The Controlled-Environment Chamber: A New Mouse Model of Dry Eye

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PURPOSE. To develop a controlled-environment chamber (CEC) for mice and verify the effects of a low-humidity setting on ocular surface signs in normal mice.

METHODS. Eight- to 12-week-old BALB/c mice were used in a controlled-environment chamber (CEC) where relative humidity (RH), temperature (T), and airflow (AF) are regulated and monitored. Mice were placed into the CEC and exposed to specific environmentally controlled conditions (RH = 18.5% ± 5.1%, AF = 15 L/min, T = 21–23°C) for 3, 7, 14, and 28 days. Control mice were kept in a normal environment (RH = 50%–80%, no AF, T = 21–23°C) for the same duration. Aqueous tear production by means of the cotton thread test, corneal fluorescein staining (score, 0–15), and goblet cell density in the superior and inferior conjunctiva were measured by a masked observer.

RESULTS. No statistically significant differences between the groups were found at baseline. Decreased tear secretion and increased corneal fluorescein staining were significantly present on day 3, 7, 14, and 28 in animals kept in the CEC. Goblet cell density was significantly decreased in the superior conjunctiva on day 7, and on day 3, 7, and 14 in the inferior conjunctiva in the CEC-kept mice compared with control animals.

CONCLUSIONS. This study indicates that exposure of normal mice to a low-humidity environment in a CEC can lead to significant alterations in tear secretion, goblet cell density, and acquisition of dry eye-related ocular surface signs. (*Invest Ophthalmol Vis Sci.* 2005;46:2766–2771) DOI:10.1167/iovs.04-1326

Dry eye syndrome, or keratoconjunctivitis sicca (KCS), affects millions of individuals—in particular, women—in the United States alone.[1] The National Eye Institute Industry Workshop on Clinical Trials in Dry Eyes produced a classification that essentially separates dry eye syndrome into two major types: tear-deficient forms (including Sjögren's syndrome and non-Sjögren's tear deficient) and evaporative forms.[2] The pathogenesis of both forms of dry eye is not thoroughly understood, but a growing body of evidence suggests that the tear film and the ocular surface (cornea, conjunctiva, and accessory lacrimal glands), Meibomian glands, and main lacrimal gland are functionally interconnected.[3] Therefore, damage to the tear film can affect other components of this functional unit, creating a series of pathologic changes that may lead to characteristic ocular surface changes and symptoms of dry eye.[4]

The tear film is constantly exposed to multiple environmental factors, including variable temperature, airflow (AF), and humidity (RH), which may stimulate or retard its evaporation. In particular, a low-humidity setting in the presence of a significant AF increases the tear evaporation rate,[5] as is frequently reported by subjects in desiccating environments.[6] Indeed, even people with a normal tear secretion rate may experience dry eye symptoms while exposed to dry environments, such as airplanes[7] and workplaces.[8] Conversely, dry eye symptoms improve in humid environments.[9] Of interest, it has been demonstrated that ocular surface tests, such as the Schirmer test and tear breakup time are decreased in subjects who live in dry climates,[10] suggesting that multiple functional facets of the tear film–ocular surface can be influenced by the environment.

Numerous animal models of dry eye have been developed to reflect the multiplicity of the pathophysiologic mechanisms involved in dry eye.[11] Unfortunately, none takes into consideration the exact effect of the environment on the tear secretion and ocular surface conditions. A controlled environmental setting in which temperature, humidity, and AF can be recorded and regulated and in which dry eye can be induced would be a useful tool for investigating pathogenic mechanisms in KCS as well as the relationship between critical environmental factors in dry eye.

The purpose of this study was to develop a controlled-environment chamber (CEC) for mice in which temperature, humidity, and AF are constantly monitored and regulated and to test the hypothesis that a low-humidity environmental setting can induce dry eye–related ocular surface signs in normal mice.

