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Lacrimal apparatus, Eyelids, Orbit

Establishment of a Beagle Dog Model of Dry Eye Disease

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Purpose: To establish a Beagle dog model of dry eye disease (DED).

Methods: DED models were induced by surgical removal of orbital lacrimal glands and entire resection of third eyelids in the left eyes of six Beagle dogs. Intact right eyes served as self-controls. Non-anaesthetized Schirmer test (STT), tear break-up time (TBUT), and fluorescein staining grading were performed monthly after operation. Interleukin (IL)-1β, IL-8, IL-10, and tumor necrosis factor–α (TNF-α) levels were detected in tears and conjunctiva tissues. Six months after surgery, conjunctiva and cornea were collected and histopathologically analyzed.

Results: Signs of DED appeared within one month after surgery and then remained stable. STT values were significantly reduced by 88% within 3 weeks after operation and remained stable over months with 1.6 $\pm$ 0.4 mm. Mean TBUT decreased significantly within two months after operation and maintained 5.2 $\pm$ 1.1 seconds. The mean fluorescein staining score was highest at the first month and then was reduced, eventually reaching a balance with 11.0 $\pm$ 1.3 points. Elevated levels of IL-1β, IL-8, IL-10, and TNF-α were detected in tears and conjunctivas of operated eyes. Hematoxylin and eosin staining showed cornea neovascularization in the corneal stroma with thickened stroma layer and disorganized collagen bundles. Periodic acid-Schiff staining revealed a reduced function of conjunctival goblet cells.

Conclusions: A combined type of DED model on the Beagle dog was established by removal of the orbital lacrimal gland and resection of the third eyelid. This DED model is easily accessible and is stable at six-month observation.

Translational Relevance: The surgery-induced Beagle dog DED model is easily accessible and stable over a relatively long time.

Introduction

Dry eye disease (DED) is a chronic ocular surface condition characterized by reduced tear secretion and altered tear film composition. Chronic irritation of an abnormally lubricated ocular surface leads to conjunctival epithelial keratinization, conjunctival goblet cell decrease, corneal epithelial defects, or even blindness.[1]

Minor salivary gland (MSG) transplantation is an effective and easily accessible surgical treatment for severe DED without Sjögren's syndrome,[2] which was first introduced by Murube et al.[3] in 1998. Transplanted MSG can improve basal secretion of tears and ocular surface conditions in the early stages.[4–6] However, the long-term viability of transplanted MSG needs to be further established as the progress of fibrosis and acinar atrophy.[2,7] Therefore establishing a proper DED animal model for MSG transplantation is essential for further exploring the mechanism underlying the regulation of transplanted MSG secretion.

So far, numerous DED models have been developed using mice, rabbit, and swine[8,9]; however, our preliminary study showed that MSGs in those species are less suitable for investigation of MSG transplantation. Recently, researchers reported that canine species shared similar MSG histological features with humans,[10] and autograft of inferior buccal MSG in treating canine spontaneous DED has been successfully performed.[11] Our preliminary study also found

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that canine inferior buccal MSG, which is located in the submucosal connective tissue and predominantly consists of mucous acini with the duct opening directly on the mucosal surface, was very similar to human labial MSG. Thus the canine species has more potential to serve as a good animal model for investigating MSG autotransplantation.

At present, there are only two dog models of DED, the spontaneous model and the main lacrimal gland ablation model.[8] The spontaneous canine DED model was more commonly used in investigations, because spontaneous DED occurs commonly in many canine breeds, including the American cocker spaniel, Lhasa Apso, and Shih Tzu, but it is rarely observed in laboratory dog breeds such as mixed-breed and Beagle dogs.[12] More importantly, spontaneous DED in dogs affects salivary gland function as well, causes xerostomia, and has multiple serum antibodies resembling human Sjögren syndrome.[13] Sjögren's syndrome is a contraindication for MSG transplantation in treating severe DED. Thus spontaneous canine DED model is not the ideal model for MSG autotransplantation.

Surgically induced DED model was relatively less used. Helper et al.[14] has introduced a surgically induced DED model using mongrel dogs, which is still applied today.[15] However, different dog breeds have morphological variations in the gross anatomy of ocular structures, including size and location of lacrimal glands.[16] The Beagle dog is a mature experimental animal strain with stable ocular anatomy, a deep orbit, and a small-size lacrimal gland with a shallow location.[16] More importantly, spontaneous DED rarely occurs in Beagle dogs (1.2%).[12] Advantageous and stable ocular structures make Beagle dogs a more proper animal model for MSG transplantation investigations. However, so far, no study has reported on the establishment of a DED model using Beagle dogs.

This study aimed to establish a new canine model of DED using Beagle dogs, modify the previously reported surgical techniques, and proceed with a long-term ocular observation of this new model. We hoped that the new model could be ideally used to study MSG transplantation in treatment of severe DED.

