

ORIGINAL ARTICLE

Sleep deprivation disrupts the lacrimal system and induces dry eye disease

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Sleep deficiency is a common public health problem associated with many diseases, such as obesity and cardiovascular disease. In this study, we established a sleep deprivation (SD) mouse model using a 'stick over water' method and observed the effect of sleep deficiency on ocular surface health. We found that SD decreased aqueous tear secretion; increased corneal epithelial cell defects, corneal sensitivity, and apoptosis; and induced squamous metaplasia of the corneal epithelium. These pathological changes mimic the typical features of dry eye. However, there was no obvious corneal inflammation and conjunctival goblet cell change after SD for 10 days. Meanwhile, lacrimal gland hypertrophy along with abnormal lipid metabolites, secretory proteins and free amino-acid profiles became apparent as the SD duration increased. Furthermore, the ocular surface changes induced by SD for 10 days were largely reversed after 14 days of rest. We conclude that SD compromises lacrimal system function and induces dry eye. These findings will benefit the clinical diagnosis and treatment of sleep-disorder-related ocular surface diseases. *Experimental & Molecular Medicine* (2018) 50, e451; doi:10.1038/emm.2017.285; published online 2 March 2018

INTRODUCTION

Sleep is a ubiquitous and fundamental biological requirement of all animals.¹ It is well accepted that high-quality sleep is requisite for optimal human health and performance. However, sleep deficiency, resulting from shortened sleep duration, irregular timing of sleep, poor sleep quality and sleep/circadian disorders, is highly prevalent in present-day society. Nearly 30% of adults and 60% of adolescents in the United States fail to obtain sufficient amounts of sleep.^{2,3} Sleep deficiency has become a common public health problem in the world. Numerous studies have demonstrated that sleep deficiency is associated with an increased risk of various diseases, including diabetes,⁴ obesity,⁵ hypertension,⁶ cardiovascular disease,⁷ psychiatric illness,⁸ substance abuse,⁹ pregnancy complications,¹⁰ depression¹¹ and neurobehavioral and cognitive impairments.¹² Moreover, sleep deprivation (SD) is also associated with declines in the health-related quality of life.¹³

Recently, sleep-deficiency-induced eye problems have drawn much attention in both the public and medical domains. A retrospective study of a United States veteran-affairs population found that sleep apnea was associated with an increased risk of dry eye.¹⁴ A recent study of a group of healthy young

male volunteers found that staying up all night could induce tear hyperosmolarity and reduce tear secretion.¹⁵ In a survey of a large Korean adult population, increases in dry eye symptom incidence were associated with declines in sleep duration.¹⁶ This association is consistent with the greater prevalence of sleep disorders among dry eye patients.^{17,18} By contrast, after treating these dry eye patients with hyaluronate, a mucin secretagogue and a steroid, their sleep quality improved.^{18,19} This corresponding effect of dry eye therapeutics on sleep quality suggests that a healthy ocular surface may produce a positive feedback on sleep. However, the cause-and-effect relationship between sleep deficiency and ocular surface health remain elusive, and the underlying mechanism is lacking.

In the current study, we implemented an SD mouse model to determine if there is an association between changes in the ocular surface system and sleep deficiency. We found that SD could trigger tear-film, ocular-surface and lacrimal gland changes within 2 days. Concomitant pathological changes became time dependently more pronounced in these tissues. These results indicate that SD can disrupt the lacrimal system function in secretion, lipid metabolism and protein synthesis,

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thereby causing dry eye disease. These findings may have important clinical significance.

MATERIALS AND METHODS

Animals

Adult C57BL/6 mice at the same age and weighing 18–22 g were purchased from the Shanghai SLAC Laboratory Animal Center (Shanghai, China); 212 animals were used in this study. Two mice were housed per cage before the study for 1 week at 25 ± 1 °C; relative humidity, $60 \pm 10\%$; and alternating 12-h light/12-h dark cycle (0800 to 2000 hours). All the animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of Animals in Ophthalmic and Vision Research, and the experimental protocol was approved by the Experimental Animal Ethical Committee of Xiamen University.

SD model establishment

The ‘platform over water’ method is widely used in rat SD.²⁰ To achieve a suitable method to deprive mice of sleep, the platform was replaced by sticks in our study to develop a ‘stick over water’ method. Briefly, two circular wooden sticks (6 mm diameter) were placed across the side walls of the cage at a height of 4.0 cm from the bottom. Water filled the cages to a level 1.0 cm beneath the sticks. The horizontal distance between the two sticks was 6.0 cm, which allowed the mice to move between the two sticks. The mice had unrestricted food and water access when they stood on the front stick (Supplementary Figure S1). When the mice fell asleep while standing the stick, the development of muscle atony caused the animal to lose balance and slip down to the water surface, which awakened the animal. Mice in the SD group were placed on this stick configuration at 1300 hours for different durations from 12 to 20 h per day and were transferred to their home cages during the resting phase. Before the experiment, each mouse was adapted to the SD procedure for 1 h on 3 consecutive days. Two mice were kept in one cage. The control group mice were housed in standard home cages.

Slit-lamp evaluation and fluorescein test

The anterior and lateral views of the mice eyeballs were observed under a slit-lamp microscope (BQ900H Haag-Streit, Bern, Switzerland). Subsequently 2 μ l of 0.1% sodium fluorescein was applied to the cornea and was washed away with saline after one blink. The corneal appearance was examined and graded under the slit-lamp microscope with a cobalt blue filter. The corneal fluorescein staining was scored as reported previously.²¹

Aqueous tear secretion test

The aqueous tear volume was measured with the phenol red thread (Zone-Quick; Yokota, Tokyo, Japan) tear secretion test at 1300 hours in the standard environment. Briefly, mice were narcotized by intraperitoneal injection of 400 mg kg⁻¹ chloral hydrate before being evaluated. The lower eyelid was pulled down gently, and the thread was placed on the palpebral conjunctiva at a specified point approximately one-third of the distance away from the lateral canthus of the lower eyelid for 15 s. One measurement was taken from each eye. The values indicated by the red color position of the aqueous front on the thread were recorded in millimeters.

Measurement of corneal sensitivity

To measure the corneal sensitivity, we applied a Cochet–Bonnet esthesiometer (Luneau, Paris, France) to the center of the cornea. When the mice were held, the esthesiometer nylon filament touched

the center of the cornea vertically and a positive response was recorded if three reflexes occurred (evoked blink) in response to five consecutive touches. Measurements were performed starting with the fully extended 60 mm filament, which applied the least amount of pressure, and then it was incrementally shortened in 10-mm steps until a response was obtained. The longest filament length causing a positive result was considered the corneal sensitivity threshold.

Hematoxylin–eosin and Oil Red O staining

Eyeballs or lacrimal gland tissues were removed immediately after the mice were killed and fixed in 4% paraformaldehyde overnight. The samples were embedded in OCT, and 6- μ m-thick cryosections were used for histological analysis. The tissue section samples were stained with hematoxylin and eosin or Oil Red O (ORO) solutions as described previously²² and examined under a light microscope.

Cell apoptosis detection

In situ cell apoptosis was determined by the TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay (DeadEnd Fluorometric TUNEL System G3250; Promega, Madison, WI, USA) in corneal frozen sections. Sections were counterstained with 4', 6-diamidino-2-phenylindole I (Vector), mounted and photo images were taken with a confocal laser scanning microscope (Olympus Fluoview 1000; Olympus, Tokyo, Japan). The images were captured and processed using Olympus Fluoview software (Olympus, Tokyo, Japan).

RNA-seq

Total RNA of the lacrimal glands was extracted with Trizol and assessed using the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) and Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). The quality of the total RNA samples was evaluated as follows: RNA integrity number >7.0 and 28S:18S ratio >1.8. Libraries for sequencing were constructed with the NEB Next Ultra RNA Library Prep Kit (NEB) for Illumina. Subsequently, a quantitative reverse transcription-polymerase chain reaction was performed to validate the microarray results. The library cDNA was subjected to paired-end sequencing with a pair end 125-base pair reading length on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA).

RNA isolation and quantitative real-time reverse transcription-PCR analysis

Total RNA was extracted from mouse corneal, conjunctival and lacrimal gland tissues with TRIzol (Invitrogen) and was reverse-transcribed to cDNA by the ExScript RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's protocol. Real-time PCR was performed using a SYBR Premix Ex Taq Kit (RR420A; Takara) on a StepOne plus Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). The primers used to amplify the specific gene products are listed in Supplementary Table S1. The results of relative quantitative PCR were analyzed by the comparative threshold cycle (Ct) method and normalized to β -actin expression as an endogenous reference.