METHODS

Animals

Eight to 12-week-old female BALB/c mice (Taconic Farms, Germantown, NY) were used in these experiments. The protocol was approved by the Schepens Eye Research Institute Animal Care and Use Committee, and all animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Procedure

A CEC was created that allowed regulation of AF and humidity and control of temperature (Fig. 1). The chamber consists of a cage (Laboratory Products Inc., Seaford, DE) modified to allow the use of desiccants. The usable floor area of our modified cage is 725 cm². The
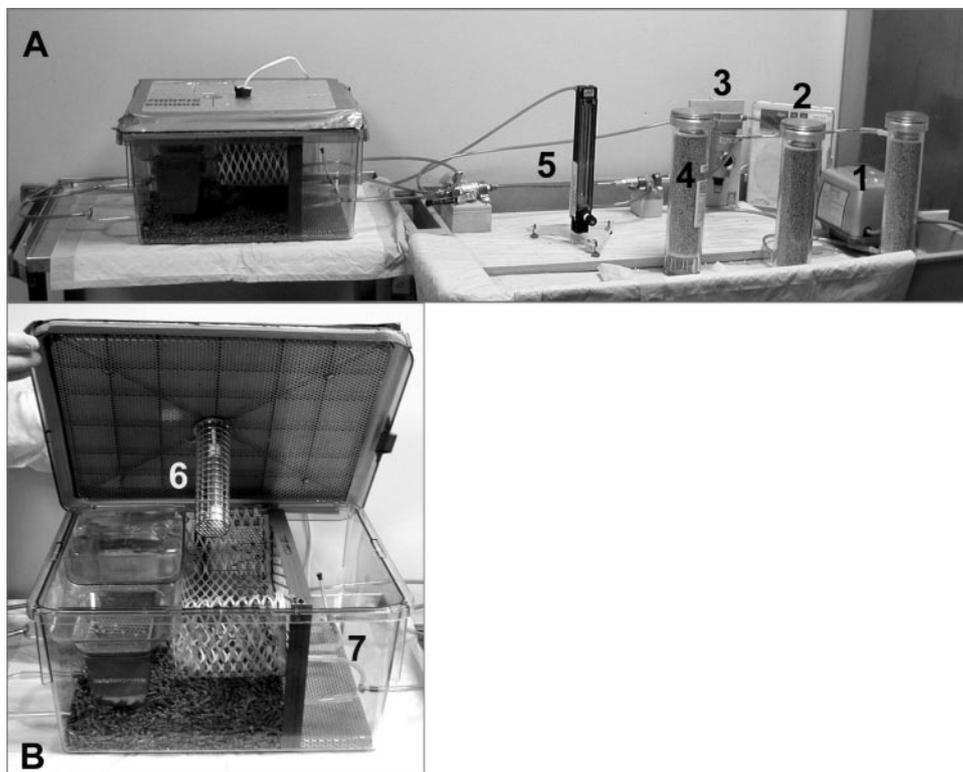
FIGURE 1. Photograph of the CEC. (**A**) Overview of the system with air pump (1), temperature–humidity recorder (2), water separator (3), desiccators (4), and flowmeter with valve (5). (**B**) Detail of the modified cage with temperature–humidity probe (6) and space available for desiccants (7).

roof of the cage is sealed with isolating material to make the chamber independent from the humidity of the room where it is placed. A hole on the roof allows the air to move outside the CEC. A stainless-steel barrier system has been placed inside the chamber, in which desiccants can be placed without the risk of any contact with the mice. For desiccants, we use indicating silica gel packed in cartridges of 114 mm diameter (Cole-Parmer Instrument Company, Vernon Hills, IL), and anhydrous $CaSO_4$ (Drierite; W. A. Hammond Drierite Co., Xenia, OH), both of which are commonly used to remove moisture from the environment and are nontoxic to humans and animals. The CEC is connected to an air line and a temperature and humidity recorder. A small, low-noise (38 dB) oilless linear pump (38 L/min open flow, 26 W; Gast Manufacturing, Inc., Benton Harbor, MI) placed 1 m from the chamber is used as the source of air. The flow is regulated by a flowmeter (0–50 L/min, accuracy ±5%) with a valve placed on the air line. The air is pumped into the chamber through four tips (1-mm diameter) placed in two opposite walls. The height of the tips (3.5 and 4.5 cm on one side, 3 and 4 cm on the other) has been chosen to correspond to the height of the mouse's eyes. The humidity of the air pumped in the chamber can be regulated by a valve that can direct the air into a desiccant system made of a water separator (SMC Corp., Tokyo, Japan), and air-drying columns containing $CaSO_4$. In the CEC, temperature (range, 5–45°C, accuracy ±1°C), and humidity (0%–100%, accuracy ±2%) are constantly monitored by a probe and recorded on circular charts by a temperature humidity recorder (Supco, Allenwood, NJ).