Methods

Animals

The study protocol was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (LA2016316) and adhered to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

Six male Beagle dogs (six months, 5–8 kg) were purchased from Peking University Laboratory Animal Center and treated according to the Peking University institutional guidelines for animal care and the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Before any canine subject was admitted to the study, the eyes of each dog were screened for spontaneous DED symptoms. Of the six canine subjects included in this study, all left eyes were surgically induced for DED, and the right eyes were intact and served as self-controls.

Surgical Induction of DED

The DED model was established by surgical removal of orbital lacrimal glands (OLGs) and nictitans lacrimal glands (NLGs) along with the entire third eyelid. This method was based on and further modified from previous studies.[14,15]

The operation was performed with the dog under general anesthesia induced by propofol (Dasheng Pharmaceutical, Shanxi, China) and maintained by isoflurane (J&K Scientific, Beijing, China). For sterile preparation of the eyes and periocular regions, 3% levofloxacin eyedrops were administered for three days before operation, and the conjunctival sac was rinsed with normal saline solution before skin disinfection. The upper and lower eyelids were sutured to prevent dryness of the ocular surface during operation (Fig. 1A). To remove OLGs, a skin incision was made over the dorsolateral orbit rim, transecting the orbital septum, and the OLGs were exposed as Figure 1B shown, bluntly separating the orbital ligament. The OLGs were sharply dissected from their supporting tissues. The orbital fascia was sutured with 5-0 absorbable thread, and the skin wound was closed using 4-0 nylon thread. For the removal of NLGs, the incision was made surrounding the base of the third eyelid, and the NLGs, along with the associated cartilage and connective tissues, were entirely removed. Then the conjunctival wound was sutured with 6-0 absorbable thread. After surgery, a total of 400,000 U penicillin (North China Pharmaceuticals, Hebei, China) was administered intramuscularly twice daily for three days. Eye drops with 3% levofloxacin were given three times daily for five days after operation.

The Schirmer Test

The Schirmer test (STT) without anesthesia was performed before any vital dye was applied to the ocular surface. A standard Schirmer strip was folded at the notch and carefully inserted into the junction of the middle and outer third of the lower lid by using

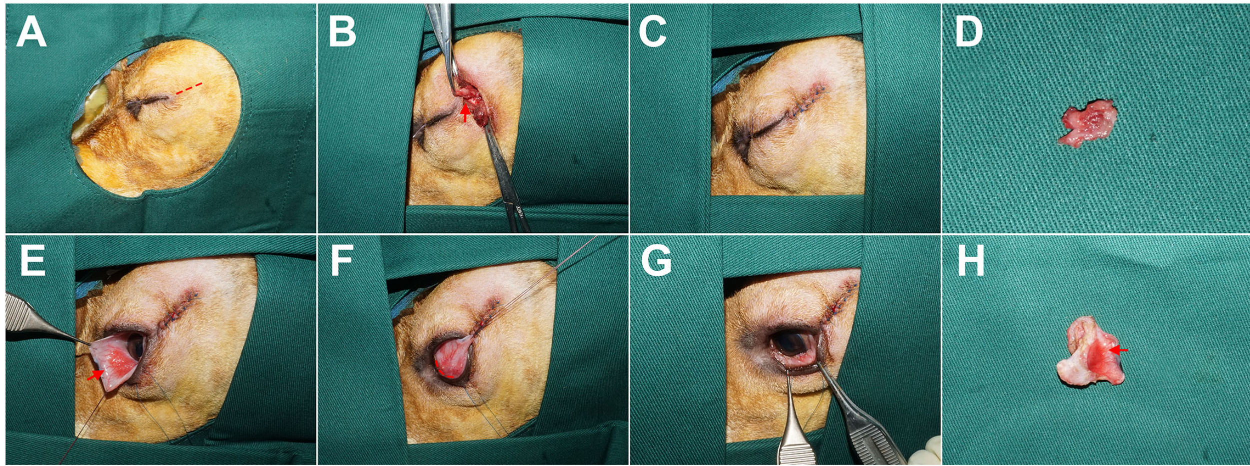

Figure 1. Surgical techniques. (**A**) Surgical approach was marked by *red dashed lines*, the upper and lower eyelids were sutured to prevent dryness of ocular surface during operation. (**B**) The orbital lacrimal glands were exposed (*red arrow*) and removed. (**C**) The skin wound was sutured. (**D**) The orbital lacrimal glands were removed. (**E**) The third eyelid was turned up, and the nictitans lacrimal glands were exposed (*red arrow*). (**F**) Part of the surgical approach surrounding the base of the third eyelid was marked by *red dashed lines*. (**G**) The third eyelid was entirely resected, and the wound was sutured. (**H**) The resected third eyelid and the nictitans lacrimal glands (*red arrow*).

a tweezer. After keeping the eyelids closed for one minute, the length of wetting strips was measured. For each eye, the Schirmer test was performed three times, and the mean value was recorded.