Immunofluorescence staining

Frozen sections (6- μ m thick) of the eyeballs and eyelid tissues were fixed with 4% paraformaldehyde for 20 min at 4 °C. Sections were rehydrated in phosphate-buffered saline and then incubated in 0.2% Triton X-100 for 10 min. After the sections were rinsed three times with phosphate-buffered saline for 5 min each and preincubated with 2% bovine serum albumin for 1 h, samples were incubated with anti-K10 (ab24638, 1:100; Abcam, Cambridge, UK), anti-CD45 (sc-

52491, 1:50, Santa Cruz, Dallas, TX, USA) and anti-ZO-1 (61-7300, 1:100, Invitrogen) antibodies at 4 °C overnight. After three separate 10-min washes with phosphate-buffered saline, they were then incubated with AlexaFluor 488-conjugated immunoglobulin G (A21206 or A21208, 1:300; Life Technologies, Carlsbad, CA, USA) for 1 h. After three additional phosphate-buffered saline washes for 10 min each, sections were counterstained with 4', 6-diamidino-2-phenylindole I, mounted with mounting medium and photographed using a confocal laser scanning microscope.

Ultrastructure of lacrimal gland and cornea

Cornea or lacrimal gland tissues were dissected and fixed in 2.5% glutaraldehyde at 4 °C for 2 h. Samples were prepared for scanning electron microscopy or transmission electron microscopy as reported

previously.²³ The ultrastructures of the corneal epithelial surface and lacrimal gland were examined and photographed with a scanning electron microscope (S-4800; Hitachi, Tokyo, Japan) or transmission electron microscope (JEM2100HC; JEOL, Tokyo, Japan).

Periodic acid-Schiff staining

The paraffin sections were deparaffinized and rehydrated. After being rinsed with deionized water for 10 min, the sections were stained in periodic acid alcohol for 10 min, and then rinsed with deionized water for 10 min. Subsequently, the sections were stained in Schiff reagent (Leica Biosystems, Nussloch, Baden, Germany) for 10 min, followed by rinses with sulfurous acid for 2 min and then tap water for 15 min. Then, each section was dehydrated four times in ethanol for 2 min and two times in xylene for 2 min. Finally, the sections were mounted with

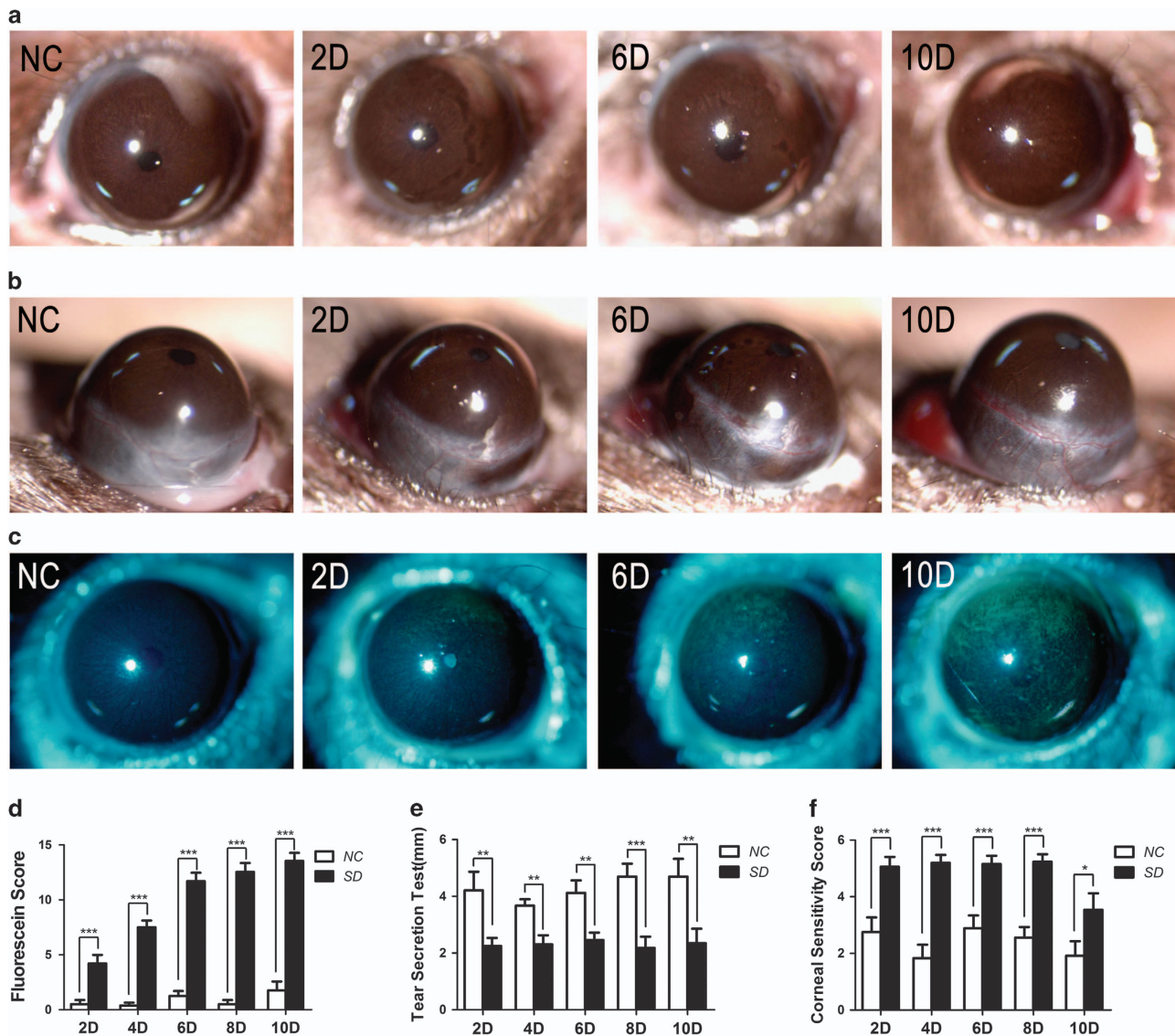


Figure 1 Ocular surface and tear film change after sleep deprivation (SD). (a) Slit-lamp image of mice cornea at days 0, 2, 6 and 10 after the SD. (b) Conjunctival and limbal hyperemia was present after 2 days of SD and increased with time. (c) Sodium fluorescein staining of the cornea showed diffused staining after 2 days of SD and became more intensive at days 6 and 10. (d) The fluorescein staining score gradually increased from day 2 to day 10 after SD. (e) The phenol red thread tear secretion test showed that the aqueous tear secretion declined after 2 days of SD and was maintained at a low level during the following 10 days. (f) Corneal sensitivity increased throughout the entire SD duration ($n=8-20$ /group, $*P<0.05$, $**P<0.01$, $***P<0.001$).

mounting medium. Conjunctival cells stained by periodic acid-Schiff were counted under the microscope. The average number of goblet cells was counted in six representative slices of homologous positions for comparison.

Liquid chromatography-tandem mass spectrometry analysis

The mice were killed after different durations of SD, and the lacrimal glands were immediately dissected and harvested. After wet weight measurement, the lacrimal gland tissues were homogenized by ultrasonic extraction in 1 ml of 20% methyl alcohol for 30 min. The homogenates were centrifuged, and the supernatants were collected, filtered and stored at -80°C until performance of the biochemical analyses. The levels of acetylcholine, dopamine and 34 amino acids were measured with a liquid chromatography-tandem mass spectrometry instrument (QTRAP 5500; SCIEX, Concord, Canada).

Statistical analysis

The statistical analysis was conducted using SPSS 16.0.0 (SPSS, Chicago, IL, USA). All summary data are reported as the mean \pm s.d. Comparisons between groups were performed by an unpaired, two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Ocular surface manifestations after SD

In the 'stick over water' cage configuration, the mice appeared sleepy and started to nap after continuously standing on the

stick for ~ 12 h. We first varied the SD time from 12 to 20 h per day in 2-h increments for 6 days. At the end of a period, the corneal fluorescein staining scores gradually increased as the SD time was prolonged. In the 20-h SD group, the staining was diffuse (Supplementary Figure S2). In this group, a slit-lamp microscopy evaluation every 2 days identified ocular surface changes reflecting progressive increases in dryness and roughness, which started to occur as early as 2 days after initiating SD (Figure 1a). Conjunctival and limbal hyperemia was also observed after 2 days of SD, and eyelid closure was normal during their sleep-deprived time (Figure 1b). The cornea did not show any signs of neovascularization after 10 days of SD (Figure 1b). Sodium fluorescein staining after 2 days was diffuse and progressively became more intense as the number of days of SD increased (Figure 1c). Fluorescein staining score results validated corneal epithelial damage progression as a function of SD duration (Figure 1d).