The CEC was initially validated by testing the temperature and humidity in its environment in different room conditions. Specifically, we tested its capacity to decrease the humidity in its chamber by placing desiccants and maintaining constant AF of 15 L/min of dehumidified air. In the second phase of our studies we evaluated the CEC with mice in it. Before initiating these experiments, animals were kept in (standard) individual cages for at least 4 days. These cages were placed in the same room, with temperature maintained at 21°C to 23°C, at a relative humidity (RH) of 40% to 60%. The animals were then randomly divided into study ($n = 30$) and control ($n = 30$) groups.

Mice in the study group were placed into the CEC ($n = 5$/cage) and exposed to controlled environmental conditions (RH, < 25%; AF, 15 L/min; temperature, 21–23°C) for 3, 7, 14, and 28 days, to determine the effect of these environmental conditions in vivo. These settings were chosen for two reasons: First, we selected environmental conditions that nearly maximized the capacity of the CEC to deliver a low-humidity and high-AF condition, to expose mice to a highly drying environment; second, we determined settings that we had determined could be reliably delivered and maintained by the CEC for prolonged periods. During these experiments, the animals' behavior, food, and water intake were not restricted. The control mice ($n = 5$/cage) were kept in standard cages with a normal environment (RH, 50%–80%; no AF; temperature, 21–23°C) for the same duration of time as the study group.

Aqueous tear production and corneal fluorescein staining were measured by a masked observer in all mice, before and after exposure to the CEC or the normal environment. Conjunctival goblet cell density was measured in the study and control groups at the end of each experiment.

Measurement of Aqueous Tear Production

Tear production was measured with the phenol red thread test (Zone-Quick; Lacrimedics, Eastsound, WA). The validity of this test in mice has been described.[12] Under a magnifying fluorescent lamp, the threads were held with jeweler forceps and placed in the lateral cantus of the conjunctival fornix of the right eye for 30 seconds after the excess tears were removed for a standard time of 4 seconds. In addition, because measurement of tear secretion in mice has not been fully standardized, at days 3 and 7 the test was also performed for 60 seconds without removing the excess tears. The tear distance (in millimeters) was read under a microscope (model S4; Carl Zeiss Meditec, Oberkochen, Germany), by using the scale of a hemacytometer.

Corneal Fluorescein Staining

Corneal fluorescein staining was performed by applying 0.5 μL of 5% fluorescein by micropipette into the inferior conjunctival sac of the

right eye. The cornea was examined with a slit lamp biomicroscope in cobalt blue light 3 minutes after fluorescein instillation. Punctuate staining was recorded in a masked fashion with a standardized (National Eye Institute) grading system of 0 to 3 for each of the five areas in which the corneal surface was divided.[2]

Histologic Analysis

The eyes, including the lids, were fixed in 10% buffered formalin and embedded in methacrylate (Technovit 7100; Heraeus, Wehrhein, Germany). Central vertical plane sections of 3-μm thickness were stained with hematoxylin-eosin or with periodic acid-Schiff (PAS). One masked observer evaluated conjunctival epithelial morphology and counted the number of goblet cells in the superior and inferior conjunctiva. The superior conjunctiva was marked with black ink after the animals were killed. Counting of goblet cells was performed under a microscope (model E800; Nikon, Melville, NY), with a 20× objective. Because the number of goblet cells was not always uniform throughout the sections, three different sections were selected for counting, and an average was calculated.

Statistical Analysis

The Student's *t*-test for the aqueous tear production, and the Mann-Whitney test for corneal fluorescein staining were used to compare the differences between the study and the control groups. Within-group changes from baseline were analyzed by the paired *t*-test (aqueous tear production) and by the Wilcoxon test. The ANOVA was performed for conjunctival goblet cell density changes, using a post hoc test of Bonferroni. All tests were two-tailed, and a $P < 0.05$ was considered to be statistically significant.

RESULTS

Controlled Environment Chamber

Without placing desiccants in the chamber, and with no AF, the RH in the CEC increased with time. In particular, the RH increased in the first 3 hours, and remained constant for 14 days (Fig. 2A). Of note, the RH in the chamber increased in a similar manner, despite the initial RH in the room where the CEC was placed. The use of an AF of 15 L/min of desiccated air reduced the RH in the CEC, regardless of the RH conditions in the room in which the CEC was placed (Fig. 2B). In both cases, the recorded temperature was 21°C to 23°C, the same as room temperature.

