Tear Production After Bilateral Main Lacrimal Gland Resection in Rabbits

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PURPOSE. This study reports tear compensation observed in rabbits with bilateral resection of main lacrimal gland (LG) and explored the potential mechanisms.

METHODS. Dry eye conditions were created by resection of nictitating membrane (NM), Harderian gland (HG), and main LG in eight (16 eyes) male New Zealand White rabbits. In addition to Schirmer test, Periodic acid-Schiff (PAS) staining, and ocular surface staining with fluorescein and rose Bengal, conjunctival impression cytology was employed before and up to 4 months after excision (AE). Using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), expression of inflammatory biomarkers (IL-1β, TNF-α, and matrix metalloproteinase-9) were monitored. Further, involvement of ion and water transporters were investigated in conjunctival epithelium.

RESULTS. Significant dry eye phenotypes in rabbits were observed 1 month AE, which corroborated with elevated biomarker mRNA expression. However, Schirmer test score and goblet cell numbers never decreased AE in conjunctival epithelium. Moreover, ocular surface staining, and biomarker expression declined to baseline in over 4 months AE. No upregulation was observed of the following conjunctival ionic transporters: cystic fibrosis transmembrane conductance regulator, sodium potassium chloride cotransporters, sodium potassium ATPase, and epithelial sodium channels. Instead, aquaporin (AQP) 4 and AQP5 were upregulated. Immunolocalization and immune blotting of AQP4 was demonstrated in rabbit conjunctival epithelium.

CONCLUSIONS. In the absence of NM, HG, and main LG in rabbits, tear secretion was not decreased and significant improvement of dry eye phenotypes observed with time AE. Conjunctival AQPs are possibly involved in a compensatory tear fluid production.

Keywords: dry eyes, conjunctiva, aquaporins

Dry eye syndrome (DES) is characterized by an immunoinflammatory response, affecting the ocular surface.1 Dry eye syndrome involves defects in aqueous, lipid, and/or mucin layers of the tear film (TF), resulting in damage to the ocular surface.1,2 Dry eye syndrome is currently classified into two categories, aqueous tear-deficient and evaporative dry eyes, all leading to increase in tear osmolarity and ocular surface inflammation.3 Ocular surface plays a significant role in the modulation of tear volume and TF composition.4 The conjunctiva, and to a much lesser degree, the cornea, are involved in basal tear production.4 Although the main lacrimal gland (LG) is considered the major source of tears,5 the conjunctiva modifies the TF by absorbing/secerting electrolytes and water and by secreting proteins such as mucin.6

Diquafosol, an activator of the P2Y2 receptor, is now used as a topical ophthalmic solution outside the United States for DES.7 Diquafosol stimulates mucin secretion from conjunctival goblet cells,6,9 activates the fluid pump mechanism of the accessory LGs on the conjunctiva,10–12 thereby promoting TF stability and rehydration.7 Human clinical studies have demonstrated its efficacy in improving aqueous tear secretion12–14 and mucin secretion,15–17 establishing a new dry eye treatment strategy: tapping into the reserve of the conjunctiva for tear and mucin augmentation.

It is believed that in the ocular epithelia, fluid transport involves secondary active Cl− transport which creates the osmotic gradient to drive transepithelial water transport.18 Besides P2Y2 receptor, ionic transporters such as cystic fibrosis transmembrane conductance regulator (CFTR), sodium potassium chloride cotransporter (NKCC), sodium potassium ATPase (NKA), and epithelial sodium channels (ENaC) across conjunctival epithelium regulate the ionic gradient across ocular surface as well.19 Additionally, the presence of aquaporin (AQP)-type water channels in ocular surface tissues suggest their role in water/fluid transport and TF homeostasis.20 Across a variety of epithelial tissues, AQP- and CFTR have been identified as the principal molecular pathways for water and Cl− transport, respectively.18 Aquaporin 3 and AQP5 have been identified in conjunctival epithelium.20–22

Previous in vitro studies have shown that rabbit conjunctival epithelium has the capacity to provide adequate tear volume.23,24 In the present study, mixed-mechanism dry eye conditions were created in rabbits by bilateral excision of the nictitating membrane (NM), Harderian gland (HG), and main
LG. The ocular surface status of these rabbits was evaluated before and after the excision for 4 months. We herein report findings of spontaneous improvement of dry eye phenotypes and ocular surface inflammation in these rabbits, as well as our initial attempt to delineate the underlying mechanism, by analyzing different ionic and water transporter proteins encoded by conjunctival epithelium.