Tear secretion and corneal sensitivity changes after SD

Aqueous tear secretion fell to approximately half the normal-control amount after 2 days of SD and remained at a low level for the following 10 days (Figure 1e). Lacrimal gland fluid secretion is innervated by both sympathetic and parasympathetic nerves, whereas the cornea is richly endowed with sensory nerve endings that respond to changes in the external

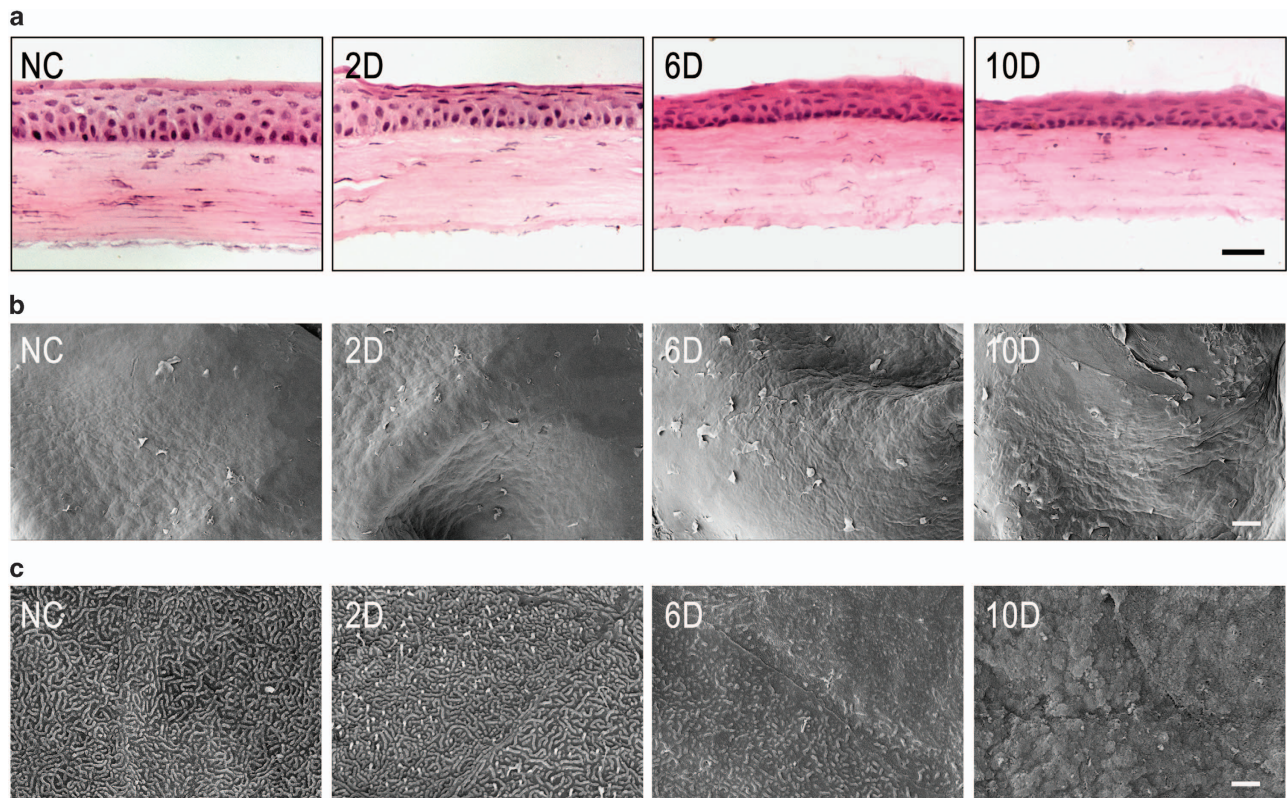


Figure 2 Histological changes of the corneal epithelium after sleep deprivation (SD). (a) Superficial layers of corneal epithelial cells became more compacted after 2 days of SD, and the corneal epithelial surface became rough at days 6 and 10. The bar represents $50\ \mu\text{m}$. (b) Scanning electron microscopy (SEM) revealed that the shedding of cells from the mouse corneal surface gradually increased from days 2 to 10. The bar represents $100\ \mu\text{m}$. (c) High magnification of SEM showed that the density and length of the microvilli on the apical surface of the corneal epithelium gradually decreased after SD for 6 to 10 days. The scale bar represents $1\ \mu\text{m}$ ($n=3/\text{group}$).

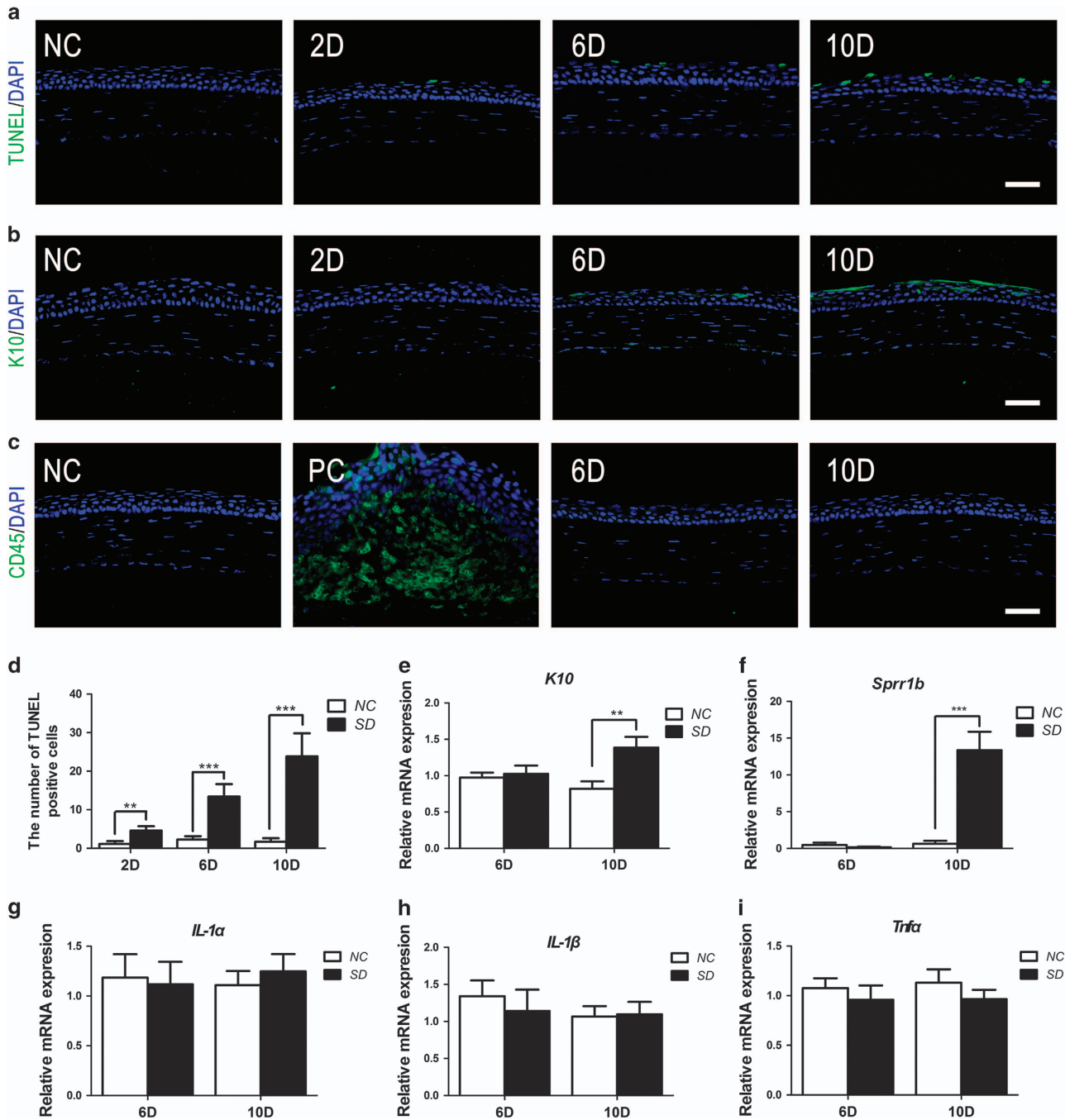


Figure 3 Corneal epithelium apoptosis, phenotypic change and inflammatory response after sleep deprivation (SD). (a) The TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay showed that apoptotic cells appeared in the superficial corneal epithelium after 2 days of SD and increased with time. The scale bar represents 50 μ m. (b) Immunofluorescent staining revealed K10-positive cells appearing 6 days after SD and increasing at day 10, whereas there was no indication of cells undergoing apoptosis in the normal cornea (NC). The scale bar represents 50 μ m. (c) CD45 immunostaining showed no CD45-positive cell infiltration in the normal control and in corneas after 6 or 10 days of SD. The mouse corneal ulcer positive control (PC) exhibited numerous CD45-positive cells in the corneal epithelium and stroma. The scale bar represents 50 μ m. (d) Cell count results showed that apoptotic cells gradually increased from days 2 to 10 of SD. Quantitative reverse transcription-PCR (qRT-PCR) results revealed that *K10* (e) and *Sprr1b* (f) gene expression levels markedly increased in the corneal epithelium after 10 days of SD. Interleukin-1 α (*IL-1 α*) (g), IL-1 β (h) and tumor necrosis factor- α (TNF- α) (i) gene expression was not upregulated after SD for 6 or 10 days, compared with the normal control ($n=6-10$ /group, ** $P<0.01$, *** $P<0.001$).

environment. We found that the corneal sensitivity markedly increased after 2 days of SD and was maintained at a high level until 8 days. Subsequently, the sensitivity slightly declined after 10 days SD, but its level was still higher than that in the normal control (Figure 1f).

