prevents the eyes from drying, thus playing a protective role against various diseases. One of the principal causes of dry eye disease (DED) is damage to the tear film; other contributors are hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities[2-3]. Air pollution, dryness, allergy, smoke, and ultraviolet (UV) light affect tear osmolarity and can consequently cause tear film damage[4]. Transmembrane and secretory mucins might additionally promote DED by increasing friction between tear fluid in epithelial cells of the eye, thereby causing tear film instability. Eyelid notching also causes poor film formation, which can equally cause DED[5]. Meibomian plays an important role in tear film stability and the protection of the ocular surface against microbial agents and meibomian gland dysfunction (MGD) as a result of functional abnormalities such as hyperosmolarity, tear evaporation, and ocular surface staining; MGD might occur if the glands synthesise insufficient meibomian containing the proper quality and quantity of lipids[1,5-6]. The most common cause of DED is MGD. Environmental stress, stem cell renewal, and ageing can also cause MGD[1,5]. Corneal damage can occur as a result of corneal injury associated with inflammation and hypersensitivity of the corneal nerves, and this can result from apoptosis, goblet cell loss, and reduced secretions[7]. Treatment of DED can be performed using lactoferrin and mimetic lactobionic acids due to their moisture-retaining capacity[2,8]. Damaged epithelial cells and environmental stress might trigger inflammatory mediators such as chemokines and cytokines on the cornea surface, causing tear film instability, which causes irritation and the vicious cycle of DED to continue[2].

DED is also associated with the inflammation of the lacrimal and meibomian glands, the cornea, aqueous tears, and conjunctiva. An increase in hormones such as androgens and oestrogen might also be correlated to the onset of DED[6,9]. It has been hypothesized that adaptive immunological reactions can appear due to prolonged exposure to external stimuli, and the patient may experience chronic diseases of the eye if the immune system fails to prevent DED pathogenesis due to tear film loss[5,9]. DED affects ocular function, corneal structure integrity, and overall eye health. Thermal pulsation therapy, intraductal probing, intense pulsed light, antibiotics, non-steroid essential fatty acid, and hormonal therapy are suitable for the treatment of MGD[10-12]. Meiboscore might help treat the symptoms associated with MGD, while other forms of management of the disease might include the treatment of the pathophysiological mechanism of MGD as an intervention against this disorder[5,10,12]. Honavar[13] claimed that each step in categorizing DED and initiating appropriate management is important and that the mere use of artificial tears will not do justice to the management of DED.

MATERIALS AND METHODS

Ethical Approval All animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the EC Directive 86/609/EEC for animal experiments using protocols approved and monitored by the State Food and Veterinary Service of Lithuania (animal license number G2-95).

Meibomian Gland Disfunction MGD is a condition that results from a malfunction of the meibomian glands. The condition is often associated with chronic inflammation of the glands, which leads to changes in the composition of the meibum, the oily substance produced by the glands. The abnormal meibum can become thicker and more viscous, making it difficult for the glands to secrete it properly. This results in clogging of the gland orifices and the formation of meibomian gland ductal cysts. MGD with the meibomitis model was induced with complete Freund's adjuvant (CFA) containing lysed *Mycobacterium* lyophilised cells (Sigma-Aldrich, USA). While MGD has been studied in rabbit models, Freund's adjuvant is not typically used as a method of inducing MGD in this model. Under general and topical anaesthesia (Oftan Obucain, 4 mg/mL Santen, Tampere, Finland), CFA (each 50 μL) was injected into the nasal, central, and temporal upper eyelid margin of the right eye. Saline was injected into the left eye as a control into the respective places of the left upper eyelid.

Lacrimal Gland Resection Lacrimal gland resection (LGR) was performed to induce a more severe form of the DED. In rabbits it can result in a significant reduction in tear production. The degree of tear production reduction may vary depending on the extent of the gland removal and the individual rabbit's physiology. Bilateral resection of the nictitating membrane (NM), Harderian gland (HG), and main lacrimal gland (LG) was performed on both eyes. The skin of both periocular regions was shaved. Induction was initiated with an intramuscular injection of ketamine (25 mg/kg; Ketamidor 10%, 10 mL, Richter Pharma, AT) and medetomidine (0.375 mg/kg; Sedator 1 mg/mL, 10 mL, Eurovet Animal Health B.V. NL). General anaesthesia was maintained by the ketamine and medetomidine composition for induction during the entire surgery. Analgesia was maintained during the operation by injecting the opioid buprenorphine (0.03 mg/kg Sol. Bupaq, multidose 0.3 mg/mL, 10 mL, Richter Pharma, UK). The cardiopulmonary status of the animals and the depth of anaesthesia were monitored every 15min with a pulse oximeter (patient monitor, PM60A, China). The surgical field was disinfected with 5% betadine (Sol. Betadine 1 mL/10 mg, Egis, HN) and draped. The NM was cut off at its base. The HG was extracted from the space between the medial rectus muscle and the anterior wall of the orbit, through the excision wound of the NM, and removed in its entirety. Haemostasis was achieved by gentle pressure tamponade. A curve-shaped linear incision was subsequently made along the inferior and lateral orbital rims to remove the infraorbital, temporal, and intraorbital lobes of the main LG. The orbit septum and skin wound were then closed separately with interrupted 4-0 polyglactin sutures. Maxitrol 5 mL ophthalmic drops (Alcon, Puurs, Belgium) were administered topically at the surgical wound thrice daily for 7d, and enrofloxacin 15 mg/kg (Enroxil, 50 mg/mL, KRKA, SL) was given subcutaneously once daily for 3d as a prophylactic agent.