METHODS

Experimental Animals and Ethics Statement

Male New Zealand white rabbits ($n = 8$, 16 eyes; Harlan Sprague Dawley, Indianapolis, IN, USA), weighing 2.0 to 2.5 kg were used for the study. The rabbits were reared under standard laboratory conditions ($22 \pm 2^\circ C$, 40% to 5% relative humidity, and a 12-hour light–dark cycle) with free access to food and water throughout the experiment. The study was conducted in compliance with the Tenets of the Declaration of Helsinki and ARVO Statement for the use of Animals in Ophthalmic and Visual Research. The protocol was approved by the University of Arizona (Tucson, AZ, USA) Institutional Animal Care and Use Committee (protocol# 14-511). All surgeries were performed by skilled surgeons (YN and MW).

Operative Procedure

The bilateral resection of the NM, HG, and main LG was performed following a modified surgical procedure of Chen et al.25 Identical procedure was performed on both eyes. Briefly, the skin of both periorcular regions was shaved. Induction was initiated with an intramuscular injection of Ketamine (25 mg/kg; Henry Schein, New York, NY, USA) + Xylazine (5 mg/kg; Akorn, Inc., Decatur, IL, USA) and each rabbit intubated with an uncuffed endotracheal tube. The general anesthesia was maintained by 1% to 4% Isoflurane gas with 1.5 L/min oxygen flow during the entire surgery. The cardiopulmonary status of the animals and the depth of anesthesia were monitored every 5 minutes. The surgical field was disinfected with 5% betadine solution used for analysis.

Conjunctival Impression Cytology

Conjunctival impression cytology (CIC) was performed on each rabbit eye after instilling 0.5% proparacaine hydrochloride eye drop (Bausch & Lomb, Rochester, NY, USA) and wiping away the excessive tear fluid. One 8-mm diameter nitrocellulose (Sartorius AG, Göttingen, Germany) filter paper disc with a pore size of 0.45 μm was gently placed on the surface of the superior temporal bulbar conjunctiva 2 mm from the corneal limbus. After applying light pressure for 5 seconds, the paper was peeled off and immediately placed in either, Trizol solution (Invitrogen, Carlsbad, CA, USA) for RNA isolation or 10% formalin solution for Periodic acid-Schiff (PAS) staining or 100 μL radio immunoprecipitation assay (RIPA) buffer (Teknova, Hollister, CA, USA) for protein isolation.

Schermer I Test

Briefly, after the rabbits were comfortably stabilized with a restraining device, standard Schirmer test filter paper strips (Alcon Laboratories, Inc., Fort Worth, TX, USA) were folded at the 5-mm notch and inserted into the lower lateral one-third of conjunctival fornix and eyelids closed by gentle force for 5 minutes. The length of the moistened paper was then recorded. The test was performed three times and the average score was used for analysis.

Conjunctival Fluorescein Staining Test

All 16 eyes of rabbits were examined under the slit-lamp microscope (GR-54; Gilras LLC, Miami, FL, USA) with a cobalt blue filter after instilling 1 drop 1% fluorescein solution. The corneal fluorescein staining was assessed by a trained ophthalmologist (YN), using the standard National Eye Institute grading system.26-27 Briefly, the cornea was divided into five areas (central, superior, nasal, inferior, and temporal); punctate fluorescein staining in each area is graded on a scale of 0 to 3, and the total score (0–15) is the sum of scores from all five areas.

Rose Bengal Staining Test

After instilling 1 drop 0.1% rose Bengal solution (HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA, USA) all rabbit eyes were examined under the yellow light of the same slit-lamp microscope by the same ophthalmologist (YN). Areas of rose Bengal staining represent dead or dying cells. Within the palpebral fissure, the exposed ocular surface was divided into three sections: nasal and temporal bulbar conjunctiva, and the central cornea. The intensity of staining in each section was scored up to three points, with a maximum possible score of nine points.