In a second phase we exposed mice to the CEC. The overall mean RH in the CEC was 18.5% ± 5.1%, with no significant differences between each set of experiments. The AF was kept constant at 15 L/min. The recorded temperature in the CEC was in the range of 21°C to 23°C. Animals demonstrated normal behavior similar to that of littermates in the standard cages. No stress responses (e.g., aggressive behavior, hair loss, mutilation, and excessive eye rubbing) were noted over 3 hours of direct observation and 4 hours of videographic recording. The weight gain and the food and water intake of the CEC and control groups were similar during the course of the study.

Aqueous Tear Production

At baseline (day 0), no statistical differences were recorded between the groups in terms of aqueous tear production. Statistically significant decreases in tear secretion were recorded after 3, 7, 14, and 28 days of exposure to the CEC environment (Fig. 3). In particular, the mean cotton thread wetting for control group and CEC was 1.8 ± 0.2 and 1.4 ± 0.3 mm respectively on day 3 ($P < 0.005$, $n = 10$), 1.8 ± 0.2 and 0.9 ± 0.2 mm on day 7 ($P < 0.0001$, $n = 10$), 2 ± 0.4 and 1 ±
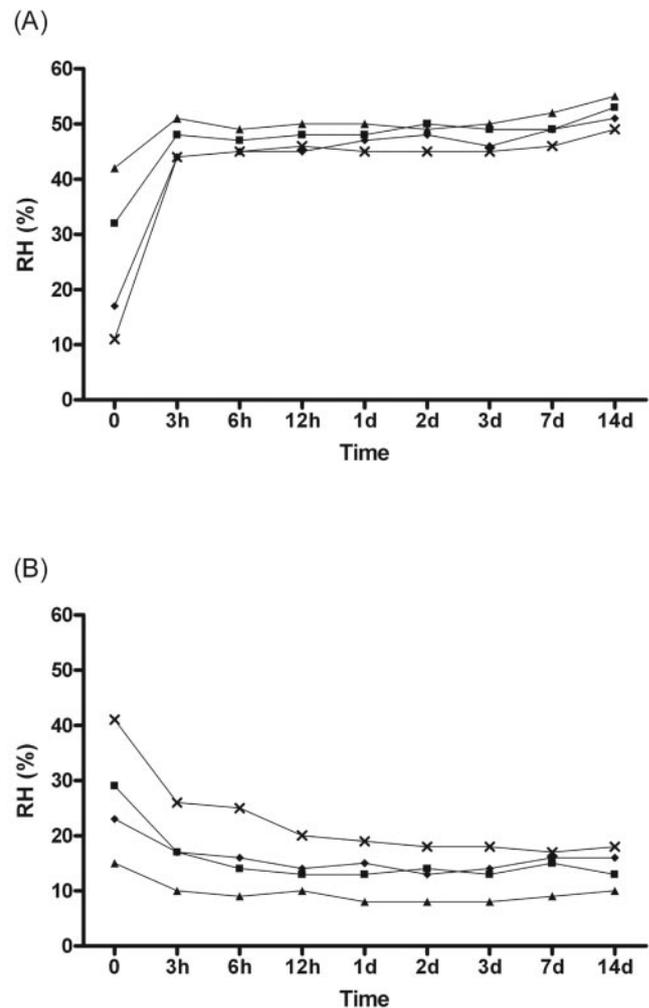

(A)

(B)

FIGURE 2. Four RH settings in the CEC, denoted by different symbols. (**A**) Without desiccants and AF, the RH increased dramatically and predictably, independent of the starting value in the room where the CEC was placed. (**B**) The use of dehumidified AF (15 L/min) in the chamber decreased the starting RHs in a well-controlled manner. Temperature: 21°C to 23°C.

0.3 mm ($P < 0.005$, $n = 5$) on day 14, 1.9 ± 0.3 and 1.3 ± 0.4 ($P < 0.05$, $n = 5$) on day 28. In the study group, significant differences in tear secretion were shown at each time point when compared with baseline. A similar pattern of tear secretion suppression (though less significant) was seen after the cotton thread test was performed for 60 seconds without removing the excess tears. In particular, cotton thread wetting was reduced by 31% at day 3 ($P < 0.05$) and 25% at day 7 ($P = 0.15$).