Dry Eye with Benzalkonium Chloride Benzalkonium chloride (BAK) is used to induce corneal epithelial damage, similar to the damage observed in human DED. BAK is a commonly used preservative in eye drops and has been shown to have toxic effects on the ocular surface, leading to decreased tear film stability and increased corneal damage. BAK (Sigma-Aldrich, USA) was received as a 10% solution, which was diluted with saline to form up to 0.1% solution. Prepared BAK 0.1% solution was used as topical drops (60 μL) twice daily on both eyes for 35d.

Experimental Animals All rabbits were housed at a constant temperature of 22°C±1°C and a humidity of 55%±10% in a light-controlled environment (lights on from 7 *a.m.* to 7 *p.m.*) with *ad libitum* access to food and water. MGD model: n=4, 4 months old, males, New Zealand white rabbits (Innovative Medicine Center, Lithuania). LGR model: n=5, 6–10 months old, males, Dutch belted rabbits (Lidkoping Kaninfarm, Sweden). BAK model: n=12, 5–7 months old, males, New Zealand white rabbits (Charles Rivers, France).

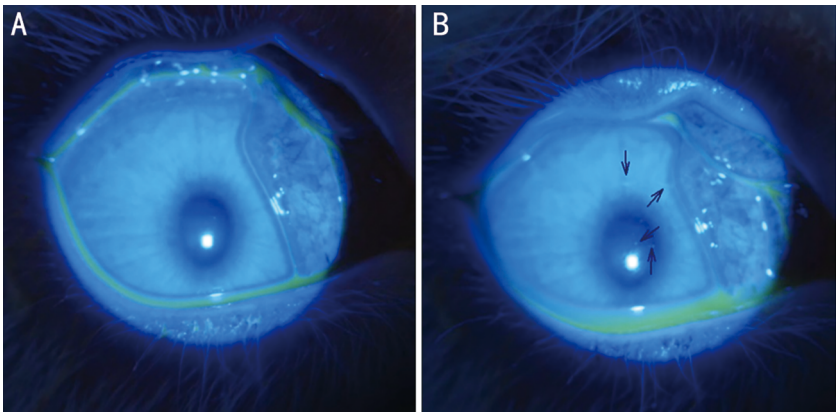

Figure 1 OD eye during a TBUT test The TBUT test is useful in diagnosing and monitoring various ocular surface diseases, such as dry eye syndrome, MGD, and corneal epithelial disorders. A: Tear film right after a blink; B: Taken after a few moments; dry spots start to appear. TBUT: Tear break-up time; OD: Right eye; MGD: Meibomian gland dysfunction.

Corneal Fluorescein Staining Test Corneal fluorescein staining is a diagnostic test used to detect various corneal abnormalities and assess the integrity of the corneal surface. The staining patterns can provide information about the location, severity, and extent of corneal damage or pathology, such as corneal abrasions, ulcers, infections, or DED. Corneal fluorescein staining was performed before injection CFA on day 0 and after injection on days 7, 14, 21, 28, and 35. Fluorescein sodium ophthalmic strips were used to stain cornea surfaces. The tip of the strip was inserted into the conjunctival sac for a second, and the eye was closed a couple of times for the stain to cover the ocular surface. The ocular surface was examined under a stereo microscope (Leica M165 FC, Germany) and imaged using a digital camera (Leica DMC6200, Germany).

Schirmer Test The Schirmer test is a diagnostic test used to measure the quantity of tears produced by the LG. This test is performed to evaluate tear production and to help diagnose conditions such as dry eye (DE) syndrome. Tear production was measured by the Schirmer tear test using ophthalmic strips (Optitech Eyecare, India) on days 0, 7, 14, and 21. The rabbits were restrained with cloth for immobilisation. The Schirmer paper strip was inserted into the conjunctival sac around the junction of the middle and outer thirds of the lower lid. The wetted length (mm) of the paper strip was read after 1min, and the procedure was repeated thrice. Paper strips, which did not wet, were ignored. The average result was recorded as a finale.