Corneal Fluorescein Staining Test

The bilateral resection of the NM, HG, and main LG was performed following a modified surgical procedure of Chen et al.25 Briefly, the skin of both periorcular regions was shaved. Induction was initiated with an intramuscular injection of Ketamine (25 mg/kg; Henry Schein, New York, NY, USA) + Xylazine (5 mg/kg; Akorn, Inc., Decatur, IL, USA) and each rabbit intubated with an uncuffed endotracheal tube. The general anesthesia was maintained by 1% to 4% Isoflurane gas with 1.5 L/min oxygen flow during the entire surgery. The cardiopulmonary status of the animals and the depth of anesthesia were monitored every 5 minutes. The surgical field was disinfected with 5% betadine solution used for analysis.

Evaluations

All rabbits were assessed before excision (BE) and monthly up to 4 months after excision (AE). To minimize slit-lamp finding artifacts, the evaluations were carried out in 2 days in the following sequence on each eye. The first day begins with corneal fluorescein staining, followed immediately by rose Bengal staining and conjunctival impression cytology. On the second day, Schirmer test I (StI; without anesthesia) was performed.
RNA Isolation and cDNA Synthesis

Total RNA was isolated from the CIC specimens in TRIzol solution according to manufacturer’s instructions (Invitrogen). RNA concentration was measured using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The purity of the RNA was assessed by absorbance ratio at 260/280 nm. A ratio greater than 1.9 was considered good quality RNA preparation and used for further experiments. The first strand cDNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) using approximately 500 ng total RNA according to manufacturer’s instructions. Reverse transcription was initiated using RT-primer mix (oligo-dT and random primers) supplied with the kit.

Primer Design and Evaluation

Primers with an annealing temperature of 60°C were designed using OligoPerfect Designer (Life Technologies) for amplifying coding sequence fragments of 100 to 135 bp in length. The primer sequences used in this study are listed in the Table.

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

The reverse transcription (RT) reactions were set using QuantiTect SYBR Green PCR kit (Qiagen). The 25 μL total volume of reaction mixture contained 12.5 μL SYBR Green PCR Master Mix (Qiagen), 0.4 μM forward and reverse primers, and 1 μL cDNA. The qPCR was performed on StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the following amplification cycling conditions: 15 minutes, 95°C initial denaturation; 40 cycles of 15 seconds, 95°C denaturation, 30 seconds, 60°C primer annealing, and 30 seconds, 72°C extension. The fluorescence was recorded during the elongation step in each cycle. A melting curve analysis was performed at the end of each PCR by gradually increasing the temperature from 60°C to 95°C while recording the fluorescence. A single peak at the melting temperature of the PCR-product confirmed primer specificity. To be able to compare between different runs, we used a fixed fluorescence threshold for derivation of the cycle threshold (Ct) value for all runs. We performed three technical replicates for each biological specimen to evaluate the relative quantification.

Relative Quantification of mRNA Levels

Relative quantification of the expression of different genes (Table) from each rabbit CIC specimen was performed at each time point BE and AE. The fold change in expression of tested genes was relative to the internal housekeeping gene, β-actin (endogenous control gene). The mean fold change in expression of genes was calculated using 2−ΔΔCT method, where ΔΔCT = (CTGene − CTβ-actin)after excision − (CTGene − CTβ-actin)before excision. Differences between Ct for each target mRNA and β-actin in each specimen was used to calculate level of target mRNA relative to that of β-actin mRNA in the same specimen.

Immunohistochemistry

To verify the expression of AQP4 in the rabbit conjunctiva, immunohistochemistry was performed. Fresh conjunctival tissues from unoperated and operated rabbits were immediately embedded in cryomolds with tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA), frozen in Isopentane (Sigma-Aldrich Corp.) cooled on dry ice, and then stored at −80°C. Conjunctiva tissues were sectioned (4 μm), fixed in cold acetone for 10 minutes, air dried, and stored at −80°C until further use. Slides were stained using Ventana Medical Systems automated platform (Roche, Tucson, AZ, USA) using standard DAB staining kit (Ventana Medical Systems, Inc., Roche) as per manufacturer’s instructions. The primary antibody was mouse monoclonal antibody (which cross reacts with rabbit) to AQP4 (Abcam, Cambridge, MA, USA) diluted at 1:30 with Ventana PSS diluent (Ventana Medical Systems, Inc., Roche). After incubation at 37°C for 30 minutes, the slides were incubated with anti-mouse IgG biotin labeled antibody (Vector Laboratories, Burlingame, CA, USA) at 1:200 dilution and incubated at 37°C for 30 minutes. Finally, the slides were counter stained with Hematoxylin II (Ventana Medical Systems, Inc., Roche) and bluing reagent (Ventana Medical Systems, Inc., Roche). The staining was observed and photographed using a light microscope (Nikon Eclipse TF; Nikon, Melville, NY, USA).

