A Murine Model of Dry Eye Induced by an Intelligently Controlled Environmental System

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PURPOSE. To establish a novel murine model of dry eye using an intelligently controlled environmental system (ICES).

METHODS. Thirty BALB/c mice aged 4 to 6 weeks were housed in the ICES in which the relative humidity, airflow, and temperature were maintained at 15.3% ± 3% (mean ± SD), 2.1 ± 0.2 m/s, and 21° ± 2°C, respectively, for 42 days. Thirty mice of similar age and housed in a normal environment were controls (relative humidity, 60%–80%; no airflow; temperature, 21°–23°C). The ocular surfaces of the animals in both groups were analyzed before and 3, 7, 14, 28, and 42 days after the experiment for aqueous tear production, corneal barrier function, conjunctival morphology, and goblet cell density. The level of apoptosis on the ocular surface also was assessed using active caspase-3 at 42 days.

RESULTS. A low-humidity environment was maintained constantly by the ICES. Animals in this environment had decreased aqueous tear production, increased corneal fluorescein staining, and marked thinning and accelerated desquamation of the apical corneal epithelium compared with control eyes. Squamous metaplasia of the conjunctival epithelium with decreased goblet cell density also developed in the animals housed in the ICES. Active caspase-3 was highly expressed on the ocular surfaces of the animals housed in the ICES at 42 days.

CONCLUSIONS. The biological and morphologic changes of dry eye induced by ICES in mice are similar to those in humans. This dry eye environment appears to upregulate apoptosis on the ocular surface. (Invest Ophthalmol Vis Sci. 2008;49: 1386–1391) DOI:10.1167/iovs.07-0744

Dry eye is a condition characterized by changes in quality, quantity, or both of the tear film. These changes may cause dysfunction of the cornea and the associated structures such as the conjunctiva, lacrimal and meibomian glands, and ocular innervation.1,2 The typical clinical manifestations of dry eye include ocular irritation, blurred vision, and recurrent corneal infection and ulceration.3

The mechanisms associated with tear film instability are multifactorial and mainly include immune dysfunction, nervous disorders, and environmental changes.4 A variety of dry eye models have been developed over the past decades to induce abnormal tear dynamics by mimicking these factors. These models are experimental immune dacryoadenitis4,5 pharmacologic blockade of cholinergic muscarinic receptors6,7 and mechanical control of tear secretion8 and evaporation.9,10 However, most models cause not only abnormal changes in the tear film but also other complications that do not result from tear film abnormality. These complications affect an understanding of the relationship between the instability of tear film and the formation of dry eye. Therefore, an ideal dry eye model should minimize changes in areas other than the ocular surface.

The ocular surface is constantly exposed to environmental challenges such as wind and low humidity that can compromise the stability of the tear film.11 An environment that causes dehydration, such as that in air conditioning, can accelerate evaporation of the tear fluids from the ocular surface, resulting in dry eye changes. It is estimated that 35% to 48% of persons who work in an environment with low humidity experience dry eye symptoms,12,13 that is, ocular irritation, ocular surface desiccation, and sick building or office eye syndrome. Based on results from these environmental factors, Barabino et al.14 in 2005 developed a dry eye model under low humidity using a controlled environment chamber (CEC). This model appears to reflect clinical conditions in patients with dry eye and provides a better approach to the study of mechanisms of dry eye disorders compared with previous models.4–10 However, this model has not been widely used because of its lack of simultaneous control of different environmental factors, relatively low efficiency, and unstable humidity (subject to impact by the external environment). These insufficiencies could compromise the consistency of the results in experimental animals.

The present study established an intelligently controlled environmental system to induce a murine dry eye model. This system provided an indoor environment with constant low humidity and high air velocity. The feasibility of the intelligently controlled environmental system (ICES) for inducing functional and pathologic changes in the animal eyes also was evaluated.

METHODS

Animals

This animal experiment was approved by the Animal Care and Ethics Committee of Wenzhou Medical College, Wenzhou, China, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Sixty female BALB/c mice (age range, 4–6 weeks) were supplied by the Animal Breeding Unit of Wenzhou Medical College.