Tear Break-up Time Test Tear break-up time (TBUT) is a clinical test used to assess the stability of the tear film on the surface of the eye. The TBUT test was used to assess for evaporative DED and was performed on days 0, 7, 14, 21, and 28. The TBUT is recorded as the number of seconds that elapse between the last blink and the appearance of the first dry spot in the tear film. A shorter TBUT time indicates a more unstable tear film, which can lead to symptoms of dryness, irritation, and visual disturbances. By measuring TBUT, we can better understand the underlying mechanisms of ocular surface diseases. Figure 1A shows a healthy, intact corneal surface right after a blink, with an evenly distributed tear film. Figure 1B displays a broken tear film, which is marked with black arrows; the time taken for this to occur is how the time of film disintegration is determined.

Histological Sample Collection Tissues (eyelids, cornea, eyeball) were dissected following euthanasia on the 28th day after the CFA injection. The tissues were fixed overnight in 4% paraformaldehyde. Later, these were cut into 8-μm layers and stained with haematoxylin-eosin (HE) and periodic acid-Schiff (PAS).

RESULTS

Meibomian Gland Dysfunction Injection of CFA into the eyelid margins showed that the orifice size of the meibomian glands correlated with the injections. After injections, inflammation of the eyelids progressed throughout the experiment. The condition is often associated with chronic inflammation of the glands, which leads to changes in the composition of the meibum, the oily substance produced by the glands. The abnormal meibum can become thicker and more viscous, making it difficult for the glands to secrete it properly. This results in clogging of the gland orifices and the formation of meibomian gland ductal cysts. During this period, the size of the orifices decreased until they were no longer visible when comparing them before the induction of CFA, as seen in Figure 2. Also, the edges of the eyelids became progressively blunter and, by the end of the experiment, became oedematous. Figure 3 shows how corneal fluorescein staining accentuated the swelling of surrounding tissues of the eye surface, which reduced the surface of the cornea. The staining got more intense and widespread thoughout cornea surface as the days went by, proving the occurrence of corneal surface damage. The average tear volume did not change after

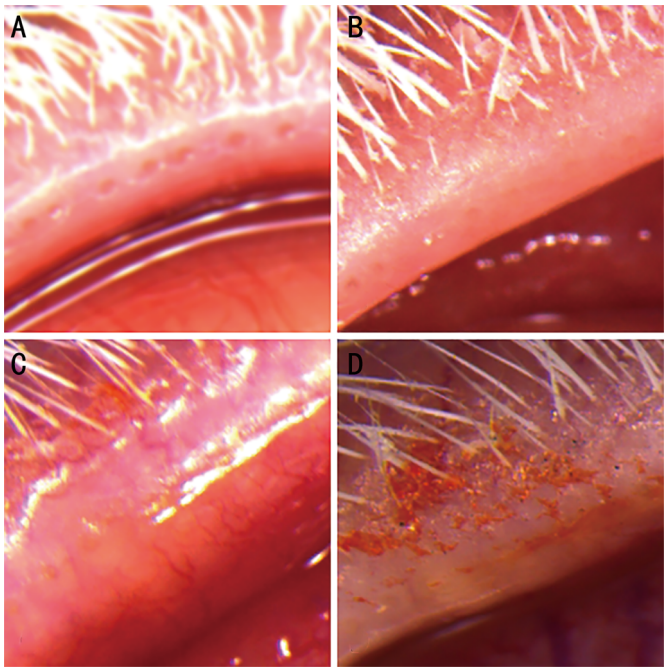

Figure 2 Edge of rabbit's eyelids A: Margin of eyelid with sharp edges and clearly visible orifices of meibomian glands before injection of CFA; B: Eyelid 7d post-injection of CFA where orifices are still visible; C: Eyelid 14d post-injection of CFA showing swelling and hyperaemia of eyelid; D: Eyelid 21d post-injection of CFA showing no reduction in swelling. CFA: Complete Freund's adjuvant.

