Dry eye disease (DED) is a highly prevalent ocular surface disease, estimated to affect about 10% of adults globally. DED has been shown to involve chronic ocular surface inflammation. Common symptoms include sensations of ocular grittiness, conjunctival redness, blurred vision, and eye pain.

Tear hyperosmolality is common to all DED subtypes and severities, with a tear osmolarity ≥316 mOsm/L being a sensitive and specific clinical diagnostic marker for DED. Clinically, chronic tear hyperosmolality has been linked to reduced tear stability and increased tear levels of the pro-inflammatory cytokine interferon-gamma. In mice, recurrent topical application of hyperosmolar solutions (i.e., 3000 mOsm/L) is pro-inflammatory to the mouse conjunctiva, increasing epithelial expression of nuclear factor kappa-B, and priming T cells to promote the development of aqueous-deficient DED. Acute (<10 minute) exposure to mild hyperosmolar stress (<500 mOsm/L) is sufficient to trigger DED-like toxicity and release of pro-inflammatory interleukin-6 in in vitro assays of human corneal epithelial cells. Thus, although mild, acute hyperosmolar stress can mimic the osmotic insult of DED to the corneal epithelium in an acute experimental setting, it is unknown whether this tear film perturbation can also modulate resident corneal epithelial immune cells in vivo. To address this, we evaluated the response of corneal epithelial dendritic cells (DCs) to topical mild hyperosmolar stimuli.

Anatomically, corneal DCs are optimally located to respond to tear hyperosmolality; they are the predominant immune cell population in the epithelium and have projections that extend up to the tear-epithelial interface while interacting with osmosensory nerves. Epithelial DCs are also recognized as a clinical marker of corneal inflammation. Using in vivo confocal microscopy (IVCM), corneal epithelial DCs have been observed to increase in density and change morphology in clinical and preclinical conditions with corneal inflammation. Changes in both DC density and morphology have been observed in...
patients with DED. However, the specific contribution of hyperosmolar stress to these changes has not been reported.

The aim of the present study was to examine whether corneal epithelial DCs alter in density, morphology, and/or phenotype in response to acute topically hyperosmolarity in vivo. Using immunofluorescent microscopy and flow cytometry, we observed early morphological and phenotypic responses to mild and severe hyperosmolar stress. Our findings support a role for tear hyperosmolarity in modulating corneal immune cell responses.

**MATERIALS AND METHODS**

**Animals**

Female C57BL/6j mice (8–12 weeks old), purchased from the Animal Resources Centre (Canning Vale, WA, Australia), were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures received ethics approval from the Florey Institute of Neuroscience and Mental Health animal ethics committee.

**Formulation of Saline Solutions**

The iso-osmolar (350 mOsm/L) and hyper-osmolar (450 and 600 mOsm/L) saline solutions were formulated by diluting sodium chloride solution (HyperSAL 6% hypertonic saline solution; Briemar Nominees Pty Ltd; Royal Children’s Hospital Pharmacy, VIC, Australia) in sterile saline (Pfizer Sodium Chloride 0.9% for Injection BP; Pfizer Australia, NSW, Australia). Osmolarity was confirmed prior to topical application based on the average at least two measurements using a freezing point osmometer (Osmette III; Precision Systems, Natick, MA, USA), at room temperature.

**Topical Application of Solutions in Anesthetized Mice**

C57BL/6j mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) and placed in cages, warmed by a heat pad. In experiment A, animals received an 8 μL topical drop of 450 (n = 6 mice) or 600 mOsm/L (n = 6) hyperosmolar saline on a randomly assigned eye. The contralateral eyes, and both eyes in the control group (n = 3), received 350 mOsm/L saline topical drops, as an iso-osmotic stimulus based on previous tear osmolarity measurements in healthy mouse populations.28,29 To assess DC activation, tissues were incubated in primary antibodies against CD45 (1:500; rat anti-mouse CD45; BD Biosciences, Franklin Lakes, NJ, USA; Cat #550539).