Corneal epithelial histological changes after SD

Hematoxylin and eosin staining showed that the nuclei in the superficial epithelial layers became more condensed 2 days after SD, and the corneal epithelial surface became less smooth between days 6 and 10 (Figure 2a). Scanning electron microscopy observation at a low magnification showed that only a few surface cells were undergoing detachment after 2 days SD. However, the number of cells being lost from the surface markedly increased by day 6. After 10 days, a large epithelial area had separated as a sheet indicating desquamation (Figure 2b). Under high magnification, we found that the density and height of the microvilli gradually decreased after SD between 6 and 10 days (Figure 2c). Tight junctional integrity after 10 days SD was evaluated by ZO-1 immunostaining. The results showed that there was marked decrease of ZO-1 expression on the surface corneal epithelium, indicating extensive disruption of the tight junctional integrity (Supplementary Figure S3).

SD-induced apoptotic and phenotypic changes

TUNEL assay evaluation of corneal epithelial apoptosis revealed that the apoptotic cells emerged in the superficial corneal

epithelium after 2 days of SD and that their frequency gradually increased up to day 10 of SD (Figures 3a and d).

Squamous metaplasia is the most common ocular surface pathological change in dry eye.²⁴ K10 and Sprr1b are validated biomarkers of this process in dry eye disease.^{25,26} K10-positive cells appeared 6 days after SD and increased with time, whereas they were undetectable in the normal cornea (Figure 3b). Quantitative reverse transcription-PCR results identified marked increases in both K10 (Figure 3e) and Sprr1b (Figure 3f) mRNA expression after 10 days SD.

For up to 10 days of SD, there was no detectable immune cell infiltration based on the absence of CD45-positive cells (Figure 3c). Similarly, proinflammatory interleukin-1 α , interleukin-1 β and tumor necrosis factor- α gene expression in the cornea was not upregulated after SD for either 6 or 10 days (Figures 3g-i).

Goblet cell change after SD

Conjunctival goblet cells secrete gel-forming mucins and contribute to tear film stability.²⁷ Periodic acid-Schiff staining revealed that there was no marked change in conjunctival goblet cell density after 10 days of SD (Figures 4a and b); this finding was not consistent with changes in other aqueous-tear-deficient dry eye conditions, such as primary Sjögren's syndrome,²⁸ which exhibits a decreased goblet cell density. We further performed quantitative reverse transcription-PCR on the conjunctival tissues, and the results showed that the conjunctival goblet cell MUC5AC gene expression after 10 days of SD also did not significantly differ in comparison with age-matched control mice (Figure 4c).

Lacrimal gland hypertrophy after SD

The lacrimal gland has a multifaceted role in maintaining a homeostatic microenvironment for a healthy ocular surface via tear secretion. As aqueous tear secretion decreased and was maintained at a low level after SD, we determined whether the lacrimal glands underwent atrophy. Unexpectedly, the lacrimal glands instead underwent obvious hypertrophy after SD. Their size (Figure 5a) and weight (Figure 5b) markedly increased after 2 days of SD and became even more prominent at days 6 and 10, accompanied by acini engorgement (Figure 5c). In the acini cells, the vesicle accumulation increased, following the same enlargement pattern (Figure 5c). These changes were not attributable to immune cell activation and infiltration because lacrimal gland CD45 immunostaining was invariant and negative irrespective of SD duration (Figure 5d). Furthermore, RNA-seq lacrimal gland tissue analysis showed no significant changes in tumor necrosis factor, interleukin and interferon family gene expression (data not shown). However, the cell proliferation-related genes *Ccnb1*, *Cdkn3*, *Cdca8*, *Bub1*, *Bub1b*, *Cdk1*, *Mad2l1*, *Ccnb2*, *Mli67*, *Cdkn2c* and *Cdk14* were strongly upregulated after 2 days of SD and returned to normal levels at 10 days of SD (Figure 5e). There were no significant increases in cell apoptosis-related genes at 2 days of SD, but *Bax*, *Trp53* and *Fas* gene expression was obviously increased at 10 days of SD (Figure 5f).

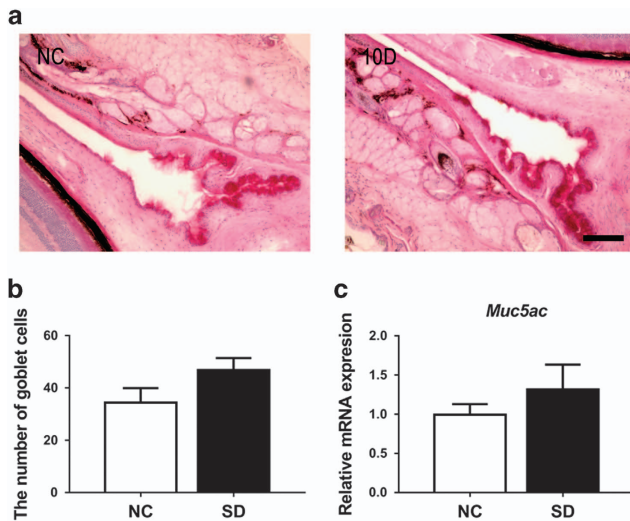


Figure 4 Goblet cell change after sleep deprivation (SD). (a) Periodic acid-Schiff (PAS) staining showed goblet cell distribution in the fornix conjunctival area in control mice (NC) and mice after SD for 10 days (10D). The scale bar represents 200 μ m. (b) Goblet cell counts revealed no significant change at day 10 compared with the normal control. (c) Quantitative reverse transcription-PCR (qRT-PCR) showed that the MUC5AC expression in the conjunctival tissue was mildly increased but not significantly different in mice after 10 days of SD, compared with age-matched control mice ($n=6$ /group).