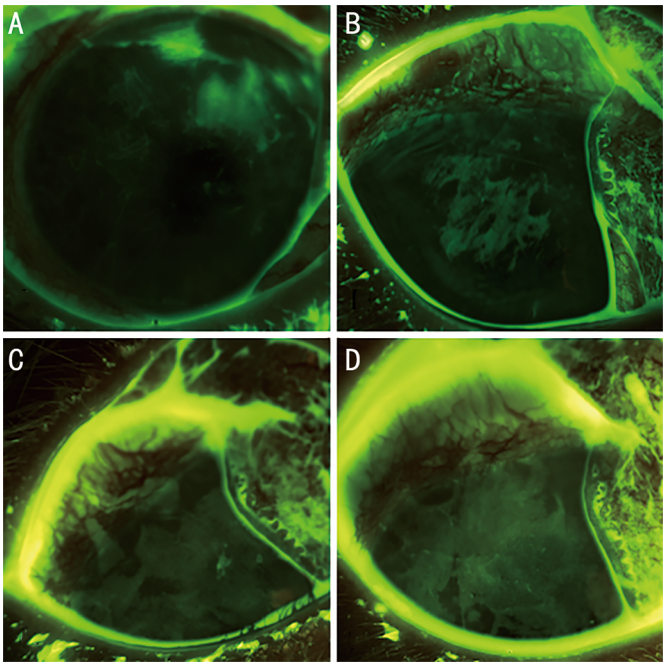

Figure 3 Images of corneal fluorescein staining patterns can provide information about the location, severity, and extent of corneal damage or pathology, such as corneal abrasions, ulcers, infections, or DED A: Staining performed at the baseline before injection of CFA, which shows some located staining areas at the edges of cornea; B: Cornea fluorescein staining 7d after injection of CFA, the staining area is spread though out the cornea surface; C: Cornea fluorescein staining 14d after injection of CFA, the staining area is spread though out the cornea surface; D: Cornea fluorescein staining 21d after injection of CFA, the staining area is spread though out the cornea surface. CFA: Complete Freund's adjuvant; DED: Dry eye disease.

injection of CFA in the treated right eye (OD) when compared with the left eye (OS) or tear volume before induction, which is presented in Figure 4. That shows that production of LGs were not impacted. TBUT was reduced by around 50%, as shown in Figure 5 and did not change further during the whole period of the experiment, which is a sign of increased tear film evoporation.

In addition, severe inflammation can be detected in the eyelid's parenchyma, as well as thinning of the anterior epithelium corneal cells, which can be seen in Figure 6A and 6B and normal epithelium cells in Figure 6C and Figure 6D. This inflammation also caused a drastic reduction in the goblet cells, as shown in the conjunctivas in Figure 7. There was a huge difference in inflammation severity between the injected eyelid OD of CFA (Figure 8A) and control eyelid OS (Figure 8B).

Lacrimal Gland Resection A reduction in tear production by around 50% was observed after the resection of LG and NM (Figure 9). During the TBUT test, a short TBUT was observed, which is a sign of poor tear film production too (Figure 10). Corneal damage can be observed with fluorescein staining (Figure 11). This is because the LG is responsible for producing a significant portion of the aqueous component of the tear film, and removing it can lead to a decrease in tear production and more severe DE. The histology samples

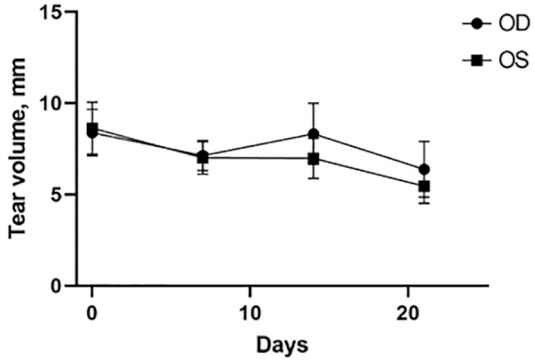

Figure 4 Average tear volume before and after injection of CFA Average tear volume increased after injection of CFA in the treated eye OD. CFA: Complete Freund's adjuvant; OD: Right eye; OS: Left eye.

showed a reduction in goblet cells of the conjunctiva (Figure 12), though no significant changes in the cornea were observed.

Benzalkonium Chloride The BAK model with rabbits showed elevated tear production during the experiment after induction with a solution of 1% BAK as eye drops

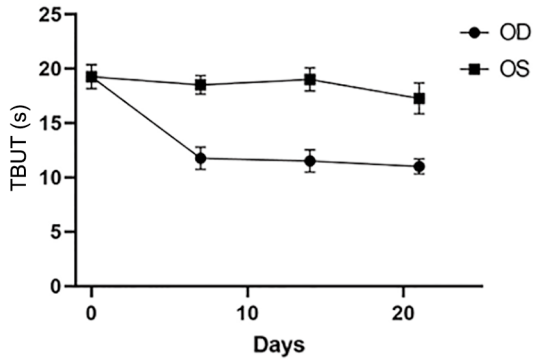

Figure 5 TBUT after CFA injection Seven days after injection of CFA into OD eyelid, the TBUT reduced by around 50% and remained that way throughout the experiment due to increased tear evaporation rate, when untreated eye OS TBUT was the same as at the baseline. TBUT: Tear break-up time; CFA: Complete Freund's adjuvant; OD: Right eye; OS: Left eye.

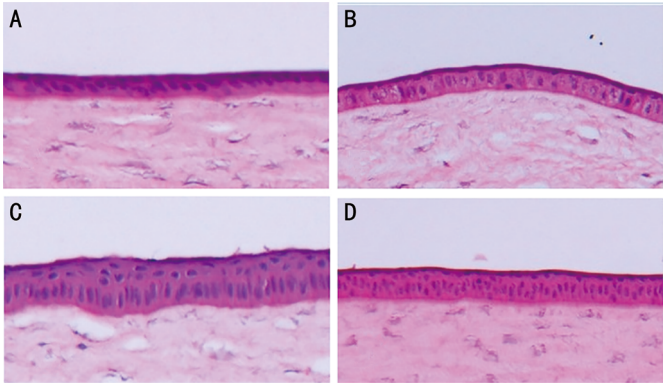

Figure 6 Anterior epithelium of cornea stained with HE A, B: Treated eyes (OD); C, D: Untreated eyes (OS). A, B: Epithelium cell layers are thinner and reduced in density than in C and D. The epithelium of the cornea can become thinner as a result of dry eye syndrome. The cornea is nourished and protected by the tear film, which is made up of various components including water, lipids, mucins, and electrolytes. In dry eye syndrome, there is a deficiency or instability in one or more of these components, leading to an unstable tear film and subsequent damage to the corneal epithelium. HE: Haematoxylin-eosin; OD: Right eye; OS: Left eye.