Tear Break-Up Time

The tear break-up time (TBUT) was conducted second. The TBUT was measured using a handheld slit lamp (MediWorks, Shanghai, China) with a cobalt blue filter over the light source, the oculars were set at magnification $\times 10$. Two minutes after the instillation of 10 μL of 0.5% fluorescein solvent (Jingming New Technological Development Co., Tianjin, China) by means of a micropipette, the TBUT, defined as the time in seconds between the last blink and a dark spot occurring on the corneal surface, was assessed by a trained ophthalmologist. The TBUT was performed three times, and the mean value was recorded.

Corneal Fluorescein Staining

The corneal fluorescein staining was graded between four and eight minutes after the instillation of 0.5% fluorescein dye to ensure reproducibility.[17] The cornea was divided into five sections: central, temporal, nasal, upper, and lower zone. Punctate epithelial erosions (PEEs) of each zone were counted and scored. If no PEE was seen, the score was 0; 1 to 5 PEEs were scored as 1; 6 to 30 PEEs were scored as 2; more than 30 PEEs were scored as 3. The possible score for each zone was 0 to 3, and for each cornea was 0 to 15. A trained ophthalmologist conducted the examination of the cornea staining.

Tissue Sampling and Processing

Conjunctival specimens were obtained by surgical biopsy from both control and experimental eyes six months after operation. All dogs, except one, were administered general anesthetic, and a 3 mm^3 piece of conjunctiva specimen was excised from the ventral anterior fornix, a most reliable sampling site for quantifying conjunctival goblet cells.[18] One canine subject was euthanized to obtain cornea and conjunctiva samples six months after operation.

The specimens were fixed with 10% formaldehyde, embedded in paraffin, and then sliced into 5 μm sections. The sections were rehydrated and stained with hematoxylin and eosin. The conjunctiva sections were stained with periodic acid-Schiff (PAS; Solarbio Science & Technology, Beijing, China) to show conjunctival goblet cells. Three fields (magnification $\times 40$) of each specimen were photographed under a light microscope (Leica, Heidelberg, Germany) and analyzed by two independent observers using the Image Pro Plus version 6.0 software (Media Cybernetics, Rockville, MD, USA). The average number of PAS staining positive cells reflects the quantity of functional goblet cells. The average PAS staining density reflects the stores of intracellular mucin, which is PAS staining positive, indicating the quality of individual goblet cells.

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Table. Primer Sequences Used for Real-Time PCR

Gene	Forward (5′-3′)	Reverse (5′-3′)
IL-1β	TACCTGTGGTCTTGGGCATC	TGTAGGGTGGGCTTTCCATC
IL-8	TGTCCTTTCTGCAGCTCTCTG	GGATGGAAAGGTGTGGAGTGT
IL-10	CAAGCCCTGTCGGAGATGAT	TGATGTCTGGGTCGTGGTTC
TNF-α	CCTCTTGCCCAGACAGTCAA	TCAGCTTCGGGGTTTGCTAC
GAPDH	TGCCCAGAACATCATCCCTG	GATACATTGGGGGTGGGGAC

Tear Collection and Inflammatory Molecules Quantitation

Tear samples were collected from all canine subjects before the surgery, and at two and six months after operation. A nonstimulated tear sample (1 μL) was collected by using a glass microcapillary tube (Drummond Scientific Company, Broomall, PA, USA) at the temporal canthus of the left eye. Samples were diluted 10 times with ice-cold phosphate buffered saline solution and stored at −80°C for the analysis of inflammatory cytokines.

The concentrations of tear inflammatory molecules including interleukin (IL)-1β, IL-8, IL-10, and tumor necrosis factor–α (TNF-α) were detected by using enzyme-linked immunosorbent assay kits according to the manufacturer's protocol. All enzyme-linked immunosorbent assay kits were purchased from MyBioSource (IL-1β, MBS2000111; IL-8, MBS2502144; IL-10, MBS2021448; TNF-α, MBS2019482; San Diego, CA, USA).

Inflammatory Molecule Transcriptional Analysis

A piece of conjunctival sample was collected from each left eye of all six dogs before surgery and at six months after operation. Transcription levels of inflammatory molecules (IL-1β, IL-8, IL-10, and TNF-α) in conjunctival samples were detected by real-time polymerase chain reaction (PCR). Total RNA of conjunctival samples was extracted by using the E.Z.N.A Total RNA Kit (Omega Bio-tek, Norcross, GA, USA) and was reversely transcribed to cDNA by using the First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The obtained cDNA (2 μL) was amplified with Fast Start Universal SYBR Green Master (Roche, Basel, Switzerland) and detected by the Quant Studio 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). GAPDH was selected as the internal control. Primer sequences are shown in the Table.

Statistical Analysis

All data were presented as mean ± SD and analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Statistical significance was calculated by using Student's t-test when comparing two groups and one-way analysis of variance followed by post hoc tests when comparing more than two groups. $P < 0.05$ was considered statistically significant.