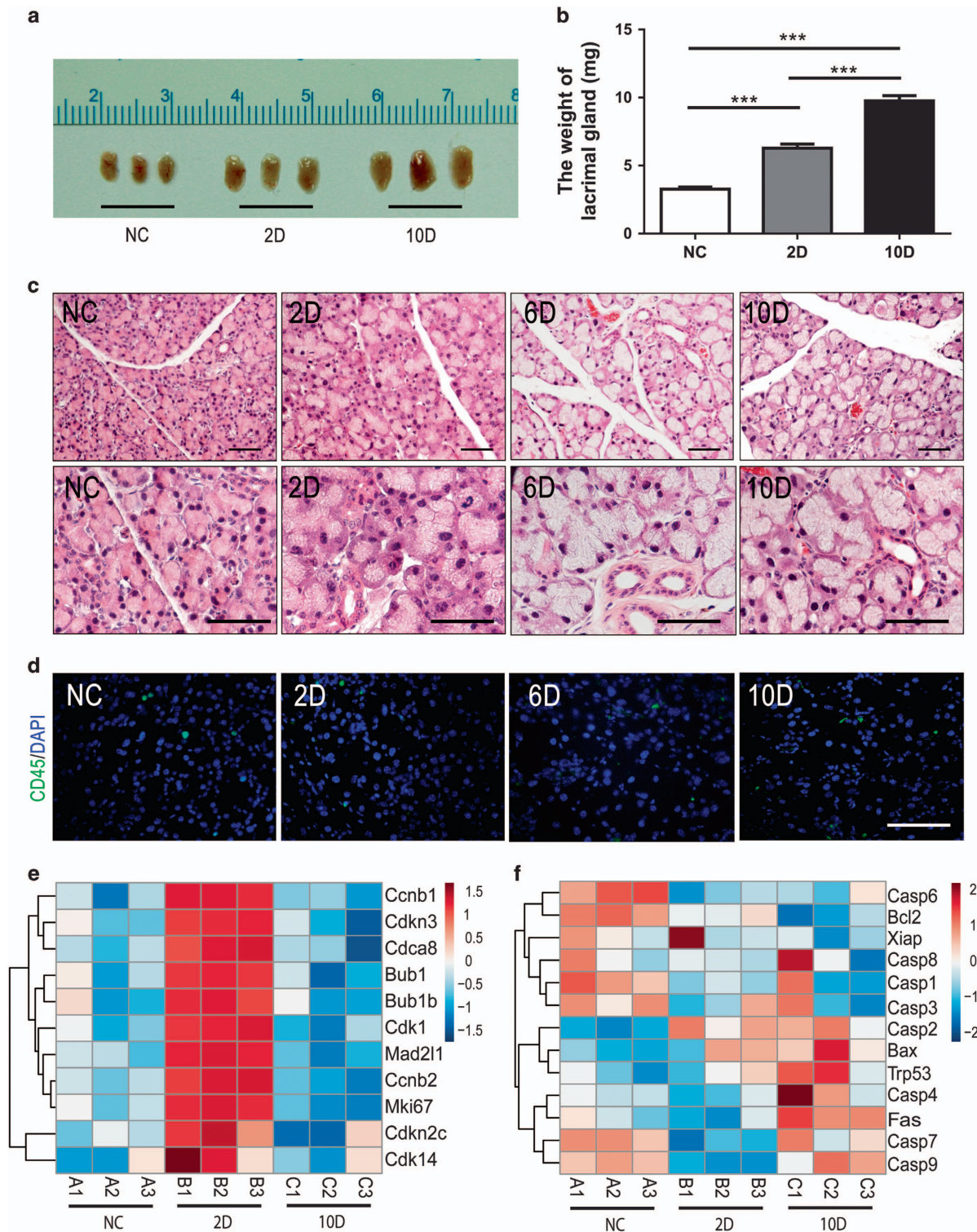


Figure 5 Morphological change and inflammation of the lacrimal gland after sleep deprivation (SD). Lacrimal gland size (a) and weight (b) both markedly increased after SD for 2 days, and these increases became more prominent at day 10 ($n=6/\text{group}$, $***P<0.001$). (c) Hematoxylin and eosin (H&E) staining showed enlarged lacrimal gland acini after SD for 2 and 10 days. High magnification pictures in the lower row show vesicle accumulation in acini cells, which was more prominent at day 10 compared with day 2. The scale bars represent 100 μm . (d) Immunostaining of lacrimal gland tissues showed no obvious CD45-positive cell infiltration after SD for different durations. The scale bar represents 100 μm . (e) RNA-seq results showed that the cell proliferation-related genes *Ccnb1*, *Cdkn3*, *Cdca8*, *Bub1*, *Bub1b*, *Cdk1*, *Mad2l1*, *Ccnb2*, *Mki67*, *Cdkn2c* and *Cdk14* were strongly upregulated at 2 days of SD but returned to normal levels at 10 days of SD. (f) The cell apoptosis-related genes *Bax*, *Trp53* and *Fas* obviously increased at 10 days of SD. The data were transferred to the Z-score $\log_2\text{-Fpk}$ by each row and clustered based on the Euclidean distance ($n=3/\text{group}$).

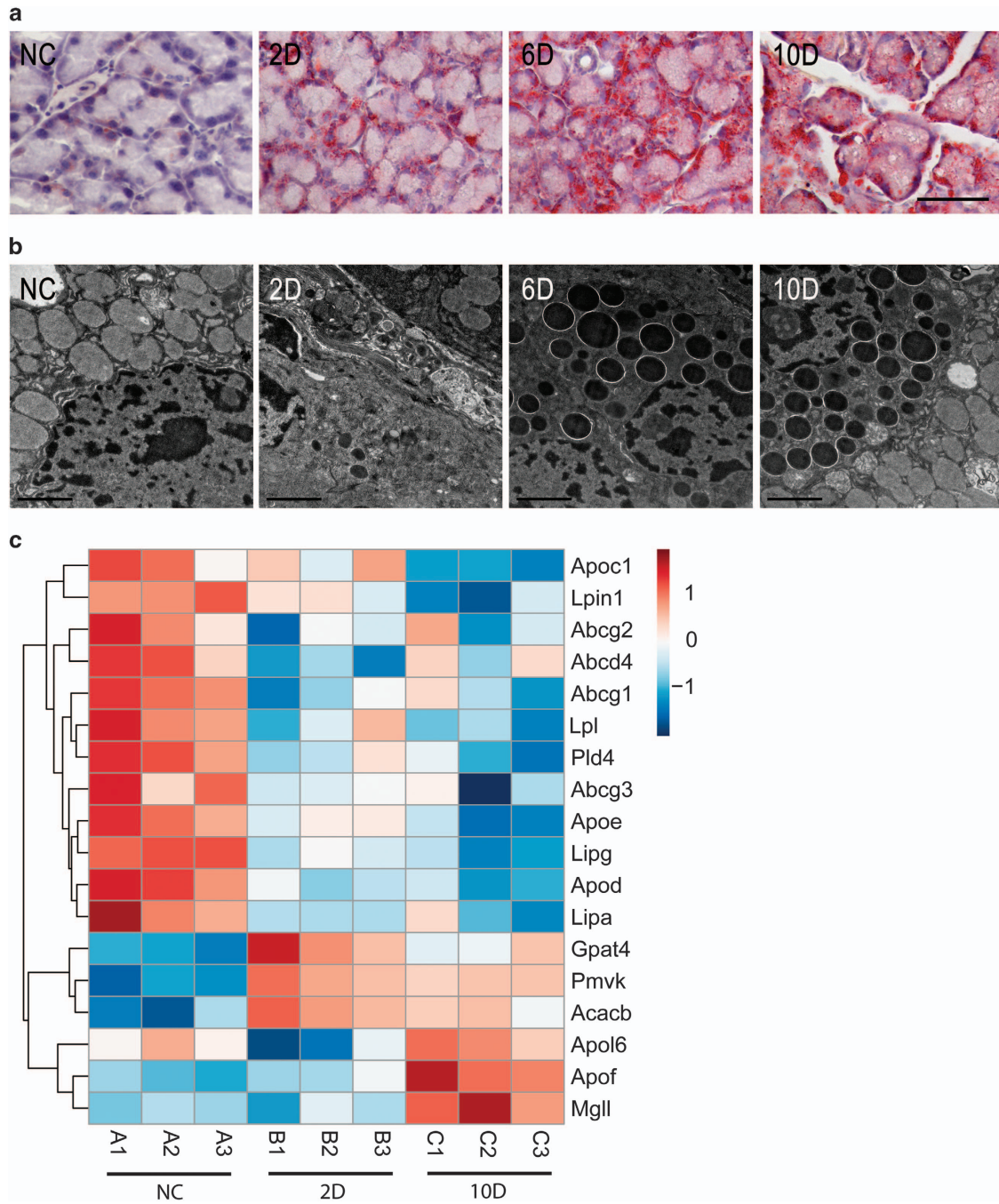


Figure 6 Abnormal lipid metabolism in the lacrimal gland after sleep deprivation (SD). **(a)** Oil Red O (ORO) staining was negative in the normal lacrimal gland; however, positive staining was displayed in both the cytoplasm of acini cells and the extracellular matrix after 2 days of SD, and gradually increased with time. The scale bar represents 50 μ m. **(b)** Transmission electron microscopy of the lacrimal gland revealed lipid droplets emerging in the acini cells after 2 days SD, and they became prominent at days 6 and 10. The scale bars represent 2 nm. **(c)** RNA-seq results showed that the lacrimal gland lipid-metabolism-related gene *Abcd4*, *Abcg2*, *Abcg1*, *Lipa*, *Apoc1*, *Lpin1*, *Abcg3*, *Lpl*, *Lipg*, *Apod*, *Apoe* and *Pld4* expression levels significantly decreased after SD, whereas those of *Acacb*, *Pmvk*, *Gpat4*, *Apol6*, *Apof* and *Mgll* significantly increased. The data were transferred to the Z-score log 2-Fpkm by each row and clustered based on the Euclidean distance ($n = 3$ /group).