ICES

The ICES included one cabinet (100 × 50 × 100 cm), an adjustable temperature-dehumidifier (ZD-890c; Ouyi Company, Hangzhou, China), an intelligent dehumidifying device (MS, 13X; Hengye Company, Shanghai, China), and a noise-free ventilator (LFA-40,529; Laxin Company, Wenzhou, China).
The mice were housed in 12 cages (five animals per cage) in normal environmental conditions (relative humidity, 60%–80%; no airflow; temperature, 21°C–23°C) for at least 5 days. Six cages (n = 30) were randomly assigned to be the experimental group, and the remaining six cages (n = 30) served as controls. Animals in the experimental group were housed in two sealed cabinets (three cages in each cabinet) in a 20-m³ room. The relative humidity in the cabinet was reduced to 40% ± 5% using the temperature dehumidifier, which was located next to the cabinet in the room, followed by a further reduction to 15% ± 5% using the intelligent dehumidifying device where 2 kg molecular sieves (MS, 13X; Hengye Company) acted as a desiccant. The air flow and temperature in the cabinet were maintained at 2.1 ± 0.2 m/s by the ventilator located 20 cm from the mouse cages in the cabinet. The temperature in the cabinet was maintained at 21°C to 23°C by the temperature dehumidifier. Relative humidity and airflow were determined based on relative humidity where 15% ± 3% was the lower limit of ambient humidity controlled by the ICES for a prolonged period and on airflow where 2.1 ± 0.2 m/s was the speed beyond which the mouse eyes could not naturally open.

An anemometer (AR816; Huxin Company, Shanghai, China) with a measurable range of 0 to 30 m/s and an accuracy of ±5% was used to monitor the airflow in the cabinet. The humidity and temperature in the cabinet were monitored and digitally displayed by a control system in the dehumidifying device. The mechanism of this control system was as follows. The intelligent alloy spring contracted when the dehumidifying device absorbed the moisture in the cabinet. This resulted in opening of the channel medial to the cabinet and closing of the channel lateral to the cabinet. Moist gas from the cabinet flowed into the lower channel of the dehumidifying device, was dehydrated by the molecular sieves, and was eliminated from the upper channel. This cycling of gas was maintained until the humidity inside the cabinet reached homeostasis (Fig. 1A). The control system was automatically switched to warming and demisturizing as long as the working status of the molecular sieves was saturated. The intelligent alloy spring then stretched; this action was followed by simultaneous closing of the medial channel and opening of the lateral channel. Cool air was absorbed by the molecular sieves after it entered the lower channel of the dehumidifying device. The air was heated and finally evaporated from the upper channel (Fig. 1B).

Animals in the control group were housed in cages under normal environmental conditions (relative humidity, 60%–80%; no airflow; temperature, 21°C–23°C). Both groups of animals were evaluated on 0, 3, 7, 14, 28, and 42 days for the following parameters, with the exception that apoptosis was assessed only at day 42.

**Measurement of Aqueous Tear Production**

Tear production was measured in 10 eyes using the phenol red thread test (Zone-Quick; Menicon, Nagoya, Japan). The threads were held using one pair of forceps and were applied to the lateral canthus of the conjunctival fornix of the right eye for 30 seconds under slit-lamp biomicroscopy. The length of the wet cotton thread was measured using a millimeter scale.

**Corneal Fluorescein Staining**

Corneal fluorescein staining was evaluated in 10 eyes by instilling 0.5 μL of 5% fluorescein solution into the inferior conjunctival sac using a micropipette. The cornea was examined using slit-lamp microscopy in cobalt blue light 5 minutes after fluorescein instillation. The stained area was assessed according to a standardized grading system ranging from 0 to 3, with the corneal surface divided into five regions.15 Then the total score from the five regions was analyzed.

**Histologic Analysis**

The animals were humanely killed with an overdose of a mixture of ketamine and xylazine; five eyes were submitted for histologic assessment. Superior conjunctivas were marked with black ink after the animals were killed. Each entire eye, including the lids, was fixed in 10% formalin. After dehydration, the specimens were embedded in paraffin, cross-sectioned, and stained with hematoxylin-eosin or periodic acid-Schiff reagent. The morphology of the corneal and conjunctival epithelium and the number of goblet cells in the superior and inferior conjunctiva were assessed under a microscope (Imager.Z1; Carl Zeiss Meditec, Oberkochen, Germany) by two independent masked observers. The thickness of the corneal epithelium was measured with an image analyzing system (Axiovision; Carl Zeiss Microimaging GmbH, Oberkochen, Germany). To reduce test variability, the mean of three measurements was used for analysis.

**Scanning Electron Microscopy**

After the animals were killed, five corneas were dissected and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer. After they were dehydrated in a graded series of ethanol, the specimens were critical-point dried in a Polaron critical-point dryer and mounted on 10-mm aluminum stubs with double-sided tape. The corneal surface was examined by scanning electron microscopy (SEM; S-3000N; Hitachi, Tokyo, Japan) with an accelerating voltage of 3 kV.