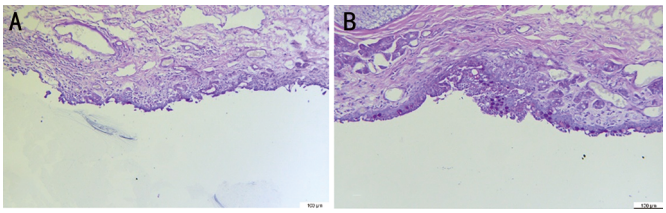

Figure 7 PAS staining of conjunctiva A: OD eyelid 28d after CFA which shows less goblet cell; B: OS eyelid which was injected with saline. DED can lead to a reduction in the number and function of goblet cells in the conjunctiva. Goblet cells are responsible for producing mucin, which is an important component of the tear film that helps to maintain the ocular surface health. PAS: Periodic acid-Schiff; CFA: Complete Freund's adjuvant; DED: Dry eye disease; OD: Right eye; OS: Left eye.

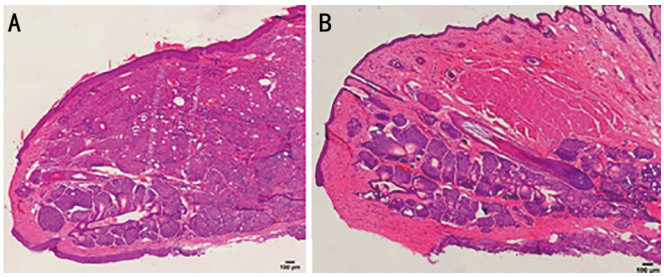

Figure 8 HE staining of the central portion of the eyelid with sections of meibomian glands A: OD eyelid 28d after CFA. With clearly visible inflammation. It is visible that sample is swollen with various inflammatory cell like macrophages, eozinofiles, plasmocytes and other. B: OS eyelid injected with saline 28d. It is more difficult to see edges between different tissues of eyelid. HE: Haematoxylin-eosin; CFA: Complete Freund's adjuvant; OD: Right eye; OS: Left eye.

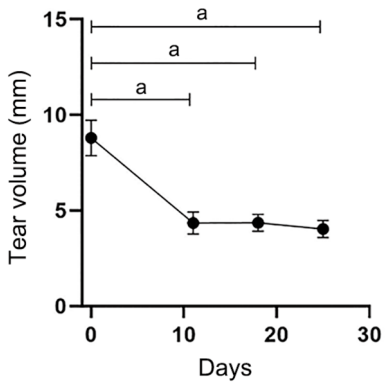

Figure 9 Tear volume before and after LG, HG, and NM resection Average tear production of all (n=5) was reduced by 50% after operation. This is because the LG is responsible for producing a significant portion of the aqueous component of the tear film. LG: Lacrimal gland; HG: Harderian gland; NM: Nictitating membrane. Parametric data was analyzed using one-way ANOVA test. ^{a}P<0.005 when compared tear volume before and after induction.

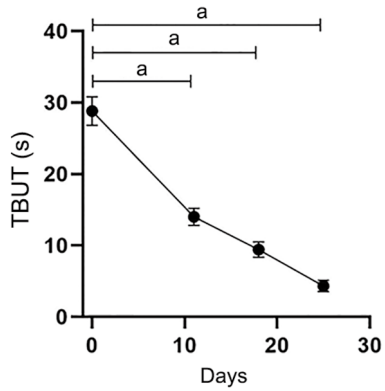

Figure 10 Lacrimal gland removal reduces TBUT significantly Inadequate tear film leads to faster evaporation and a shorter TBUT. After LGR, the reduced tear production leads to a decreased volume of tears, and a shorter TBUT. TBUT was reducing in each measurement day. Parametric data was analyzed using one-way ANOVA test. ^{a}P<0.005 when compared tear volume before and after induction. TBUT: Tear break-up time; LGR: Lacrimal gland resection.