Immunoblotting

Total cell lysate proteins were isolated from CIC in RIPA buffer with 1× HALT protease and phosphatase inhibitor single use inhibitor cocktail (ThermoScientific, Rockford, IL, USA) by incubating on ice for 30 minutes. Protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, New York, NY, USA). Samples were mixed with Laemmli sample buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) containing β-mercaptoethanol and heated at 95°C for 10 minutes. Proteins (15 μg) were resolved on 10% Mini PROTEAN TGX precast gels (Bio-Rad Laboratories, Inc.) in electrophoresis cell (Bio-Rad Laboratories, Inc.) using 1× Tris/Glycine/SDS buffer (Bio-Rad Laboratories, Inc.). Resolved proteins were transferred to immuno-Blot PVDF membrane using 1× Glycine/SDS buffer (Bio-Rad Laboratories, Inc.) and 20% methanol (Sigma-Aldrich Corp.). The membrane was
blocked at room temperature for 1 hour with 5% nonfat dried milk prepared in 1× TBS-T (50 mM Tris-HCl [pH: 7.5], 150 mM NaCl, 0.05% Tween20) buffer. The membrane was then thoroughly washed with 1× TBS-T buffer and incubated at 4°C for overnight with the primary antibody mouse monoclonal antibody to AQP4 (Abcam) at 1:200 dilution prepared in 3% bovine serum albumin (Sigma-Aldrich Corp.). Next, the membrane was thoroughly washed with 1× TBS-T buffer and incubated at room temperature for 1 hour with secondary antibody goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc., Fort Worth, TX, USA) at 1:10,000 dilution prepared in 5% nonfat dried milk. The membrane was finally washed thrice with 1× TBS-T buffer, incubated with super signal west pico chemiluminescent substrate (Thermoscientific) for 1 minutes, exposed to Amersham Hyper film TM ECL (GE Health Care Ltd., Pittsburgh, PA, USA), and developed using SRX-101A medical film processor (Konica Minolta Medical and Graphic, Inc., Shanghai, China). Apparent molecular weights were determined using precision plus protein all blue standards (Bio-Rad Laboratories, Inc.).

**Data Analysis and Statistics**

Data in figures are presented as mean standard error method, the bars representing standard errors. Statistical significance between two groups (BE and AE) was evaluated using unpaired 2-tailed t-test. A probability of $P$ equal to 0.05 was considered significant (where applicable, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

**RESULTS**

**Ocular Surface Changes**

Significantly higher scores for both fluorescein and rose Bengal staining demonstrated the presence of dry eye phenotypes in as early as 1 month AE (Fig. 1). Interestingly, both fluorescein and rose Bengal staining on the cornea and conjunctiva progressively reduced with time AE (Fig. 2).

**Tear Secretion as Measured by Schirmer I Test**

Interestingly, there was no significant reduction in tear production over the 4-month period AE (Fig. 3). The SIt score was 20.49 BE and slightly reduced to 18.18 at 1 month AE, but such a reduction was not significant ($P = 0.18$). Schirmer I test scores were 22.89 at 2 months ($P = 0.083$), 25.70 at 3 months ($P = 0.0004$), and 20.96 at 4 months AE ($P = 0.799$; Fig. 3). Our data shows that the tear secretion increased significantly at 3 months, and then returned to baseline level at 4 months AE. It appears that the level of tear secretion at each time point was

![Tear Production After Bilateral Main LG Resection](https://example.com/tear-production.png)

**FIGURE 1.** Clinical examination of the rabbit eyes before (BE) and after (AE). Comparing with BE, increased staining of fluorescein on the cornea (top panels) and that of rose Bengal on both the cornea and conjunctiva (bottom panels) was observed 1 month AE.