Corneal Fluorescein Staining

Minimal punctuate corneal fluorescein staining was recorded at baseline (day 0) in both the control and the study groups, without significant differences between the two groups (Fig. 4). There was a statistically significant increase in staining in the CEC-kept mice at day 3 (5.8 ± 2.2, $P < 0.0001$, $n = 10$), day 7 (5.5 ± 2.6, $P < 0.0001$, $n = 10$), day 14 (3.3 ± 1, $P < 0.05$, $n = 5$), and day 28 (4.6 ± 2.3, $P < 0.05$, $n = 5$), compared with control mice in standard cages in which corneal staining averaged 1.2 ± 1 throughout the course of the studies (Fig. 4). At all time points, the corneal staining of the
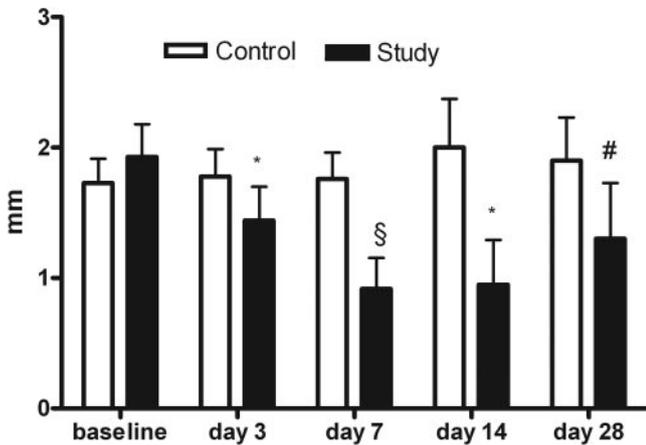
FIGURE 3. Tear production in the study and control groups at different time points. Statistically significant differences were found in the study group at days 3, 7, 14, and 28 when compared with the control group (*t*-test). Data show mean ± SD (error bars). *$P < 0.005$, §$P < 0.0001$, #$P < 0.05$.

study group was significantly higher than at baseline. The corneal staining was significantly higher in the central and nasal area when compared with the other areas in which the corneal surface had been divided.[2]

Conjunctival Epithelial Morphology

In the control group, the conjunctiva had a cuboidal basal layer with three to four layers of epithelial cells flattened toward the free surface and not cornified (Fig. 5A). In the CEC-exposed mice, the conjunctival epithelial morphology showed four or more epithelial layers with a marked thickening of the outer layer on days 3, 7, and 14 (Figs. 5B–D). On day 28, the morphology of the conjunctiva showed limited areas of thickening (Fig. 5E).

Goblet Cell Density

The number of goblet cells in the superior conjunctiva in the CEC exposed mice was significantly lower than in control mice (47 ± 7, $n = 8$) at day 7 (31 ± 11, $n = 8$, $P < 0.05$), whereas it did not show statistically significant differences at day 3 (33 ± 16, $n = 5$), day 14 (38 ± 3, $n = 4$), and day 28 (39 ± 3, $n = 4$; Fig. 6A). In the inferior conjunctiva, goblet cells density was significantly lower than in the control at all time points studied (Fig. 6B, compare Fig. 5A with Figs. 5B–D), except on day 28 (Fig. 5E). No significant differences were recorded between days 3, 7, and 14.

DISCUSSION

The control of environmental conditions and the study of their influence on the ocular surface is a critical element for a better understanding of the pathophysiology of dry eye. In this study, we showed the efficacy of our CEC to control humidity, AF, and temperature to which mice were exposed. Furthermore, we demonstrated that in normal mice a low-humidity and high-AF environment can induce ocular surface signs of dry eye, as reflected by significant reductions in conjunctival goblet cell density, and lacrimal secretion, as reflected by cotton thread wetting and an increase in corneal fluorescein staining.