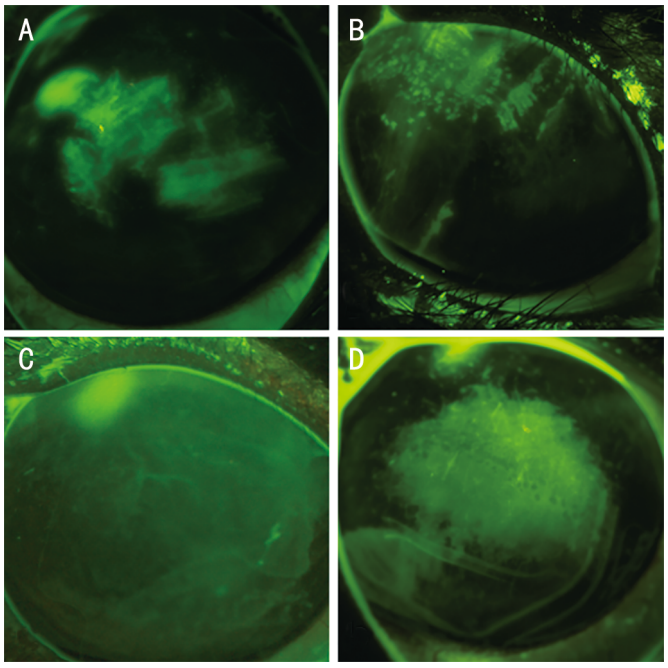

Figure 11 Images of corneal fluorescein staining A: Corneal fluorescein staining performed at the baseline before operation, which shows some located staining area in the center of the cornea; B: Cornea 11d after operation, shows more spread staining at the saides of cornea; C: Cornea staining after 18d post-operation, staining of cornea is spread though out the surface of cornea; D: Cornea staining 25d after operation, when compared with baseline, the staining of conrea is spread much wider around the surface.

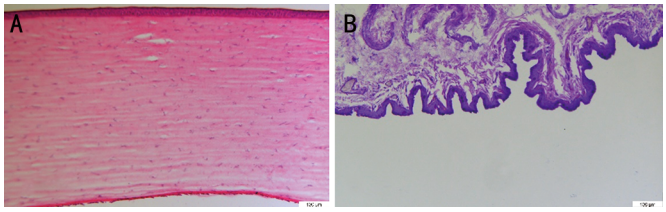

Figure 12 Histology of cornea (A) and conjunctiva (B) of LGR model There is no evidence in any macro structural changes of cornea. LGR: Lacrimal gland resection.

(Figure 13). The initial response to BAK exposure increased tear production as a compensatory mechanism to the damage caused by the compound. Although, the TBUT gradually decreased 3wk after induction (Figure 14). It may suggest that elevated tear production was not enough to compensate the quality of the tear film because of induced toxicity which lead to the alteration of tear film components, such as lipids and mucins, which can also contribute to increased evaporation of tear film after using 1% BAK solution as an eye drops. In Figure 15, fluorescein staining of the ocular surface shows how corneal lesion was induced by 1% BAK solution an eye drops in rabbits. Throughout the experiment, the corneal damage deepened as the staining got brighter and more widespread with time. Histological samples (Figure 16) show a decrease in conjunctival goblet cells and a thinning of the corneal layer

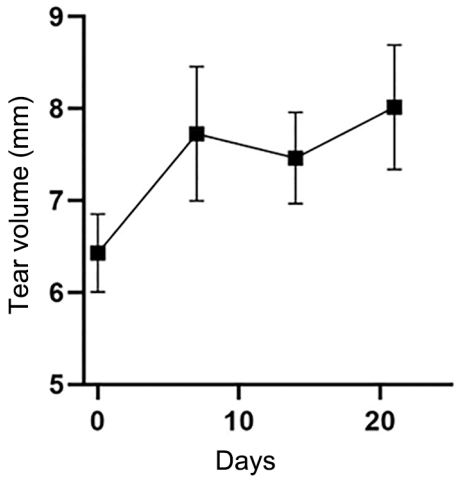

Figure 13 Tear volume increased during the 3-week experiment The increase in tear production after BAK exposure may be due to a compensatory response to the ocular surface irritation caused by BAK, as the body tries to lubricate and protect the eyes. BAK: Benzalkonium chloride.

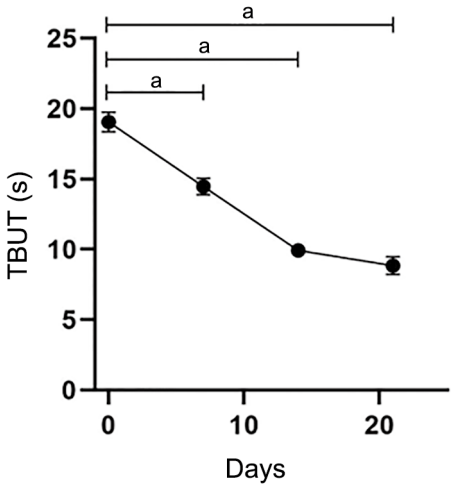

Figure 14 BAK has an impact on the reduced TBUT BAK-induced toxicity can lead to the alteration of tear film components, such as lipids and mucins, which can contribute to decreased TBUT. Parametric data was analyzed using one-way ANOVA test. $^{a}P<0.005$ when compared tear volume before and after induction. TBUT: Tear break-up time; BAK: Benzalkonium chloride.

and in contrary no fenotype of inflammation was observed in the histopathology samples of lower eyelid.