![Fluorescein and rose Bengal staining scores of rabbit ocular surface before (BE) and after (AE) excision.](https://example.com/staining-scores.png)

**FIGURE 2.** Fluorescein and rose Bengal staining scores of rabbit ocular surface before (BE) and after (AE) excision. Fluorescein and rose Bengal staining scores are compared BE with each time points AE.
Interestingly, none of the ionic transporters showed any expression of AQP4, AQP5, CFTR, NKA, NKCC1, and ENaC. Progressively declined to the baseline level (Fig. 5). Interleukin-1β (IL-1β), TNF-α, and ocular surface injury marker matrix metalloproteinase (MMP)-9 showed significant elevation at 1 month. IL-1β was upregulated at 96-fold at 1 month AE ($P = 0.0027$) and then declined to 24-fold at 2 months ($P = 0.0002$), 13-fold at 3 months ($P = 0.0088$), and finally to 4-fold at 4 month AE ($P = 0.0533$). Similarly, TNF-α was 9-fold upregulated at 1 month AE ($P = 0.0064$), then declined to 3-fold ($P = 0.122$), 2-fold ($P = 0.265$), and 1.5-fold ($P = 0.31$) at 2, 3, and 4 months AE, respectively. Matrix metalloproteinase-9 followed a similar trend from 13-fold upregulation at 1 month AE ($P = 0.0289$) to a progressive reduction to the baseline level over 4 months AE ($P = 0.162$).

### mRNA Level of Dry Eye Biomarkers

To estimate the changes of ocular surface inflammation, the mRNA level of DES-associated inflammatory cytokines was assessed from the CIC specimens. The cytokine markers, IL-1β, TNF-α, and ocular surface injury marker matrix metalloproteinase (MMP)-9 showed significant elevation at 1 month AE, but over the following 3 months, their expression progressively declined to the baseline level (Fig. 5). Interleukin-1β was upregulated 96-fold at 1 month AE ($P = 0.0027$) and then declined to 24-fold at 2 months ($P = 0.0002$), 13-fold at 3 months ($P = 0.0088$), and finally to 4-fold at 4 month AE ($P = 0.0533$). Similarly, TNF-α was 9-fold upregulated at 1 month AE ($P = 0.0064$), then declined to 3-fold ($P = 0.122$), 2-fold ($P = 0.265$), and 1.5-fold ($P = 0.31$) at 2, 3, and 4 months AE, respectively. Matrix metalloproteinase-9 followed a similar trend from 13-fold upregulation at 1 month AE ($P = 0.0289$) to a progressive reduction to the baseline level over 4 months AE ($P = 0.162$).

### Assessment of Goblet Cells by Impression Cytology

Periodic acid-Schiff staining demonstrated abundance of goblet cells within the conjunctival epithelium of rabbit eyes (Fig. 4). However, due to inconsistency of sampling between discs and even in different areas of one disc, reliable quantitative analysis was not feasible to assess the changes in conjunctival goblet cell number BE and AE. No obvious morphologic change of the goblet cells was noted BE and AE.

### Expression of AQP4 in Rabbit Conjunctival Epithelium

It has not been previously reported that AQP4 is expressed in the conjunctival epithelium. Because the upregulation of AQP4 mRNA was detected, we set out to verify the posttranslational expression of this water channel molecule. Immunostaining against AQP4 demonstrated the abundant presence of the AQP4 in all layers of rabbit conjunctival epithelium except for the subepithelial stroma (Fig. 7). Both the squamous epithelial and the goblet cells of rabbit conjunctiva showed positive staining. There were no distinct differences in the AQP4 expression in the conjunctival tissue in rabbits without surgical excision (Fig. 7A) and AE (Fig. 7B).

Immunoblotting against AQP4 for the total protein isolated from the conjunctival epithelium showed the presence of the dimeric form of AQP4 of size approximately 65 kDa (Fig. 8) in rabbits without surgical excision (lane 1) and AE (lane 2).