Results

Parameters On Tear Secretion And Ocular Surface

The mean values of presurgical STT of control eyes and operated eyes were, respectively, 21.6 ± 4.5 mm and 21.7 ± 4.2 mm, which is consistent with normal aqueous tear levels in dogs.[18,19] There was no significant difference in STT values between control eyes and operated eyes before surgery ($P > 0.96$). For control eyes, STT values remained constant over all six-month periods, and no significant difference was found ($P > 0.78$) (Fig. 2A). For operated eyes, surgical intervention induced a sharp decline in STT values. At the first, second, third, and fourth weeks after operation, mean STT values decreased to 34.7%, 16.2%, 12.0%, and 10.3%, respectively. The tear secretions of the operated eyes were eventually stabilized at four weeks after operation, and STT values of all six operated eyes ranged from 1 to 4 mm (with a mean of 2.2 ± 0.5 mm), which was significantly lower than the self-control ($P < 0.0001$).

The tear film stability of operated eyes also significantly decreased after surgery. Because of the swelling and pain caused by the surgery, tear break-up time and corneal fluorescein staining scores were unable to be measured during the first three weeks after operation. However, one month after the operation, the mean TBUT of operated eyes decreased significantly to 10 to five seconds (with a mean of 8.2 ± 1.6 seconds) compared with the control eyes (20 to 15 seconds with a mean of 16.9 ± 2.0 seconds) ($P < 0.0001$), and further

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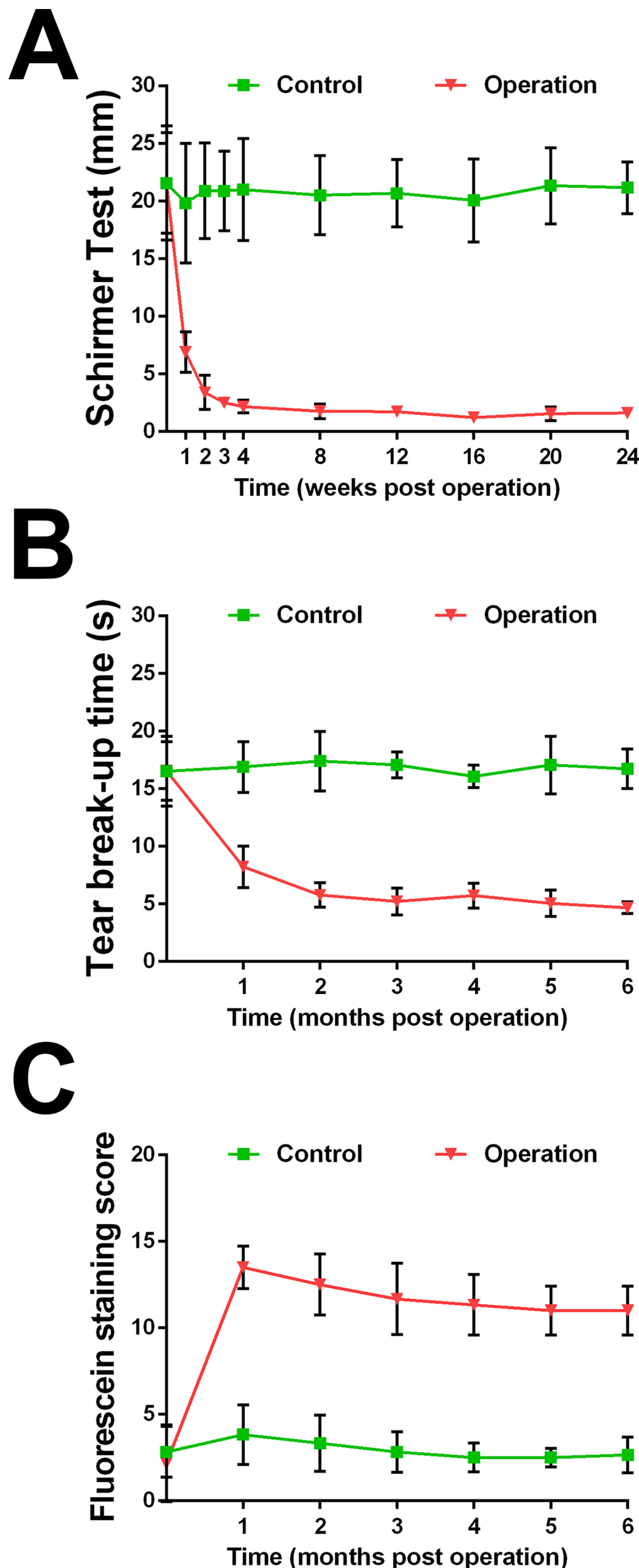

Figure 2. Comparison of STT, TBUT ,and fluorescein staining scores between the operated eyes and intact eyes six months after operation. (**A**) Schirmer test values of the operated eyes significantly reduced by 88% within three weeks after operation ($P < 0.0001$) and remained stable over the subsequent months. (**B**) Mean tear break-up time of the operated eyes decreased significantly from 16.6 to 5.8 seconds within two months after operation ($P < 0.0001$) and maintained an average of 5.2 seconds. (**C**) Mean fluorescein staining score of the operated eyes was highest in the first month after operation, a slight amelioration occurred and eventually reached a balance with a mean score of 11 points. Data are shown as means ± SD (n = 6).

decreased until the second month after operation and then stabilized at four to eight seconds with a mean of 5.8 ± 1.0 seconds (Fig. 2B).