DISCUSSION

Lacrimal Gland Resection Model Bilateral resection of the NM, HG, and main LG proved that it can create symptoms of DED. This is because the LG is responsible for producing a significant portion of the aqueous component of the tear film, and removing it can lead to a decrease in tear production and more severe dry eye symptoms. Therefore, after LGR, the reduced tear production leads to a decreased volume of tears, and a shorter TBUT. The experiment showed a reduction in tear volume was around 50%, which comfirms in a significant

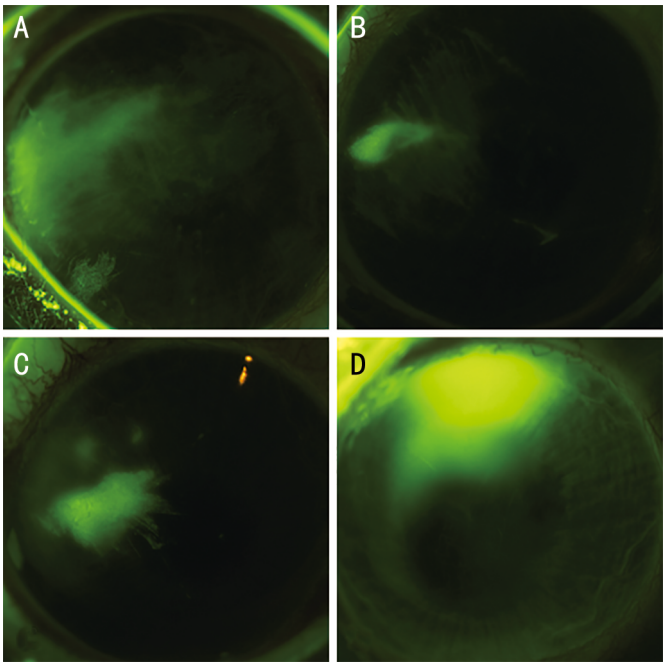

Figure 15 Fluorescein staining on ocular surface in the rabbit model with BAK BAK is a commonly used preservative in eye drops and has been shown to have toxic effects on the ocular surface and can course corneal damage. A: Corneal fluorescein staining was performed at the baseline before induction; B: Cornea staining after 7d of induction, where intensity of staining is localised in small patch; C: Fluorescein staining 14d post-induction where fluoresceine staining bit enlarged; D: Cornea staining 21d post-induction, which shows intensive fluoresceine staining in the cornea. BAK: Benzalkonium chloride.

reduction in tear production. TBUT was significantly shorter than at the baseline of the measurements too Fluorescein staining confirmed damage on the corneal surface. These findings are similar to dry eye symptoms, such as discomfort, irritation, and even corneal damage.

Ali *et al*[14] carried out assessments on the effect of DED on the central corneal thickness (CCT) of the eyes of individuals affected with DED in comparison to the eyes of age-matched healthy individuals (70 patients with DED and 70 controls). Patients that were diagnosed with DED showed a lower CCT (mean: 536.5) compared to the control group (mean: 561.3) at $P<0.01$. Reduced corneal thickness can be attributed to chronic dehydration due to high levels of inflammatory mediators[14].

A study by Fujimoto *et al*[15] sought to determine if there existed differences in corneal thickness between patients with DED and normal individuals using a rotating camera and anterior segment optical coherence tomography. The outcome of the study revealed that there existed a strong correlation between the measurement of CCT and thinnest corneal thickness in patients with DED. The difference in central (non-DED: 11.8 and severe DED 19.6) and thinnest corneal (non-DED: 13.1 and DED 20.7) thickness was significant. As such, clinicians should consider the morphological assessment of the cornea for the diagnosis of DED[15].

Another physiological feature that might be of importance in the detection of DED is the ocular surface thickness. In a study by Liang *et al*[16] that sought to examine and compare the ocular surface thickness including central corneal limit and bulbar conjunctival epithelium thickness using optical coherence tomography, the limbal epithelium in DED patients was found to be thinner and the bulbar conjunctival epithelium was found to be thicker compared with normal controls. The degree of changes in the central corneal limit and the bulbar conjunctival epithelium thickness was directly correlated to alterations in the tear film, and this was associated with the varying symptoms of DED in the patients[16].

A study by Abou Shousha *et al*[17] sought to evaluate the importance of using corneal epithelial profile maps for the diagnosis and management of DED. The prospective case-control study used ultrahigh-resolution optical coherence tomography (UHR-OCT) for assessing corneal epithelial thickness. The study included 71 subjects, out of which 52 had DED. The symptoms of DED were assessed, and it was reported that patients with DED had a highly irregular corneal thickness compared with the corneal thickness of control subjects.

Unfortunately, with our experiment, we were not able to confirm if the increased thickness of the cornea has any impact on DED.