### Discussion

In the present study, it is tantalizing to see that the tear secretion never decreased over time in the rabbits after the NM, HG, and main LG were surgically ablated. At 1 month AE, the rabbits indeed demonstrated significant dry eye phenotypes clinically (increased fluorescein and rose Bengal staining), which were corroborated by the elevated ocular surface inflammatory biomarker expression in the conjunctival epithelium. However, with no external intervention, the clinical signs of dry eye improved and ocular surface inflammation decreased to the baseline level (BE) in over 4 months. This seems to suggest that in acute dry eyes, as created in our experiment, ocular surface injury, and inflammation can be
mostly reverted as long as the tear volume is maintained. This should be distinguished from human DES caused by an underlying autoimmune disorder such as Sjögren’s syndrome, a chronic dry eye condition, in which both the main LG and the conjunctiva are affected by inflammatory infiltration.36–38 This can explain why no compensatory conjunctival tear secretion has been observed in Sjögren’s syndrome patients, even at the early stage of this disease. Hence, it makes sense that treatment strategy for DES caused by an underlying autoimmune disorder needs to be targeting disease-relevant inflammatory mediators.36,39,40 It is not surprising to see some residual dry eye phenotypes due to the mixed-mechanism dry eye conditions created in our study. The HG secretes lipid to the ocular surface which retards tear evaporation. The NM, as a third eyelid, helps to spread the tears over the ocular surface and to contain evaporation.

Our observations that tear secretion were not affected in the absence of the main LG suggest the presence of a compensatory mechanism in the remaining ocular surface tissues. In previous studies,25,41 approximately 30% to 45% reduction in tear secretion was seen upon unilateral resection of the NM, HG, and main LG, as compared with the unoperated side. The retention of 55% to 70% tear secretion capacity seen in these previous studies also suggests alternative sources of ocular surface tears. In our study, three of the eight rabbits were later randomly chosen and euthanized for autopsies. Careful inspection of the ocular orbits of all six eyes did not reveal any LG or HG tissues to suggest either an incomplete excision or regeneration. Hence, the alternative sources of tears would have to come from the remaining ocular surface tissues: the conjunctiva and the accessory LGs.

There are several possible explanations to our finding of robust tear secretion in the absence of the HG and main LG. First, we specifically chose male rabbits to study in order to minimize possible influence from sex hormones on the conjunctival function. Sex hormone alteration, especially androgen deficiency, adversely affects ocular surface homeostasis.3 Whereas previously, Chen et al.25 used Japanese albino rabbits of both sexes in their studies, and Li et al.41 used female New Zealand white rabbits only. Female rabbits, under the influence of hormones, possibly have a less robust capacity to compensate for the surgically induced dry eye condition. Second, bilateral surgery could also contribute to the discrepancy seen in our study. We believe bilateral surgery is important because DES is an immune mediated ocular surface disorder involving both eyes.32,42 In addition, the regulation of lacrimal secretion involves the central nervous control: afferent trigeminal sensory fibers from the ocular surface (cornea and conjunctiva), superior salivary nucleus in the pons and efferent fibers through the pterygopalatine ganglion.1 The neural pathway control, such as blink reflex to noxious ocular surface stimuli, involves bilateral somatosensory cortex.55 As a result, the response to the unilateral, surgically induced dry eye would ultimately lead to a compensatory over secretion on the unoperated side. In our study, comparisons were made not between eyes of the same animal, but against the same eyes BE

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**Figure 5.** The expression of inflammatory biomarker genes in rabbit conjunctival epithelium before (BE) and after (AE) excision. Significant upregulation of IL-1β, TNF-α, and MMP-9 was seen at 1 month AE, but the upregulation declined with time to the baseline level BE. The fold change in expression of an inflammatory biomarker gene is relative to internal housekeeping gene, β-actin (endogenous control gene).
and AE at different time points. Third, there have been reports supporting that the main LG is not indispensable under certain conditions. Unilateral removal of the main LG did not result in keratoconjunctivitis sicca (KCS) in squirrel monkeys, although tear secretion decreased significantly. In patients who underwent palpebral dacryoadenectomy for the treatment of epiphora, up to 86% did not develop dry eyes and up to 50% continued to have epiphora. In this procedure only the palpebral lobe of the main LG is surgically removed through a conjunctival incision. However, because the tear ducts from the orbital lobe of the LG traverse the palpebral lobe, removal of the palpebral lobe essentially blocks orbital lobe secretion to the superior lateral conjunctival fornix. Fourth, even in some rabbit dry eye models created with similar surgeries as ours, significant reduction in tear secretion was not seen in all studies. Gilbard et al. cauterized bilateral lacrimal excretory ducts in their model, in addition to excising the NM and HG. Although tear osmolarity increased and goblet cell density decreased significantly, tear secretion was unchanged. This was thought by others to be due possibly to incomplete cauterization of the excretory ducts, but it is very unlikely that incomplete cauterization took place in all animals. In a study by Odaka et al., not all rabbits had evidence of dry eyes (inadequate tear volume, decreased goblet cell density, and KCS) 4 weeks after resection of the NM, HG, and main LG. Moreover, laboratory studies also supported that rabbit conjunctival epithelium has the capacity to be the primary source of TF. It was measured in one study that in rabbits, the basal conjunctival fluid secretory rate is 0.79 μL/min, whereas the basal tear production rate is 0.72 μL/min. Another study demonstrated that the conjunctival fluid secretion was 175% greater than tear turnover. Taken together, it is plausible to speculate that, at least in rabbits, alternative tear secretion can be augmented in compensation for the deficiency of the main LG. The conjunctiva and the accessory LGs underneath it are most likely responsible for such compensatory tear production.