**Corneal Immunofluorescent Staining**

Mouse eyes were fixed in 4% paraformaldehyde (PFA) for 2 hours at 4°C, after which corneas were dissected and processed for whole-mount immunostaining. Corneas were incubated in 20 mM EDTA at 37°C for 1 hour, then blocked in 1× PBS containing 3% BSA and 0.3% Triton X-100. For morphological analyses, tissues were incubated in primary antibodies against CD45 (1:500; rat anti-mouse CD45; BD Biosciences, Franklin Lakes, NJ, USA; Cat #550539). To assess DC activation, tissues were incubated in primary antibodies against CD86 (1:300; rat anti-mouse CD86; Invitrogen, Eugene, OR, USA; Cat #14-0862-81) or CD68 (1:300; rat anti-mouse CD68; AbD Serotec, Raleigh, NC, USA; Cat #MCA1957), and counterstained with Iba1 (1:300; rabbit anti-mouse Iba1; FUJIFILM Wako, Osaka, Japan; Cat #019-19741) as an equivalent60 marker to CD45 to identify DCs in the corneal epithelium that is raised in a different species to the rat CD86 and CD68 antibodies. All primary antibodies were mixed in a formulation of 50% 1× PBS + 0.1% Triton X-100 and 20% 1× PBS + 3% BSA + 0.3% TritonX-100, and incubated overnight at 4°C. The samples were then incubated in PBS containing goat anti-rat Alexa Fluor 488 (1:500; Invitrogen; Cat #A11006) and goat anti-rabbit 647 (1:500; Invitrogen; Cat #A21244) secondary antibodies along with Hoechst (1:1000; Hoechst; Sigma-Aldrich, St. Louis, MO, USA; Cat #14553) for 2 hours at room temperature, then cover-slipped using low fade mounting medium.

**Image Acquisition**

Corneas stained with CD45 were imaged on the Leica SP8 Confocal Inverted Laser Scanning Microscope (Leica Microsystems) using a 20× objective. Based on the reported topographic differences in mouse DC morphology during homeostasis and inflammation,30 both the central and peripheral cornea were analyzed separately. For morphological analysis, 2 epithelial z-stacks were captured in the central-most 1/3 of each corneal flatmount (to define “central DCs”), and 3 epithelial z-stacks were captured in the peripheral 2/3 of the corneal flatmount (to define “peripheral DCs”), excluding the limbus (Supplementary Fig. S1a). Hoechst staining was used to define the z-boundaries of the epithelium. Higher-resolution z-stacks were also collected using a 63× objective to compare nuclear morphology in DCs. To examine DC immunophenotype, two epithelial z-stacks were taken from the central and peripheral regions of CD86- and CD68-stained tissue samples (see Supplementary Fig. S1b) using a 40× objective (290 × 290 μm field, 512 × 512 pixels per frame). All imaging settings (i.e. laser power, gain and offset, image format, and 1 μm step size) were consistent for all samples.

**Corneal Dendritic Cell Morphological Analyses**

Morphological features of corneal epithelial DCs were analyzed using two-dimensional maximum projection images on ImageJ software (version 1.52d; National Institute of Health, USA) by a masked observer. CD45+ DCs were selected for morphological analysis based on their characteristic dendritiform morphology, and location within the epithelium31, and other non-dendritiform immune cells were excluded. DC density and viability were then manually counted and averaged between two 20× images (581 × 581 μm field) from the central and peripheral cornea.
Hyperosmolarity and Corneal Dendritic Cells

FIGURE 1. Methods for quantifying two-dimensional corneal dendritic cell (DC) morphology, including relevant parameter definitions. Representative 20x confocal maximum projection z-stack image (A) showing how DC density and viability were quantified with the identification of viable dendritiform CD45-positive cells (green arrowheads) and nonviable dendritiform CD45-positive cells (red arrowheads). Representative higher-magnification confocal maximum projection z-stack (B) showing key quantification features, including tracing the DC perimeter with the ImageJ Polygon tool (green border), the Threshold tool (white) applied over the CD45-labeled DC (magenta), and the location of dendritic tips (blue arrowhead), as used to quantify DC complexity, cell area, field area, and circularity. Scale bar for all images is 50 μm.