To determine if their hypertrophy was associated with increases in neutral triglycerides and lipids, lacrimal gland frozen sections were stained with ORO. It did not stain normal lacrimal gland tissue, but stained both the cytoplasm of acini cells and the extracellular matrix after 2 days of SD,

followed by time-dependent increases in intensity (Figure 6a). Transmission electron microscopy images clearly demonstrated the accumulation of non-membrane delimited lipid droplet vesicles in the acini cells (Figure 6b). To further confirm whether there was abnormal lipid metabolism in the lacrimal

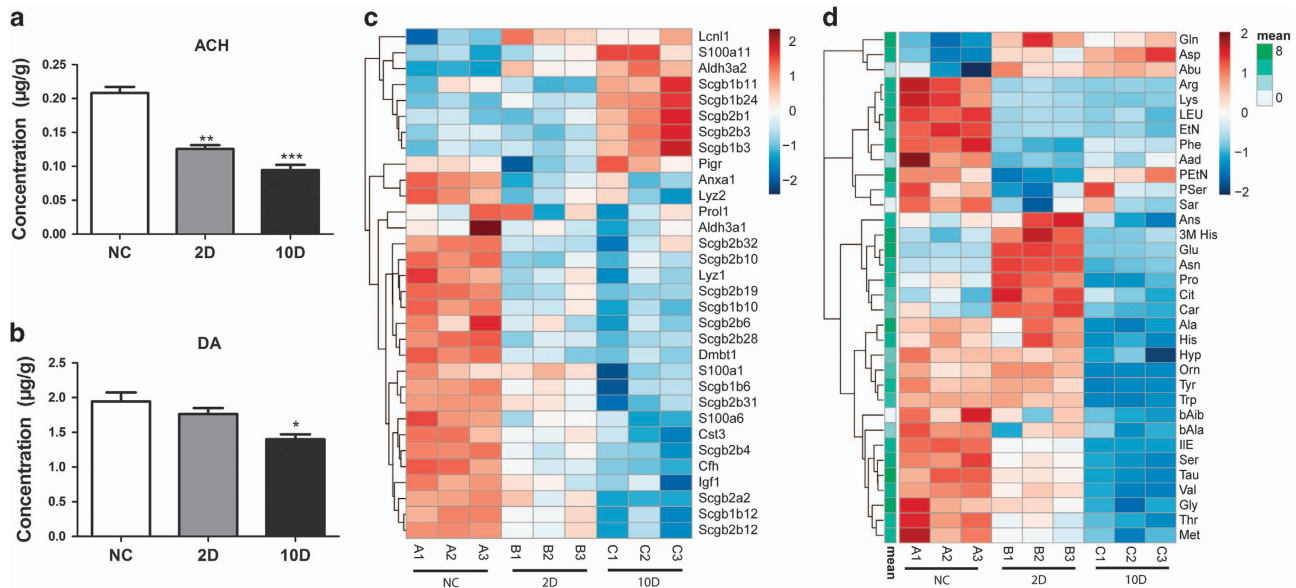


Figure 7 The lacrimal neurotransmitter, secretory proteins and free amino-acid changes after sleep deprivation (SD). **(a)** Acetylcholine (ACH) and **(b)** dopamine (DA) concentrations in the lacrimal gland tissues were significantly decreased after SD, compared with the normal control (NC). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with the NC). **(c)** The lacrimal secretory-related genes were differentially expressed after SD. **(d)** There was a marked change in the levels of 34 free amino acids in the lacrimal gland tissues after SD. The data were transferred to the Z-score \log_2 -Fpkm and \log_2 -Concentration ($\mu\text{g g}^{-1}$) by each row and clustered based on the Euclidean distance ($n = 3/\text{group}$).

gland after SD, RNA-seq analysis confirmed that SD down-regulated some lipid-metabolism-related genes, such as *Abcd4*, *Abcg2*, *Abcg1*, *Lipa*, *Apoc1*, *Lpin1*, *Abcg3*, *Lpl*, *Lipg*, *Apod*, *Apoe* and *Pld4*, while *Acacb*, *Pmvk*, *Gpat4*, *Apol6*, *Apof* and *Mgl1*, underwent upregulation, although the starting time point of this increase was variable (Figure 6c).

Alternations in lacrimal secretory-protein-related genes and free amino acids after sleep deprivation

Abundant nutrients and functional proteins are secreted from the lacrimal gland in tears and function on the ocular surface tissue.²⁹ We assessed that sleep deprivation not only influenced tear secretion but also the tear components. Parasympathetic and sympathetic nerves regulate tear secretion. Neurotransmitters acetylcholine and dopamine in the lacrimal gland are both important stimulators of aqueous tear and lacrimal protein secretion.³⁰ The liquid chromatography-tandem mass spectrometry results indicated that concentrations of both acetylcholine (Figure 7a) and dopamine (Figure 7b) were significantly decreased during SD.

Numerous functional tear proteins were significantly changed in dry eye patients.^{31,32} On the basis of their clinical study, we analyzed the effect of SD on lacrimal secretory-protein gene expression. The RNA-seq results showed that SD upregulated *Lcn11*, *S100a11*, *Aldh3a2*, *Scgb1b3*, *Scgb1b11*, *Scgb1b24*, *Scgb2b1* and *Scgb2b3* but downregulated *Pigr*, *Anxa1*, *Lyz1*, *Lyz2*, *Prol1*, *Aldh3a1*, *Scgb1b6*, *Scgb1b10*, *Scgb1b12*, *Scgb2a2*, *Scgb2b4*, *Scgb2b6*, *Scgb2b10*, *Scgb2b12*, *Scgb2b19*, *Scgb2b28*, *Scgb2b31*, *Scgb2b32*, *Dmbt1*, *S100a1*, *S100a6*, *Cst3*, *Cfh* and *Igf1* (Figure 7c).

In the ocular system, at least 23 free amino acids were found in human tears, and their concentration was altered in dry eye.³³ We measured the free amino-acid level in the lacrimal gland to evaluate the effects of SD on lacrimal gland protein synthesis and its secretory function during SD. The results showed that Gln, Asp, and Abu levels increased after 2 days and were maintained at high levels after 10 days of SD. Another group of amino acids transiently increased after 2 days and then decreased after 10 days. These amino acids included Ans, 3M His, Glu, Asn, Pro, Cit and Car. By contrast, most of the other amino acids, including Arg, Lys, LEU, EtN, Phe, Aad, PEtN, PSer, Sar, Ala, His, Hyp, Orn, Tyr, Trp, bAib, bAia, iIE, Ser, Tau, Val, Gly, Thr and Met, underwent marked declines after SD (Figure 7d).

Reversibility of the ocular surface changes after the termination of SD

To further determine whether the pathological changes of the ocular surface are reversible after the elimination of SD, we terminated the maneuver on the mice after 10 days of SD and kept them in the home cages under normal feeding conditions for 14 days. The ocular surface fluorescence staining showed that corneal staining markedly decreased to a mild dotted staining level after 14 days of rest (Figure 8a). Statistical analysis of the fluorescein score confirmed reduced corneal staining at day 14, but it was still higher than that in the normal control group (Figure 8c). ORO staining of the lacrimal gland tissues revealed that positive staining significantly decreased after 14 days of rest; however, the staining did not return to the normal control level (Figure 8b). The phenol red thread