Corneal injury was monitored by fluorescein staining score in real time and recorded in Figure 2C. One month after the operation, the mean fluorescein staining score of operated eyes intensely increased to 15 to 13 points (with a mean of 13.5 ± 1.1) and then improved afterward. Although there were some improvements in the two months, the mean fluorescein staining scores of the operated eyes remained at a higher level of nine to 13 points (with a mean of 11 ± 1.3) until the sixth month, which were different obviously from the control eyes (with a mean of 2.7 ± 0.9) ($P < 0.0001$).

Morphological Changes

Within the first month after surgery, conjunctival hyperemia rapidly occurred with an accumulation of tenacious discharge, and the cornea was affected subsequently. These pathological changes ceased to progress but began to ameliorate one month after operation and began to stabilize two months after surgery. At the sixth month after operation, there were still varying degrees of confluent staining patches observed on the cornea under fluorescein staining (Fig. 3B). Furthermore, hematoxylin and eosin (H&E) staining indicated neovascularization in the corneal stroma with thickened stroma layer and disorganized collagen bundles (Fig. 3C). H&E staining of conjunctival specimens showed a dense lymphocytic infiltration in the conjunctival epithelia (Fig. 4B). H&E and PAS staining also showed that the mean number of conjunctival goblet cells was significantly reduced six months after surgery, compared with the control eyes (Fig. 4E), and histological analysis of PAS staining density also revealed a significantly reduced intracellular mucin stores, indicating a decreased function of conjunctival goblet cells (Fig. 4F).

Tear Inflammatory Molecule Analysis

In tear samples of the operated eyes, all cytokines and chemokines measured in this study (IL-1β,