The existing animal models of dry eye mimic different pathogenic mechanisms of KCS, but, at the moment, only one of them incorporates environmental factors.[11] In 2002, Dursun

et al.[12] made a significant contribution to the field by reporting on a mouse model of KCS in which wild-type mice were exposed to an AF. They found that exposure alone to air from a fan for 1 hour, three times per day for four consecutive days did not induce signs of KCS, but did cause worsening of ocular surface signs when used in conjunction with systemically administered scopolamine (anti-cholinergic). We too can replicate dry eye in scopolamine-treated mice (data not shown), but do not find its administration to be absolutely necessary to induce ocular surface changes in mice exposed to the CEC. Perhaps, the difference between our observations and those of Dursun et al. can be explained by their limited control of environmental conditions in the cage and exposure to an AF limited to a short period. Data on the temperature and humidity in the cages where the mice were kept were not provided. In our CEC we were able to create a low-humidity setting and to monitor, maintain, and reproduce it in each experimental setting. Moreover, we were able to control the AF and temperature to have complete control of the environmental conditions to which the mice were exposed. We tested the cage before the use of animals and found that the control of the environment in the CEC was independent of the humidity of the room in which the CEC was placed. This feature of the CEC makes it suitable for use in any animal facility, reducing the costs for control of the environment in the room where the CEC is placed. In addition, we monitored the behavior of the animals in the CEC, because stress reactions have been noted in mice exposed to a fan (Barabino S, unpublished data, 2003); however, none was noted in this case when dry air was introduced by means of a low-noise air pump.

The cotton thread test has been suggested as a useful and valid technique for measuring tear production in mice.[13] We performed the test for 30 seconds after removal of excess tears, and for 60 seconds without any manipulation, to measure the total tear secretion over a longer period. This test was performed under a magnifier lamp to avoid touching the ocular surface—contact that can itself increase reflex tearing. In addition, the reading of cotton thread wetting was performed under a microscope for accuracy. In our opinion, it is particularly important to adopt strict standards in the conduct of such tests, as is done in humans, to maximize reproducibility and minimize confounding.[13]
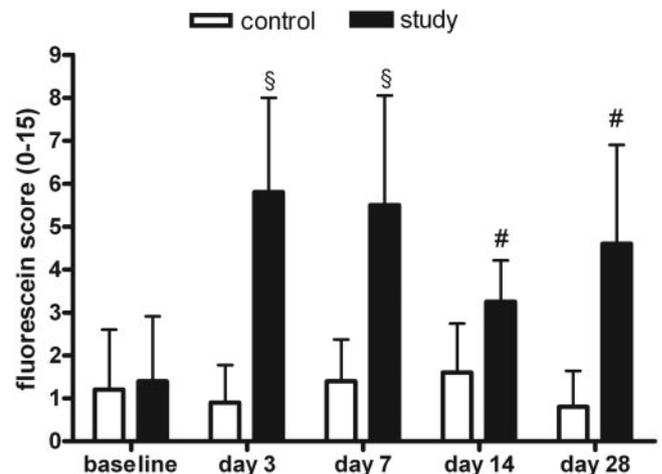