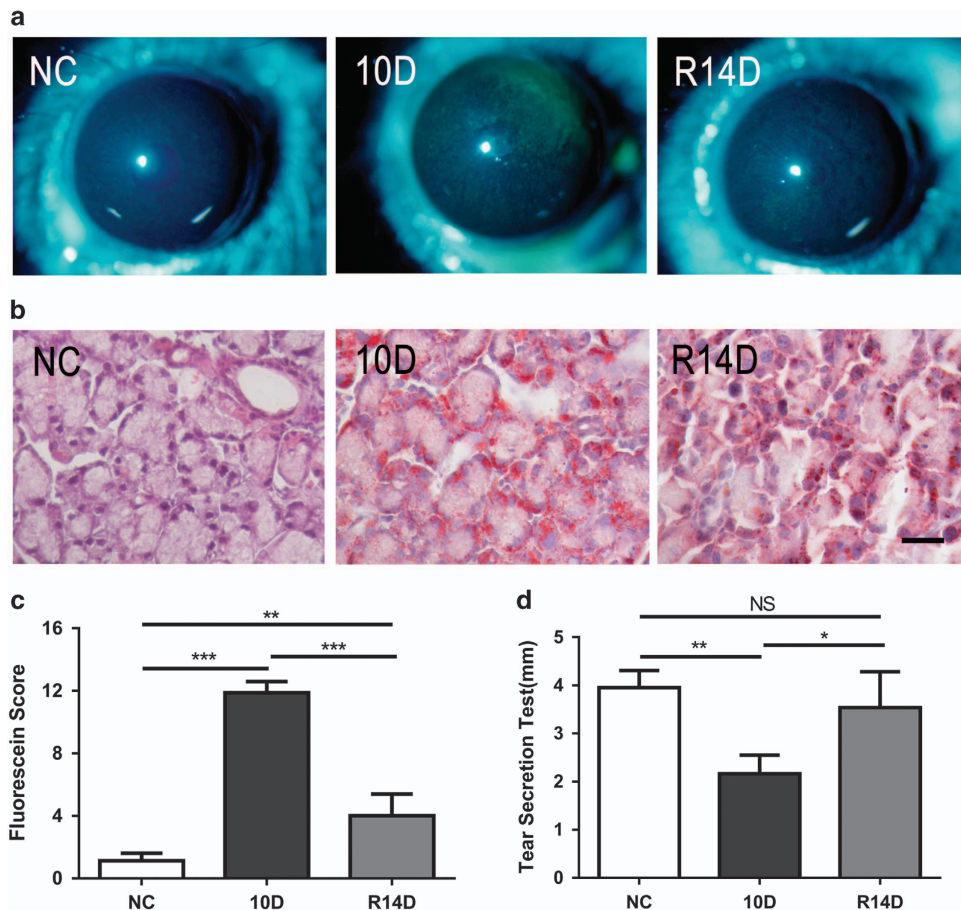


Figure 8 Reversibility of the ocular surface changes after the termination of sleep deprivation (SD). (a) The mice were put in the home cages under normal feeding conditions for 14 days after the 10 days of SD (R14D). The ocular surface fluorescein staining showed diffused and merged corneal staining after 10 days of SD (10D) but was markedly reduced to mild dotted staining after 14 days of rest (R14D). (b) Oil Red O staining of the lacrimal gland tissues showed intensive positive staining after 10 days of SD and mild staining after 14 days of rest. The scale bar represents 50 μm . (c) Statistical analysis of the fluorescein score revealed a dramatic increase at 10 days of SD and a decrease at day 14 of rest (** $P < 0.01$, *** $P < 0.001$). (d) The phenol red thread tear secretion test showed reduced tear secretion after 10 days of SD, which was restored after 14 days of rest (* $P < 0.05$, ** $P < 0.01$, NS stands for $P > 0.05$) ($n = 4/\text{group}$).

tear-secretion test showed that tear secretion increased after 14 days of rest compared with that of 10 days of SD and was restored to the normal level (Figure 8d, NS stands for $P > 0.05$). Collectively, these data suggested that the ocular surface and lacrimal gland changes resulting from 10 days of SD in the mice could revert to a relatively normal situation within 14 days.

DISCUSSION

Dry eye is a multifactorial disease with a high morbidity. We show here that SD can induce pathophysiological changes of the ocular surface in mice. Progressive changes that mimicked those described in dry eye patients occurred in the corneal epithelium and lacrimal gland during 10 days. Specifically, the prominent changes included decreased aqueous tear secretion, corneal hypersensitivity, superficial-layer epithelial desquamation, apical microvillus loss from the surface corneal epithelium, apoptosis of the corneal epithelial cells, compromised superficial-layer tight-junctional integrity and squamous metaplasia.

SD-induced dry eye, however, has unique pathologic features. First, there was a mild increase, although no significant difference, of goblet cell density and MUC5AC expression in the conjunctival tissue after 10 days of SD. This is not consistent with the findings reported in other dry eye clinical studies and animal models,^{34–36} which have shown decreased goblet cell density and MUC5AC gene expression. We presume that this may be an adaptive response to the decreased aqueous tear secretion occurring during the early stage of dry eye. Another possibility is that SD-induced stress may promote the differentiation of goblet cells, which has also been found in human airway epithelium goblet cell metaplasia under oxidative stress.³⁷ Further study is necessary to observe the goblet cell differentiation over an even longer duration of sleep deprivation to investigate the effect of chronic SD on goblet cells.

Second, as reported, inflammatory cell infiltration into the ocular surface tissues and lacrimal gland occurs in various Sjögren-syndrome-related dry eye diseases.^{38,39} However, we failed to detect inflammatory cell infiltration in both the

cornea and the lacrimal gland in our SD model. Moreover, the inflammatory-cytokine gene expression did not increase in the corneal tissues. These results indicate that the inflammatory response in the various ocular surface tissues was not obvious in the SD model, which might be due to the short duration of SD or because the aqueous tear deficiency in SD mice was not severe enough to trigger the inflammation cascade. A longer duration of observation in this model may reveal whether ocular surface inflammation is only present at a certain stage or certain degree of dry eye.

Third, for the first time, we characterized histological lacrimal gland changes induced by SD. After 2 days of SD, the lacrimal gland underwent hypertrophy, which became more evident at later times without any acinar cell apoptosis. Instead, the acinar cells became enlarged due to lipid droplet accumulation. A similar pathological change has been found in the lacrimal glands of non-obese diabetic mice,⁴⁰ which are also a Sjögren-syndrome-related dry eye model, and its lacrimal gland lipid metabolism and deposition are both abnormal. Substantial increases in *Acacb*, *Pmvk*, *Gpat4*, *Apol6*, *Apof* and *Mgll* gene expression were implicated in disrupting the homeostatic control of lacrimal gland lipid metabolism after SD. The significance and mechanism of lacrimal gland lipid accumulation requires further study.

Tears have important roles in optical function, immunity and nutrition as well as prevent dryness of the ocular surface tissue.⁴¹ Without stable and adequate tears, the ocular surface tissue will be damaged. Interestingly, aqueous tear secretion declined after 2 days by ~50% from the value before inducing SD and was maintained at this level after 10 days. This effect suggests an initial partial compromise of lacrimal gland function, whereas the corneal sensitivity instead increased after SD, indicating corneal sensory nerve stimulation. Corneal mechanical hypersensitivity is also found in individuals with Sjögren's syndrome.⁴² Because corneal nerve stimulation can induce lacrimal gland secretion through a trigeminal parasympathetic reflex arc,⁴³ this effect may contribute to maintaining a low level of aqueous tear secretion after SD.

Proteomics analysis of tears provides a possible way to subcategorize dry eye disease. In our SD model, a majority of lacrimal secretory-protein gene expression changes corresponded to tear analysis from dry eye patients. However, we can see the opposite increased or decreased trends in several genes, such as *Lcnll*, which is decreased in Sjögren's syndrome-related dry eye disease.³² This result also indicates that SD-induced dry eye has unique characteristics compared with other types of dry eye.

This study provides the first evidence that lacrimal gland and ocular surface health is sensitive to SD. The ocular surface pathological changes induced by SD mimic most of the typical characteristics of dry eye disease. However, the specific features of SD-induced dry eye also require attention in the clinic. As anti-inflammation therapy is commonly acceptable for dry eye treatment, the decision regarding whether to administer anti-inflammatory eye drops to SD-induced dry eye patients should be significant in the future. Because SD *per se* can be considered

a stressful situation, irrespective of which deprivation method is used, this model can also be used to study stress-related dry eye disease. Our study also found that the aqueous tear secretion could be restored to normal levels and that the pathological changes to the corneal epithelium and lacrimal gland could be reversed to a large extent in SD mice after 14 days of rest, further proving that SD-induced dry eye may be reversible. Since the longest duration of SD was 10 days in our study, it remains unknown whether the pathological changes in chronic dry eye induced by SD can be reversed. Further study is also needed to investigate the treatment of SD-induced dry eye as opposed to the improvement of sleep quality and quantity.

In conclusion, we herein demonstrated that SD can induce dry eye disease. Moreover, the disruption of lacrimal gland function with respect to tear secretion, lipid metabolism and protein synthesis provided a mechanism for the occurrence of SD-induced dry eye disease. These findings will benefit the clinical diagnosis and treatment of sleep-disorder-related ocular surface disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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- 1 Siegel JM. Do all animals sleep? *Trends Neurosci* 2008; **31**: 208–213.
- 2 McKnight-Eily LR, Eaton DK, Lowry R, Croft JB, Presley-Cantrell L, Perry GS. Relationships between hours of sleep and health-risk behaviors in US adolescent students. *Prev Med* 2011; **53**: 271–273.
- 3 Beaucauge KL, Xiao A, Pollmann SI, Grol MW, Beach RJ, Holdsworth DW *et al*. Loss of P2X7 nucleotide receptor function leads to abnormal fat distribution in mice. *Purinergic Signal* 2014; **10**: 291–304.
- 4 Aurora RN, Punjabi NM. Obstructive sleep apnoea and type 2 diabetes mellitus: a bidirectional association. *Lancet Respir Med* 2013; **1**: 329–338.
- 5 Knutson KL, Van Cauter E. Associations between sleep loss and increased risk of obesity and diabetes. *Ann NY Acad Sci* 2008; **1129**: 287–304.
- 6 Knutson KL, Van Cauter E, Rathouz PJ, Yan LL, Hulley SB, Liu K *et al*. Association between sleep and blood pressure in midlife: the CARDIA sleep study. *Arch Intern Med* 2009; **169**: 1055–1061.
- 7 King CR, Knutson KL, Rathouz PJ, Sidney S, Liu K, Lauderdale DS. Short sleep duration and incident coronary artery calcification. *JAMA* 2008; **300**: 2859–2866.