Nonviable DCs were identified using basic pathological signs of membrane lysis as a marker for cell death.24–26

Two-dimensional DC parameters of dendritic complexity, cell area, field area, and circularity were also calculated, as previously described in human27 and murine30,32 DC morphometry studies (see Fig. 1 for further detail). The Polygon tool was used to define the borders of each DC at each dendritic tip, after which the DC morphology was outlined by a fluorescence intensity threshold (manually set for each image and each fluorophore) and the “Measure” function was applied. Values were calculated separately for the central and peripheral cornea and each DC is presented as an independent unit.7,19

Corneal Dendritic Cell Immunophenotypic Analysis

DC phenotype was assessed using the activation markers, CD86 and CD68, which are associated with corneal inflammation.31,33–35 CD86 is a costimulatory cell surface molecule that is upregulated on corneal DCs following inflammatory stimulation.36 CD68 is an endosomal/lysosomal glycoprotein that is typically used to identify macrophages but is upregulated in DCs exposed to hyperosmolar stress.37,38 To quantify CD86 and CD68 expression within DCs, a masked observer created a binary maximum projection of the 40x epithelial z-stacks based on Iba1 staining only (manually adjusted threshold). Whole individual cells were selected with the “Rectangle” tool and a mask was created based on the Iba1-stained outline to exclude all background fluorescence. CD86 expression was measured within each DC mask using the “Mean Gray Value” tool. The number and percent-age area of CD68high particles were quantified in each cell mask with additional thresholding of the CD68-labeled channel (set equally in all groups) and the “Analyze Particles” tool - similar to the protocol described by Cope et al. (2018).39 Due to low cell numbers in the central cornea, DCs in the peripheral and central regions were pooled together as a single “corneal epithelial DC” population and presented as independent samples.7,19

Flow Cytometry

Flow cytometry was performed to assess the immunophenotype of corneal epithelial DCs after the 2-hour osmotic stress. Anaesthetized C57Bl/6J mice received topical application of 350, 450, or 600 mOsm/L saline (n = 5 mice per experimental group) for 2 hours on both eyes. Corneas were then analyzed using the same flow cytometry protocol described by Jiao et al.32 Both corneas from each mouse (total of 10 corneas per treatment, split into 4 replicates) were carefully dissected to remove the iris and limbus and digested in Dulbecco’s modified Eagle’s medium F12 (DMEM F-12, 0.1 mM Ca2+) containing 2 mg/mL Collagenase-D and 0.5 mg/mL DNAase-I (Roche, Indianapolis, IN) for 40 minutes at 37°C. Single cell suspensions were then filtered and labeled with conjugated primary antibodies against CD45 (CD45-PE; BioLegend, San Diego, CA, USA; Cat #103105 1:200), CD11c (CD11c-PeCy7; Invitrogen; Cat #25011482; 1:200) and either CD86 (CD86-APC; Invitrogen; Cat #17086281; 1:200) or CD68 (CD68-APC; BioLegend; Cat #25011482; 1:200) for 30 minutes on ice. Unstained controls omitting the above antibodies were also included. All samples were counterstained with DAPI (1:100) and then assessed on CytoFlex S Flow Cytometer (Beckman Coulter).
Hyperosmolarity and Corneal Dendritic Cells

FIGURE 2. Dendritic cell (DC) density in the central and peripheral cornea. Plots (A) and representative images (B–G) of DCs in the central (B, D, F) and peripheral cornea (C, E, G) show no differences between eyes that received topical applications of 350 mOsm/L (B, C), 450 mOsm/L (D, E) and 600 mOsm/L (F, G) saline for 2 hours. DC density was quantified based on CD45-positive staining, dendritiform morphology, and location within the epithelial plane of focus. Nonviable cells (yellow arrowhead) were included in the counts. Each data point represents the average value taken from three images per cornea. N = 18, 6, and 6 corneas in the 350, 450, and 600 mOsm/L groups, respectively. Data are presented as mean ± SEM. Scale bar for all images is 100 μm.

with 30,000 events recorded per sample. FlowJo software (version 10) was used to analyze the data in all groups.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism software (version 8.0.1; GraphPad Software, La Jolla, CA, USA). After confirming data were normally distributed, inter-group comparisons were analyzed using a 1-way ANOVA with Tukey’s post hoc test for multiple comparisons. Additionally, data from 350 mOsm/L-treated eyes in experiment A were confirmed to have no contralateral effect using a Student’s t-test before combining into a single 350 mOsm/L baseline group. Unless otherwise stated, all reported statistics refer to the inter-group comparisons from the Tukey’s post hoc test, with data expressed as mean (M) ± SEM. An alpha of 0.05 was adopted for statistical significance. The P values in plots are summarized using: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