**Evaluation of Apoptosis**

Immunohistochemistry was used to study apoptosis on the ocular surface (n = 5 eyes). After three washes in phosphate-buffered saline (PBS; pH 7.2), the tissue samples were incubated with 5 μg/mL polyclonal rabbit anti-active caspase-3 primary antibody (PharMingen, San Diego, CA) or PBS as a negative control for primary antibody at 4°C overnight. The samples were blocked with 10% goat serum for 30 minutes and incubated with goat anti-rabbit conjugated antibody (PharMingen) for 45 minutes at room temperature, followed by three washes in PBS. Apoptotic cells in different regions of the ocular surface were assessed by epifluorescence microscopy (Imager.Z1). Photographs at 200× magnification were taken of the cornea and conjunctiva. All active caspase-3–positive cells were counted in the conjunctiva over an area covering 100 μm (length) × 100 μm (width) in sagittal sections. The same method was used in the cornea over an area measuring 100 μm × 20 μm in sagittal sections.

**Statistical Analysis**

The Mann-Whitney U test was used to compare the differences in fluorescein staining scores between the experimental and control groups. Intragroup changes between different time points were analyzed using the Wilcoxon test. The unpaired sample t test was used to...
compare tear production, apoptosis, corneal thickness, and goblet cell density between the groups.

RESULTS

Environmental Conditions under ICES

Relative humidity in the cabinet remained at 15% ± 3% under the ICES during the experimental period. However, the relative humidity could increase to 283% when the cabinet was opened for approximately 5 minutes daily to provide fresh food and water for the animals. This relative humidity returned to 15% ± 3% within 2 hours because of the feedback regulation by the dehumidifying device of the ICES. Intrinsic cycling at airflow of 2.1 ± 0.2 m/s created by the ventilator accelerated the development of dry eye but did not affect the relative humidity level in the cabinet. Temperatures in the cabinet and room were at the same level (21°–23°C) during the experimental period.

Animal Behavior under ICES

Animals in the experimental and control groups did not exhibit abnormal behavior such as aggression, hair loss, body injury, or frequent eye rubbing during the experimental period. Body weights and amounts of food and water consumed by the animals in the two groups were similar at each time point.

Aqueous Tear Production

Phenol thread wetting showed 2.1 ± 0.3 mm versus 2.1 ± 0.2 mm at day 0, 2.2 ± 0.2 mm versus 2.2 ± 0.2 mm at day 3, and 2.2 ± 0.2 mm versus 2.1 ± 0.1 mm at day 7 (control vs. experimental, P > 0.05 days 0–7; Fig. 2). Results from the same measurement were 2.1 ± 0.2 mm versus 1.8 ± 0.2 mm at day 14, 2.2 ± 0.3 mm versus 1.6 ± 0.2 mm at day 28, and 2.2 ± 0.3 mm versus 1.3 ± 0.3 mm at day 42 (control vs. experimental, P < 0.01 days 14–42).

Corneal Fluorescein Staining

Fluorescein staining showed minimal punctate on the corneal surface in the experimental and control groups from day 0 to day 7. Staining was graded as 1.7 ± 0.3 versus 1.8 ± 0.2 at day 0, 1.6 ± 0.2 versus 1.7 ± 0.3 at day 3, and 1.8 ± 0.4 versus 1.6 ± 0.3 at day 7 (control vs. experimental, P > 0.05 days 0–7; Fig. 3A). However, the difference in corneal staining between experimental and control mice was 8.8 ± 2.2 versus 1.7 ± 0.2 at day 14, 10.2 ± 2.3 versus 1.8 ± 0.2 at day 28, and 12.2 ± 2.6 versus 1.7 ± 0.3 at day 42 (P < 0.01 days 14–42; Figs. 3B–D).

Morphology of the Corneal Epithelium

The corneal epithelium in the control mice was well stratified with four to five cell layers. The basal layer was lined with cuboidal cells. These cells flattened when differentiated toward the corneal surface (Fig. 4A). In contrast, the corneal epithelium in the experimental mice was less homogeneous in cell size, cell staining, and stratification after day 14 (Figs. 4B–D). Epithelial thickness did not differ significantly between the two groups (P > 0.05) from day 0 to day 7 (21.89 ± 2.16 μm vs. 20.56 ± 1.84 μm at day 0, 20.12 ± 1.86 μm vs. 21.56 ± 3.12 μm at day 3, 22.32 ± 2.59 μm vs. 20.85 ± 1.69 μm at day 7; control vs. experimental). However, the corneal epithelium in the experimental mice was significantly thinner than in the control mice since day 14 (from 17.17 ± 1.40 μm vs. 20.89 ± 2.67 μm at day 14 to 13.43 ± 1.63 μm vs. 21.86 ± 2.78 μm at day 42; P < 0.05).