- 8 Sutton EL. Psychiatric disorders and sleep issues. *Med Clin N Am* 2014; **98**: 1123–1143.
- 9 Conroy DA, Arnedt JT. Sleep and substance use disorders: an update. *Curr Psychiatry Rep* 2014; **16**: 487.
- 10 Oyiengo D, Louis M, Hott B, Bourjeily G. Sleep disorders in pregnancy. *Clin Chest Med* 2014; **35**: 571–587.
- 11 Short MA, Louca M. Sleep deprivation leads to mood deficits in healthy adolescents. *Sleep Med* 2015; **16**: 987–993.
- 12 Goel N, Basner M, Rao H, Dinges DF. Circadian rhythms, sleep deprivation, and human performance. *Prog Mol Biol Transl Sci* 2013; **119**: 155–190.
- 13 Paiva T, Gaspar T, Matos MG. Sleep deprivation in adolescents: correlations with health complaints and health-related quality of life. *Sleep Med* 2015; **16**: 521–527.
- 14 Galor A, Feuer W, Lee DJ, Florez H, Carter D, Pouyeh B *et al*. Prevalence and risk factors of dry eye syndrome in a United States veterans affairs population. *Am J Ophthalmol* 2011; **152**: 377–384 e372.
- 15 Lee YB, Koh JW, Hyon JY, Wee WR, Kim JJ, Shin YJ. Sleep deprivation reduces tear secretion and impairs the tear film. *Invest Ophthalmol Vis Sci* 2014; **55**: 3525–3531.
- 16 Lee W, Lim SS, Won JU, Roh J, Lee JH, Seok H *et al*. The association between sleep duration and dry eye syndrome among Korean adults. *Sleep Med* 2015; **16**: 1327–1331.
- 17 Ayaki M, Kawashima M, Negishi K, Tsubota K. High prevalence of sleep and mood disorders in dry eye patients: survey of 1,000 eye clinic visitors. *Neuropsychiatr Dis Treat* 2015; **11**: 889–894.
- 18 Ayaki M, Kawashima M, Negishi K, Kishimoto T, Mimura M, Tsubota K. Sleep and mood disorders in dry eye disease and allied irritating ocular diseases. *Sci Rep* 2016; **6**: 22480.
- 19 Ayaki M, Toda I, Tachi N, Negishi K, Tsubota K. Preliminary report of improved sleep quality in patients with dry eye disease after initiation of topical therapy. *Neuropsychiatr Dis Treat* 2016; **12**: 329–337.
- 20 Bergmann BM, Kushida CA, Everson CA, Gilliland MA, Obermeyer W, Rechtschaffen A. Sleep deprivation in the rat: II. Methodology. *Sleep* 1989; **12**: 5–12.
- 21 Pauly A, Brignole-Baudouin F, Labbé A, Liang H, Warnet J-M, Baudouin C. New tools for the evaluation of toxic ocular surface changes in the rat. *Invest Ophthalmol Vis Sci* 2007; **48**: 5473–5483.
- 22 Wang YC, Li S, Chen X, Ma B, He H, Liu T *et al*. Meibomian gland absence related dry eye in ectodysplasin a mutant mice. *Am J Pathol* 2016; **186**: 32–42.
- 23 Zhang L, Zou D, Li S, Wang J, Qu Y, Ou S *et al*. An ultra-thin amniotic membrane as carrier in corneal epithelium tissue-engineering. *Sci Rep* 2016; **6**: 21021.
- 24 Tseng SC. Staging of conjunctival squamous metaplasia by impression cytology. *Ophthalmology* 1985; **92**: 728–733.
- 25 Nakamura T, Nishida K, Dota A, Matsuki M, Yamanishi K, Kinoshita S. Elevated expression of transglutaminase 1 and keratinization-related proteins in conjunctiva in severe ocular surface disease. *Invest Ophthalmol Vis Sci* 2001; **42**: 549–556.
- 26 Li S, Nikulina K, DeVoss J, Wu AJ, Strauss EC, Anderson MS *et al*. Small proline-rich protein 1B (SPRR1B) is a biomarker for squamous metaplasia in dry eye disease. *Invest Ophthalmol Vis Sci* 2008; **49**: 34–41.
- 27 Inatomi T, Spurr-Michaud S, Tisdale AS, Zhan Q, Feldman ST, Gipson IK. Expression of secretory mucin genes by human conjunctival epithelia. *Invest Ophthalmol Vis Sci* 1996; **37**: 1684–1692.
- 28 Pflugfelder SC, Huang AJ, Feuer W, Chuchofski PT, Pereira IC, Tseng SC. Conjunctival cytologic features of primary Sjogren's syndrome. *Ophthalmology* 1990; **97**: 985–991.
- 29 Zoukhri D. Effect of inflammation on lacrimal gland function. *Exp Eye Res* 2006; **82**: 885–898.
- 30 Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res* 2009; **28**: 155–177.
- 31 Perumal N, Funke S, Pfeiffer N, Grus FH. Proteomics analysis of human tears from aqueous-deficient and evaporative dry eye patients. *Sci Rep* 2016; **6**: 29629.
- 32 Li B, Sheng M, Li J, Yan G, Lin A, Li M *et al*. Tear proteomic analysis of Sjogren syndrome patients with dry eye syndrome by two-dimensional-nano-liquid chromatography coupled with tandem mass spectrometry. *Sci Rep* 2014; **4**: 5772.
- 33 Nakatsukasa M, Sotozono C, Shimbo K, Ono N, Miyano H, Okano A *et al*. Amino acid profiles in human tear fluids analyzed by high-performance liquid chromatography and electrospray ionization tandem mass spectrometry. *Am J Ophthalmol* 2011; **151**: 799–808 e791.
- 34 Choi W, Lian C, Ying L, Kim GE, You IC, Park SH *et al*. Expression of lipid peroxidation markers in the tear film and ocular surface of patients with Non-Sjogren syndrome: potential biomarkers for dry eye disease. *Curr Eye Res* 2016; **41**: 1143–1149.
- 35 Zhang X, De Paiva CS, Su Z, Volpe EA, Li DQ, Pflugfelder SC. Topical interferon-gamma neutralization prevents conjunctival goblet cell loss in experimental murine dry eye. *Exp Eye Res* 2014; **118**: 117–124.
- 36 Lin Z, Liu X, Zhou T, Wang Y, Bai L, He H *et al*. A mouse dry eye model induced by topical administration of benzalkonium chloride. *Mol Vis* 2011; **17**: 257–264.
- 37 Casalino-Matsuda SM, Monzon ME, Forteza RM. Epidermal growth factor receptor activation by epidermal growth factor mediates oxidant-induced goblet cell metaplasia in human airway epithelium. *Am J Respir Cell Mol Biol* 2006; **34**: 581–591.
- 38 Inaba T, Hisatsune C, Sasaki Y, Ogawa Y, Ebisui E, Ogawa N *et al*. Mice lacking inositol 1,4,5-trisphosphate receptors exhibit dry eye. *PLoS ONE* 2014; **9**: e99205.
- 39 Bian F, Barbosa FL, Corrales RM, Pelegrino FS, Volpe EA, Pflugfelder SC *et al*. Altered balance of interleukin-13/interferon-gamma contributes to lacrimal gland destruction and secretory dysfunction in CD25 knockout model of Sjogren's syndrome. *Arthritis Res Ther* 2015; **17**: 53.
- 40 Wu K, Joffe C, Li X, MacVeigh-Aloni M, Hom M, Hwang J *et al*. Altered expression of genes functioning in lipid homeostasis is associated with lipid deposition in NOD mouse lacrimal gland. *Exp Eye Res* 2009; **89**: 319–332.
- 41 Moshirfar M, Pierson K, Hanamaikai K, Santiago-Caban L, Muthappan V, Passi SF. Artificial tears potpourri: a literature review. *Clin Ophthalmol* 2014; **8**: 1419–1433.
- 42 Tuisku IS, Konttinen YT, Konttinen LM, Tervo TM. Alterations in corneal sensitivity and nerve morphology in patients with primary Sjogren's syndrome. *Exp Eye Res* 2008; **86**: 879–885.
- 43 Yasui T, Karita K, Izumi H, Tamai M. Correlation between vasodilatation and secretion in the lacrimal gland elicited by stimulation of the cornea and facial nerve root of the cat. *Invest Ophthalmol Vis Sci* 1997; **38**: 2476–2482.



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