It is known that the genetic construction of mouse or rat ocular surface is closer to that of humans. We instead chose rabbits for our study mainly for the following reasons.

![Figure 6. Upregulation of aquaporin 4 and 5 in rabbit conjunctival epithelium AE. AQP4 gene showed a trend of upregulation (P > 0.05 at all time points) AE as compared with BE, whereas AQP5 gene showed significant upregulation at 1, 2, and 3 months AE. The fold change in expression of a gene is relative to internal housekeeping gene, β-actin (endogenous control gene).](image1)

![Figure 7. Immunolocalization of AQP4 on rabbit conjunctival epithelium. Immunostaining against AQP4 demonstrated the abundant presence of AQP4 in the membrane of squamous epithelial and goblet cells of the conjunctiva in rabbits without excision (A) and AE (B) of the main lacrimal gland, Harderian glands, and nictitating membrane. The subepithelial conjunctival stroma stained negative. Inset (B): It appears that the localization of AQP4 is on all aspects of the cell membrane in the basal layer of the epithelium (arrowheads). In the apical layer, AQP4 staining appears more predominantly on the basolateral aspect of the cells (arrows).](image2)
surface area of mouse conjunctiva occupies a much smaller fraction of the entire ocular surface. Therefore, the functional contribution of mouse or rat conjunctiva to the TF may not be comparable to that of humans. On the other hand, the surface area occupied by rabbit conjunctiva is closer to that of humans. In addition, mouse eyes are small and their lacrimal apparatus is not amenable to surgical maneuver. In particular, the intra-orbital LG and the HG are all located in the retrobulbar compartment. It is near impossible to extract these glands without severely damaging the conjunctiva and even the optic nerve. Moreover, the histarchitectural features of the rabbit LG more closely resemble those of human LG, in comparison to mice and rats. The functional implication of such resemblance is not yet clear, but could suggest that the main LG contributes similarly to the ocular surface tear fluid in both rabbits and humans. Likewise, the remaining tear contributers at the ocular surface, including the conjunctiva, could function similarly as well.

Ocular surface, lined by stratified corneal and conjunctival epithelia, lies in contact with TF. Net ionic fluxes are determined by mass action and by transmembrane Na⁺ and K⁺ concentration gradient. Net fluxes of Na⁺, K⁺, and Cl⁻ into cells and that of H⁺ and HCO³⁻ out of cells establish an osmotic gradient, which dictates the direction of the water flow across the plasma membranes. Corneal and conjunctival epithelia secrete Cl⁻ and absorb Na⁺. In conjunctival epithelium, Cl⁻ secretion (basolateral to apical movement) accounts for approximately 60% and Na⁺ reabsorption (apical to basolateral) accounts for approximately 40% of net ionic transport. It was suggested that this ionic transport may determine the amount of tears collected in the conjunctiva sac, which potentially affect the stability of TF. The ENaC in mouse, rabbit, and human conjunctiva mediates active Na⁺ reabsorption to maintain the electrolyte/water homeostasis. Similarly, CFTR (involved in secretion/transepithelial conductance of Cl⁻) has a potential role in the major pathways for Cl⁻ transport at the ocular surface. Other transporters such as NKCC and NKA have also been implicated in maintaining the ionic balance of the ocular fluid.