Morphology of the Conjunctival Epithelium

The fornix conjunctiva in the control mice consisted of multi-layers of epithelial cells that were cuboidal and columnar with homogeneous staining (days 0–42). Goblet cells were distributed mainly among the fornix epithelial cells (Fig. 5A). In contrast, the fornix conjunctival epithelium in the experimental mice was less homogeneous in cell size and cell staining with the loss of goblet cells since day 14 (Figs. 5B–D). Furthermore, squamous metaplasia appeared to develop in the conjunctiva, and the conjunctiva lost stratification and became irregular in thickness from day 14 to day 42.

The number of goblet cells from the superior and inferior conjunctiva did not differ significantly between the two groups from day 0 to day 7 (superior: 28 ± 3 vs. 30 ± 4 at day 0, 26 ± 4 vs. 34 ± 7 at day 3, 32 ± 6 vs. 30 ± 5 at day 7; inferior: 28 ± 5 vs. 33 ± 4 at day 0, 29 ± 6 vs. 34 ± 6 at day 3, 31 ± 5 vs. 28 ± 6 at day 7; control vs. experimental; P > 0.05 each region). However, this number in the experimental mice was significantly lower than in control mice at days 14, 28, and 42 (Table 1).

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933446/ on 04/29/2017)

**Figure 2.** Tear production measured using the phenol red thread test. Tear secretion decreased significantly at days 14, 28, and 42 in the experimental group compared with the control group (*P < 0.01, **P < 0.001; unpaired two-sample t-test; n = 10 eyes).
FIGURE 4. The corneal epithelium in the control mice is well stratified and differentiated, with four to five cell layers at all time points examined (A). In contrast, the epithelium in the experimental mice is thinner and less homogeneous in cell size, cell staining, and stratification at days 14 (B), 28 (C), and 42 (D).

SEM Analysis of the Corneal Epithelium

Dark and bright superficial cells were detected in the control corneas (Fig. 6A). Dark cells had smooth surfaces, and bright cells were covered by high-density microvilli and microplicae. There was no difference in cell morphology between the two groups from day 0 to day 7. The number of ruffled and desquamated cells in the experimental mice was higher than in the control mice, and most superficial cells were dark in the experimental mice from day 14 onward (Figs. 6B–D). Intact tight junctions were seen between the superficial cells in the control mice at all time points (Fig. 6E). However, these structures were disrupted in the experimental mice at day 14. SEM showed that the number of desquamated superficial cells in the experimental mice increased within 14 days compared with the number in control mice (Figs. 6B–D). In the experimental mice, loss of epithelial microvilli with disrupted tight junctions was seen at day 14 (Fig. 6F), and most superficial cells were lost by day 28.

Evaluation of Apoptosis

Apoptosis in the control mice was minimal on the ocular surface (cornea and conjunctiva) at day 42. In the experimental mice, the number of active caspase-3–positive cells significantly increased compared with the control mice (Fig. 7). Mean numbers of active caspase-3–positive cells between the experimental and control groups, respectively, were 30 ± 9 versus 5 ± 2 (P < 0.01) for the corneal epithelium and 59 ± 9 versus 4 ± 3 (P < 0.01) for the conjunctival epithelium. Staining was not assessed in the negative controls, in which only the secondary antibody was used.

DISCUSSION

We studied a low humidity-induced dry eye model using the ICES, which was developed from the CEC reported by Barabino et al.14 based on evidence that the rate of tear film evaporation from the ocular surface depends mainly on environmental temperature, humidity, and airflow.15 The low humidity in the CEC is created by a water separator and desiccants (CaSO4 and silica gel), whereas the high airflow is produced by an air compression pump. The CEC has several technical limitations in the induction of dry eye conditions. The water separator, CaSO4, and silica gel in the CEC are of low efficiency to produce a low-humidity environment, and neither CaSO4 nor silica gel can be recycled after use. Furthermore, the low capacity of the desiccant system limits the size of the controlled chamber (a usable floor area restricted to only 725 cm2), making a study with a large sample size of animals impossible. Airflow from the extrinsic cycling cannot regulate the humidity and temperature simultaneously. The humidity especially is easily affected by the external environment. For example, low airflow (≤15 L/min) from the extrinsic cycling cannot efficiently accelerate tear evaporation because increasing airflow will change the humidity level in the CEC.