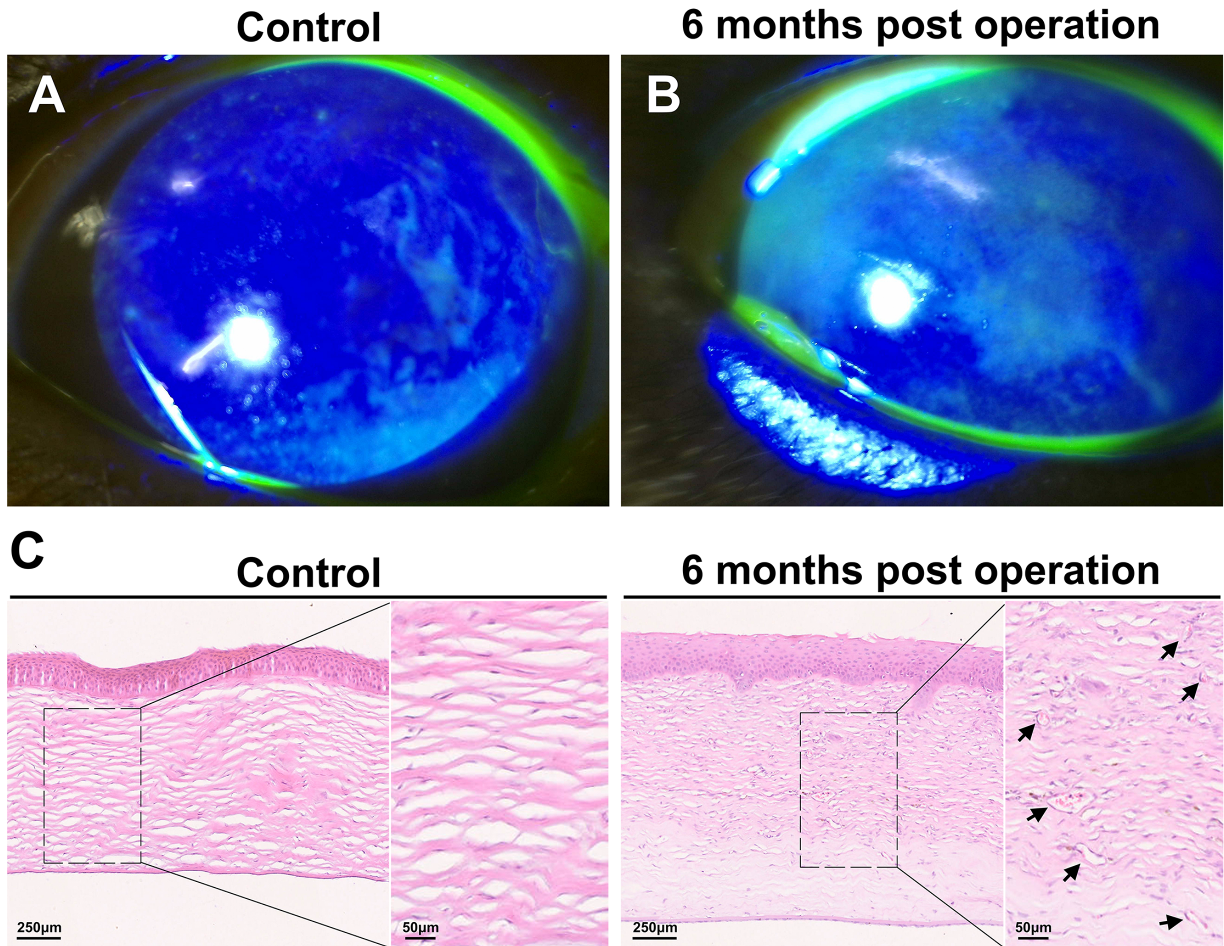

Figure 3. Fluorescein staining and representative histological images of the corneas from the operated eyes and intact eyes. (**A**) Control eye shows a normal appearance of rabbit cornea under fluorescein staining. (**B**) Confluent staining patches could be observed on cornea of operated eye. (**C**) Histologic examination of the cornea from the control eyes and the operated eyes. In the control eyes, the stroma is composed of uniform collagen without any blood vessels. In the operated eyes, neovascularization (*black arrows*) presents in the corneal stroma with a thickened stromal layer and disorganized collagen bundles.

IL-8, IL-10, and TNF-α) showed dramatic elevation of concentration at the second month after operation versus before operation ($P < 0.001$), as shown in Figure 5A. Up to the sixth month, all four inflammation entities measured continued to demonstrate a significant increase in concentration compared with the preoperative levels ($P < 0.001$). Consistent with the amelioration and stabilization of the ocular surface symptoms at six months after operation, the concentrations of IL-1β, IL-8, and TNF-α reduced partially compared with the second month, except that IL-10 showed no decrease but continued to increase six months after operation. In conjunctival samples (Fig. 5B), all four inflammatory entities measured also demonstrated a significant increase in transcription levels versus before operation ($P < 0.05$).

Discussion

DED is a multifactorial disease that could be generally categorized into three types (aqueous-deficient type, evaporative type, and combined type), as well as dry-eye animal models. The Beagle dog is a commonly used experimental animal, which was recently discovered to be a potential animal model for investigating minor salivary gland transplantation.[11] However, no dry eye model using the Beagle dog has been proposed. Therefore, based on the other canine species dry eye models,[14,15] we established and validated a combined type of dry eye model on the Beagle dog by removing the orbital lacrimal gland and complete resection of the third eyelid. Parameters of tear secretion and ocular surface of this dry eye model were stable, repeatable,