FIGURE 4. Corneal fluorescein staining. Statistically significant differences were found in the study group at days 3, 7, 14, and 28, when compared with the control group (Mann-Whitney test). Data show the mean and SD (error bars). §$P < 0.0001$, #$P < 0.05$.
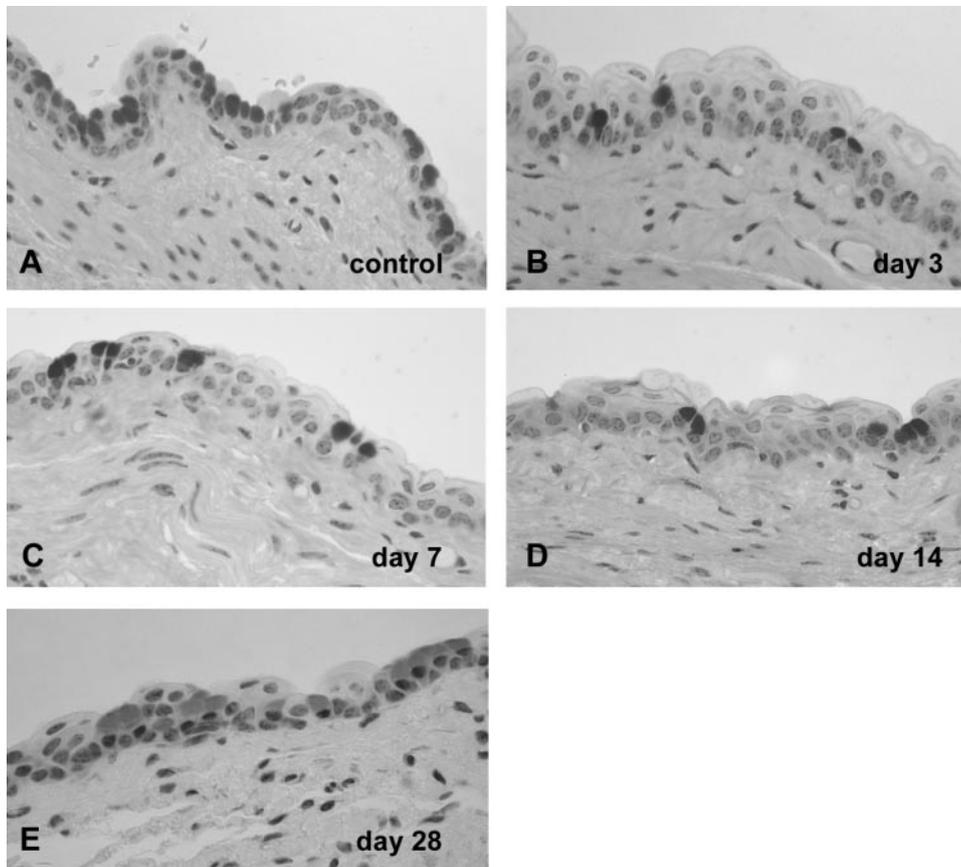
FIGURE 5. Histologic sections of the inferior tarsal conjunctiva of BALB/c mice stained with PAS. (A) Control mouse. The conjunctiva is composed of three layers of epithelial cells and contains numerous goblet cells. After exposure to the CEC for 3 (B), 7 (C), or 14 (D) days, the conjunctival epithelial morphology showed a marked thickening of the outer layer and reduced goblet cell numbers. (E) On day 28, the morphology of the conjunctiva showed limited area of thickening and a relative increase in the number of mucin-containing cells. Magnification, ×40.

The classic paradigm of experimental and clinical dry eye is primarily based on the effect of decreased lacrimal secretion on the ocular surface. In a striking finding in our study, the ocular surface damage related to a low-humidity and high-AF setting could itself precipitate alterations in tear volume and secretion. Similar apparently paradoxical (for one may expect an increased tearing response in desiccated eyes) results have been demonstrated in humans exposed to a dry climate.[10] The exact mechanisms by which these ocular surface changes affect lacrimal function remain unknown; however, it is possible that the continuous AF and low humidity that increase evaporative tear loss from the ocular surface may induce a stress response that in turn suppresses lacrimal gland function. This could explain the symptoms and signs of dry eye in employees in buildings with a high ventilation flow and low humidity[14] and in commercial airline crews exposed to pressurized cabins.[15] Indeed, alterations in tear secretion in association with ocular surface dryness further confirms the functional relevance and interdependence of the lacrimal gland–ocular surface functional unit.[3]

Precisely evaluating the condition of the ocular surface is a critical aspect of verifying signs of KCS in humans and animals alike. Use of fluorescein represents an important diagnostic tool in our hands to study the corneal epithelium. The use of a determined quantity and concentration of fluorescein, and its delivery by means of a micropipette, allowing for a 3-minute time interval between dye instillation and observation, and use of a standardized grading scheme specific for the cornea have been adopted by us to assess ocular surface epitheliopathy in mice. As in nearly all forms of significant human KCS,[4] mice exposed to the dry, high-flow environment of the CEC also showed punctuate epitheliopathy. Fluorescein staining has been described to be due to disruption of epithelial cell–cell junctions,[16] and/or damaged corneal epithelial cells.[17] The decrease of corneal staining observed after 14 and 28 days compared with 3 and 7 days of exposure to the controlled environment chamber could be related to the increasing number of goblet cells in the superior conjunctiva at day 14 and in the inferior conjunctiva at day 28.