The ICES in this study overcomes most insufficiencies from the CEC. Molecular sieves are used in the ICES to control environmental humidity, which can selectively absorb water and carbon dioxide on the surfaces of the crystals by a physically attractive force. Because the dehumidifying capacity of the ICES depends on the internal surface of the molecular sieve, the 2-kg molecular sieves used in this system provide an internal surface of 2000 g × 750 m2. Therefore, the dehumidifying system in the ICES is much more efficient than in the CEC. Further, the cabinet humidity is unaffected by the airflow speed from the intrinsic cycling. Therefore, the ICES can maintain the three environmental settings at an expected level simultaneously. The design and mechanisms allow a large sample size of animals used in an experimental setting, precise control environmental factors, and therefore increased consistency of results.

The mice experienced significant declines in tear production and significant increases in corneal epithelial defects within 14 days of entering the ICES compared with the control group. Tear and epithelial deficiencies on the corneal surface progressed throughout the experimental period. The time course for the occurrence of histologic and clinical abnormalities was similar (7–14 days), indicating that clinical assessment is sufficiently sensitive for detecting subtle changes at the...
The corneal epithelium in the experimental mice gradually thinned, with loss of normal stratification (Figs. 4B–D) and increased numbers of desquamated superficial cells (Figs. 6B–D) within 14 days of entering the ICES. Loss of epithelial microvilli with disrupted tight junctions was found at day 14, and most of the superficial cells were lost by day 28. The conjunctival epithelium in the experimental mice underwent hyperproliferation, as evidenced by the obvious development of squamous metaplasia after day 28 (Figs. 5C, 5D). Conjunctival squamous metaplasia is a histologic feature in a variety of dry eye diseases in humans and is likely related to the epithelial response to chronic stimuli, though the exact mechanism remains unknown. The number of goblet cells in the experimental mice rapidly decreased after day 7 of the experiment (Table 1), indicating that the integrity of the tear film was compromised.

In the present study, the changes in tear production and the corneal superficial cells at all time points in the control mice are available. Those in the of Barabino et al., who used the CEC. However, evidence related to the compromised tear film in the present study appears to be more consistent than that from the CEC. For instance, the present study showed progressive reduction in the goblet cell population over time during the experiment. However, this population fluctuated across different time points, with no substantial change at most time points in the CEC. These factors suggest that the constant environmental factors in the ICES contributed to the consistent results in the present study. Furthermore, squamous metaplasia found in the eyes of animals in the CEC was more obvious than that in the eyes of the animals in the CEC, indicating that the dry eye model induced by the ICES is closer to the phenotype of human dry eye.

Levels of apoptosis in the cornea and conjunctiva of the experimental mice were significantly higher than in the control mice at day 42. Upregulated apoptosis also was found on the ocular surface cells of other dry eye models, although the exact mechanisms are not fully known. Apoptosis may be a biological response to abnormal proliferation on the conjunctiva, such as squamous metaplasia, to inhibit excessive growth of the ocular surface cells. Therefore, further understanding of the apoptotic pathways in dry eye may provide a new therapy to control the development of dry eye disease.

The etiology of dry eye is multifactorial. A low-humidity environment is only one of many factors that cause dry eye. Therefore, mechanisms involved in the development of dry eye in the present study are not necessarily the same as other dry eye models, such as with immunologic, pharmacologic, or surgical induction of dry eye conditions. However, the tear dynamics and the morphologic and biological changes induced by the ICES share some features with other dry eye models, although the exact mechanisms are not fully known. Apoptosis may be a biological response to abnormal proliferation on the conjunctiva.

Table 1. Number of Goblet Cells in the Superior and Inferior Conjunctiva in Experimental Mice Compared with Control Mice at Days 14, 28, and 42

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Data are expressed as the mean ± SD (n = 5 eyes; unpaired two-sample t-test).

Figure 6. SEM shows high-density microvilli and microplicae over the corneal superficial cells at all time points in the control mice (A). The number of ruffled and desquamated cells in the experimental mice increases with time in the experimental mice. (B) Day 14. (C) Day 28. (D) Day 42. The tight junctions between the superficial cells are intact in the control mice (E). However, there is loss of epithelial microvilli with disrupted tight junctions at day 14 (F) in the experimental mice. Scale bars: (A–D) 10 μm; (E, F) 1 μm.
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References

FIGURE 7. There is minimal apoptosis on the ocular surface (cornea and conjunctiva) at day 42 in the control mice. The number of active caspase-3–positive cells in the experimental mice has significantly increased compared with that in the control mice. (A) Cornea. (B) Conjunctiva.

Murine Model of Dry Eye