To delineate the compensatory tear secretion mechanisms seen in our study, we assayed the mRNA expression of different ionic transporters (CFTR, NKA, NKCCI, and ENaC) in the conjunctival epithelium. To our surprise, none of the conjunctival ionic transporters studied showed any evidence of upregulation by 4 months AE. Because the tissue procured by CIC contains only the conjunctival epithelium, this could mean that the conjunctival epithelium is not actively involved in the compensatory tear secretion at the ocular surface. However, previous studies have indicated that basal CFTR activity may be very low under normal conditions, so that ocular surface hydration does not depend on CFTR function. Reduced CFTR function produces only minor effects on ocular functions. It was therefore proposed that upregulation of alternative secretory pathways either in tear producing glands, or within the cornea or conjunctiva, may compensate for an impaired CFTR Cl⁻ channel function. Thus, lack of evidence for upregulation of the ionic transporters assayed in our study cannot exclude the conjunctiva as a candidate tissue for compensatory tear secretion.

AQP-type water channels are a family of small transmembrane proteins that facilitate osmotically driven water transport across cell plasma membranes, providing the major molecular pathway for water movement. Ocular surface AQP channels facilitate osmotically driven water transport across cell plasma membranes in maintaining the tear volume and osmolarity. Of all the AQP4 and AQP5 functions primarily as water selective transporters and AQPs, particularly is associated with tear formation by the LG. Interestingly, it has been concluded that water transport facilitated by conjunctiva encoded AQP 3 does not play any role in transconjunctival fluid movement. In our study, AQP4 and AQP5 indeed showed an upregulation from 1 to 3 months, and returned to near baseline by 4 months AE. This could be an important mechanism to explain the maintained tear secretion capacity at the ocular surface after the resection of the main LG. Because major transporters for Cl⁻ (CFTR) and Na⁺ (ENaC) showed no evidence of mRNA upregulation, over expression of AQP in the conjunctiva epithelium suggests that the increase of water flow to the ocular surface is achieved mainly through the increase of highly permeable pathways (predominantly AQP5), not through the increase of net movement of electrolytes across the cell membrane.

It has been previously shown that AQP 5 provides the principal route for osmotically driven water flux across the intact corneal epithelium. Histologically, the corneal epithelium has all the transporters, and it can be mobilized to transport fluid when its intercellular junctions are immature and permeable, although at a very low rate. Our preliminary in vivo data in rabbit conjunctiva suggests a transcellular water flux mechanism rather than a paracellular one.

The role of accessory LGs (such as the gland of Wolfring) was not investigated in this study due to technical challenges. The function of accessory LGs could be important for ocular surface biology and may contribute significantly to the tear fluid components. In fact, it has been shown that these glands function similarly to the main LG in contributing to the electrolytes and volume of the tear fluid. Gland of Wolfring expresses most of the same channels and transporters related to K⁺ secretion that have been identified in the LG thus indicating a possible contribution to the elevated K⁺ concentration in tears. Bergmann et al. demonstrated that the rabbit conjunctiva contains glands similar to the gland of Wolfring. Therefore, it is very important to be able to study the accessory lacrimal glands in the future. We plan to procure accessory lacrimal gland cells using laser microdissection and further study the mRNA profiles of the different ionic and water transporters in these cells.

To summarize, a mixed-mechanism dry eye condition was surgically created in rabbits by resection of NM, HG, and main LG. Significant dry eye phenotypes in association with elevated ocular surface inflammation were observed at 1 month AE although tear secretion was not decreased. Interestingly, with no intervention, dry eye phenotypes and ocular surface inflammation gradually improved over a period of 4 months AE. This indicates compensatory tear secretion from the remaining ocular surface tissues and adequate tear fluid alone can alleviate ocular surface damage in an acute dry eye situation. AQP 4 and AQP 5, among other conjunctival water transporters, appear to be involved in restoring the ocular surface hydration.
surface fluids. Our study demonstrates the pivotal role played by the conjunctiva in the maintenance of ocular surface homeostasis. In addition, the conjunctiva could be a target tissue, as in the case of Diquafosol, for therapeutic interventions of DES.

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