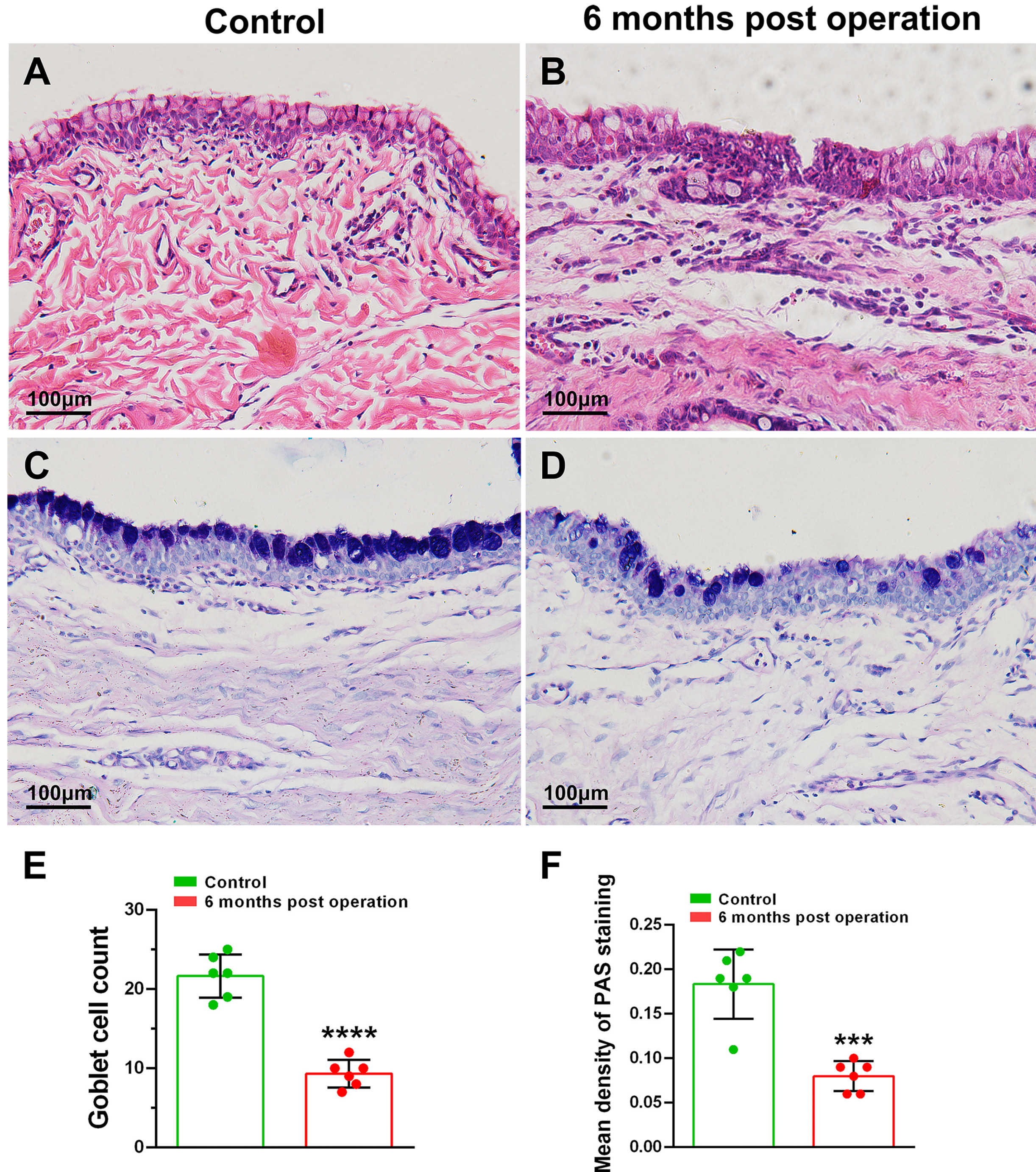

Figure 4. Histologic analysis of conjunctival epithelia. H&E staining of the conjunctiva from the control eyes (**A**) and the operated eyes (**B**). H&E staining showed a dense lymphocytic infiltration in the conjunctival epithelia (**B**). PAS staining of conjunctiva from the control eyes (**C**) and the operated eyes (**D**). (**E**) Conjunctiva goblet cells number in the operated eyes was significantly lower than in the control eyes ($P < 0.0001$). (**F**) Mean density of conjunctiva goblet cells was decreased significantly in the operated eyes, compared with the control eyes ($P < 0.001$). Data are shown as means ± SD (n = 6).

and consistent among all canine subjects in a six-month observation.

The third eyelid is not only responsible for tear secretion, but also for the function of physical protection, immunologic defense, and tear retention of the corneal surface.[20] Instead of merely removing the nictitans lacrimal glands on the third eyelid,[14,15] in this study, we resected the entire third eyelid, leading to a

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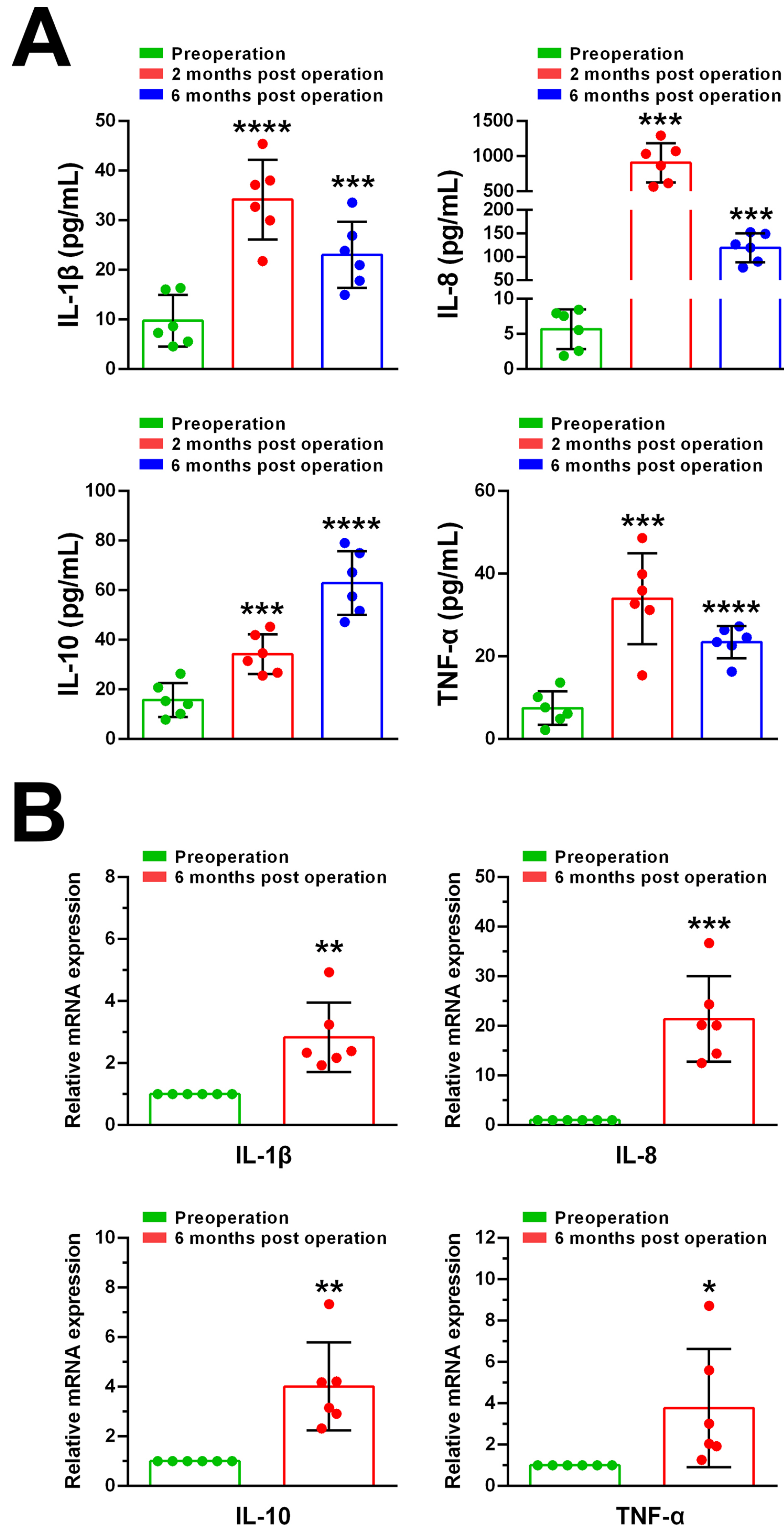