Meibomian Gland Dysfunction Model Among the various causative factors of DED, MGD is the most frequently observed cause. Also known as meibomitis, MGD appears as a heterogeneous condition that involves inflammation and hyperemia in the conjunctiva and eyelids, damage and staining to the cornea, and dry eyes due to the instability of the tear film, increased tear evaporation rate and decreased tear volume. Structural changes of these glands and inflammation are two simultaneous events that cause MGD.

The triple injection with CFA (each 50 μL) triggered inflammation of meibomian glands (meibomitis is a subtype of obstructive MGD) in New Zealand rabbits. In our rabbit model of MGD experiment, tear production was slightly increased despite the presence of MGD-related symptoms. This could be due to a compensatory response by the LG in an attempt to maintain adequate tear film stability. However, the tear film quality was compromised as shown by the decreased TBUT and widespread corneal fluorescein staining, which indicates increased ocular surface damage. The clear inflammation of the eyelid detected in the histopathological HE slides is consistent with the known inflammatory component of MGD. The inflammatory process can damage the meibomian glands and lead to the production of altered meibum that contributes

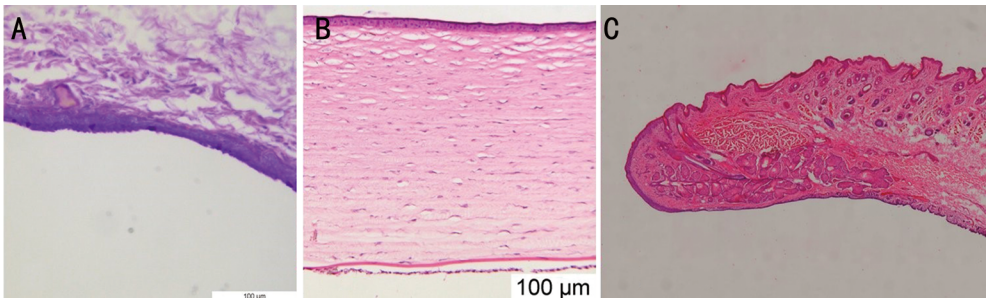

Figure 16 HE histopathology The absence of eyelid inflammation after BAK exposure to rabbit eyes does not necessarily mean that there was no inflammatory response in the eyelids. A: Conjunctiva with with less than normal goblet cells; B: Cornea, with visibly thinner epithelium than in the healthy cornea; C: Lower eyelid, with no signs of inflammation though less than normal goblet cells in the conjunctiva and thinner epithelium of cornea suggest that damaged was caused by BAK and inflammation processes may be present. HE: Haematoxylin-eosin; BAK: Benzalkonium chloride.

to tear film instability and ocular surface damage. Therefore, the combination of increased tear production with reduced tear film stability and increased ocular surface damage in the rabbit model of MGD suggests a complex interplay between different factors involved in the pathophysiology of this disease.

Aragona *et al*[5] and Liu *et al*[18] focused on MDG as a pathological event in DED. They reported that instability of the tear film causes inflammation and hyperosmolarity, both of which lead to DE syndrome[5]. A study on aqueous-deficient DE patients with punctal plugs insertion performed by Liu *et al*[18] showed improvement in DE parameters and meibomian gland function for at least 6mo in both groups, except for meiboscore.

Benzalkonium Chloride Model When administered topically to the eyes of rabbits, BAK at a concentration of 0.1% has been shown to increase tear production, possibly as a result of irritation to the ocular surface. However, the decreased tear film stability (as indicated by the decreased TBUT) and increased fluorescein staining observed in the cornea suggest that the increased tear production may not be sufficient to maintain the normal ocular surface function. The reduction of goblet cells and thinning of corneal epithelium seen in the histopathological HE slides may be due to the direct toxicity of BAK on these tissues. It is important to note that while inflammation was not observed in the eyelids, it is possible that other inflammatory markers or cytokines were present, but not detected by the HE staining.

The results differed depending on the DE syndrome model, which explains that the composition of tear film can differ depending on which part of the eye was targeted by DE syndrome induction methods. In our first model, triple injection with CFA (each 50 μL) triggered inflammation of the meibomian glands (meibomitis is a subtype of obstructive MGD) in New Zealand rabbits. The MGD model changed the lipid layer of the tear film. The bilateral resection of the NM, HG, and main LG reduced the watering layer of the tear film by reducing its production. Topical administration of the prepared BAK 0.1% solution impacted the mucosal layer of the tear film by reducing goblet cells of the conjunctiva.

Overall, the three rabbit models of DED have different underlying mechanisms and pathologies, leading to different manifestations of the disease. MGD and BAK-induced DED models primarily affect the meibomian glands and ocular surface, while the LG removal model is an aqueous-deficient DED model. The differences in tear production, TBUT, corneal staining, and histopathological findings reflect the different pathologies and mechanisms involved in each model. These models provide a valuable tool for studying the pathogenesis of DED and testing potential therapeutic agents. However, caution should be exercised when interpreting the results obtained from different DED models, as the pathologies and underlying mechanisms may differ significantly.

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