Goblet cell density is a critical parameter that reflects the overall health of the ocular surface.[18] These cells synthesize, store, and secrete large gel-forming mucins that lubricate and protect the ocular surface.[19] Previous studies have demonstrated that decreased numbers of mucin-containing goblet cells is characteristic of human dry eye.[20] In our study, we have likewise shown a decrease in goblet cell density in the conjunctiva, similar to results of other mouse models of dry eye.[12,21] Furthermore, the conjunctival epithelium of CEC-exposed mice was observed to be thicker than in the control group, similar to that shown by Dursun et al.[12] in a mouse model of dry eye, and in patients with non-Sjögren's dry eye.[22] Exposure of mice to dry environmental conditions also produces an increase in the thickness of the epidermis.[23] The increase in goblet cell density and the relative decrease in conjunctival thickening, after day 14 raise important questions regarding how conjunctival cells compensate and/or proliferate in response to exposure to a dry environment. Our CEC provides an excellent tool to study goblet cell proliferation in both normal and other mouse models (pharmacologically and genetically modified) in response to a controlled stressful environment.

In conclusion, we emphasize that adoption of the mouse model has been invaluable in better understanding of the pathophysiology of myriad pathologic ocular and nonocular conditions. However, as we have recently reviewed,[11] the current murine models of dry eye have significant limitations
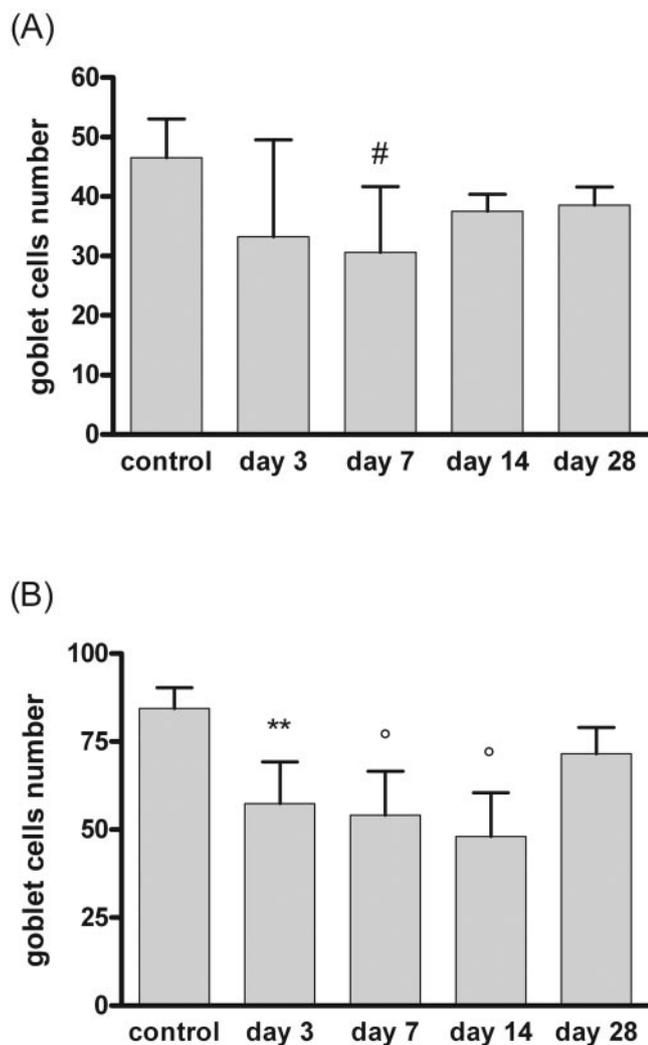
FIGURE 6. (A) Superior conjunctival goblet cell density. Statistically significant differences were found in the study group at day 7 ($n = 8$) when compared with the control group ($n = 8$; Bonferroni test). (B) Inferior conjunctival goblet cell density. Statistically significant differences were found in the study group at day 3 ($n = 5$), day 7 ($n = 8$), and day 14 ($n = 4$) when compared with the control group ($n = 8$; Bonferroni test). Data show mean $\pm$ SD (error bars). [#]$P < 0.05$, [**]$P < 0.01$, [°]$P < 0.001$.

due to the disconnect that exists in many between lacrimal and ocular surface signs. The murine CEC described herein represents a new tool to control and manipulate the environmental factors that can be responsible for changes in the lacrimal gland–ocular surface functional unit, as we have demonstrated. Given the wide array of murine reagents for study of pathophysiologic mechanisms and the availability of inbred strains with defined gene knockout and of transgenic strains, this model can play an important role in further studies regarding the physiology and pathobiology of the ocular surface in dry eye.

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