Figure 5. Inflammation molecule levels in tear samples and conjunctiva samples in the Beagle dog dry eye model. (**A**) IL-1β, IL-8, IL-10, and TNF-α concentrations in tear samples were significantly elevated two months after operation compared with before operation ($P < 0.001$). IL-1β, IL-8, and TNF-α concentrations partly declined six months after operation versus two months after operation ($P < 0.001$), whereas IL-10 showed no decrease but was elevated at six months after operation. (**B**) Gene expressions of IL-1β, IL-8, IL-10, and TNF-α in conjunctival samples were significantly increased six months after the surgery ($P < 0.05$). Data are shown as means ± SD (n = 6). ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

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complete purge of nictitans lacrimal glands and creating an environmental stress similar to that seen in humans.

Within the first month after surgery induction, STT values were reduced gradually but not immediately, which we preferred to attribute to inflammatory exudation rather than tear secretion. Secondary infection is considered an important precipitating cause of ocular surface injury, including corneal epithelial injury, conjunctival epithelial necrosis, conjunctival goblet cell reduction, and more, and also the critical cause of severe corneal injury involving the stroma layer, which is undesired. Therefore perioperative administration of antibacterial eye drops is necessary, prolonging the application time or adding artificial tears might be considered to protect the cornea from irreversible damage.

Ocular surface mucins, synthesized and secreted primarily by conjunctival goblet cells (CGCs), provide a transparent covering to the cornea, enhance the wettability of the cornea, and serve as a barrier to microorganisms and a shield of debris and noxious substances.[15,21] Significant reduction in quantity and quality of CGCs in this model could result from the abundant release of proinflammatory factors after surgical intervention and chronic irritation. As a consequence, deficiency of CGC mucins leads to instability of the tear film, chronic inflammation, and persistent corneal injury.

In this model, dramatical elevation of inflammatory cytokines and dense conjunctival lymphocytic infiltration were revealed, which have been well documented as key drivers in the pathogenesis of human DED.[22,23] In tear samples of this DED model, the concentrations of IL-1β, IL-8, IL-10, and TNF-α were dramatically increased, and significantly upregulated mRNA expressions of all four entities were also detected in conjunctival tissues, indicating that excessive inflammatory factors in tears may be produced by conjunctiva. IL-1β and TNF-α are typical proinflammatory cytokines that contribute to ocular surface inflammation,[23] whereas IL-8 is a chemokine that stimulates the release of inflammation factors like interferon-γ and damages CGCs,[24] and IL-10 is an anti-inflammatory cytokine that suppresses the above proinflammatory cytokines.[25] At six months after operation, compared with at the second month, decrease of proinflammatory cytokines (IL-1β, IL-8, and TNF-α) and increase of anti-inflammatory cytokines (IL-10) were consistent with the amelioration and stabilization of the ocular surface symptoms at six months after operation, supposing that the balance of inflammatory cytokines is closely related to effect of this DED model.

Although the surgery-induced inflammation subsided, the pathological changes in the cornea were improved after surgery. However, reduction of aqueous secretion and mucins synthesis still leaves the ocular surface in an irritant condition, which is probably why the corneal epithelial injury remained six months after operation. However, no significant difference was found in fluorescein staining scores on operated eyes from the second to the sixth month, indicating that a balance was reached between inflammation caused by tear film deficiency and self-restorability. This homeostasis makes it possible to continue therapeutic research based on this dry eye model.

This study has some limitations. First, conjunctival staining was not performed for the reason that dogs could not cooperate to expose bulbar conjunctiva staining without anesthesia, and conscious canine subjects cooperated with all other noninvasive assessments. Second, this study only sacrificed one canine subject and sampled only one pair of corneas. Hence, the corneal histopathological changes were less likely to fully represent the actual situations.

In conclusion, we have successfully established a new surgery-induced dry eye model using the Beagle dog. In this model, tear secretion and tear break-up time of the operated eyes decreased significantly, and corneal epithelial injury existed in a relatively stable pattern over a relatively long time, which is beneficial for objectively evaluating the therapeutic efficacy on intervening DED.

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