RESULTS

Corneal Epithelial DC Density

CD45 staining allows visualization of the resident DC population in the corneal epithelium.31,32,40,41 Mean corneal DC density across all osmotic groups ranged from 4 to 11 cells/mm² centrally, to 33 to 50 cells/mm² peripherally. As an increase in epithelial DC density is an indicator of corneal inflammation in clinical42 and laboratory31,36 settings, this parameter was quantified in corneas exposed to different osmotic stimuli. After 2 hours of topical 450 mOsm/L or 600 mOsm/L stress, corneal epithelial DC density did not differ from the iso-osmotic 350 mOsm/L condition, in the central (M350 = 8.27 ± 1.07 cells/mm², M450 = 10.55 ± 2.32 cells/mm², M600 = 4.85 ± 1.56 cells/mm², P350 vs 450 = 0.55, P350 vs 600 = 0.28) or peripheral cornea (M350 = 38.07 ± 2.44 cells/mm², M450 = 49.42 ± 4.56 cells/mm², M600 = 33.69 ± 4.87 cells/mm², P350 vs 450 = 0.08, P350 vs 600 = 0.66; Fig. 2). DC density was also similar in naïve mice and those that received 350 mOsm/L treatment or vehicle treatment, in central (Mnaive = 5.73 ± 2.09 vs. M350 = 10.81 ± 3.89 cells/mm², P = 0.56) and peripheral regions (Mnaive = 28.34 ± 3.98 vs. M350 = 41.11 ± 4.90 cells/mm², P = 0.26; see Supplementary Fig. S3a).

Corneal Epithelial DC Morphology

In response to topical hyperosmolar stress, corneal DCs showed morphological differences irrespective of eccentricity. DCs in mice treated with 450 mOsm/L saline had a more enlarged morphology than mice treated with 350 mOsm/L, with a larger field area (central: M350 = 1670 ± 89.94 vs. M450 = 2233 ± 187.5 μm², P = 0.005; peripheral: M350 = 1552 ± 36.5 vs. M450 = 1723 ± 61.98 μm², P = 0.037; Fig. 3a) and lower circularity (central: M350 = 0.072 ± 0.004...
vs. M_{450} = 0.051 \pm 0.007, P = 0.026; peripheral: M_{350} = 0.089 \pm 0.002 vs. M_{450} = 0.077 \pm 0.003, P = 0.013; Fig. 3b). Meanwhile, cell area (central: M_{350} = 154.8 \pm 7.42 vs. M_{450} = 144.4 \pm 16.40 \mu m^2, P = 0.78; peripheral: M_{350} = 174.6 \pm 3.32 vs. M_{450} = 196.9 \pm 6.023 \mu m^2, P = 0.15) and dendritic complexity remained similar (central: M_{350} = 4.62 \pm 0.18 tips vs. M_{50} = 4.79 \pm 0.27 tips, P = 0.86; peripheral: M_{350} = 5.25 \pm 0.08 vs. M_{50} = 5.56 \pm 0.12 tips, P = 0.11; Fig. 3c). Together, these findings suggest that the change in DC field area and circularity may result from cytoplasmic extension, rather than an altered dendrite configuration. Following a higher (600 mOsm/L) hyperosmolar stress, there were no overt changes to DC morphology. Both central and peripheral DCs exposed to 600 mOsm/L saline showed lower viability in the central cornea relative to iso-osmotic controls (M_{naive} vs 350 mOsm/L: P_{naive v 350} = 0.01, P_{naive v 500} = 0.001) and peripheral regions (M_{naive} vs 350 mOsm/L: P_{naive v 350} = 0.001) and peripheral regions (M_{naive} vs 350 mOsm/L: P_{naive v 350} = 0.001; see Supplementary Fig. S4a).}

**Corneal Epithelial DC Phenotype**

We next assessed the immunophenotypes of DCs in both confocal microscopy and flow cytometry using CD86 and CD68. Confocal microscopy was the primary method in which phenotypic analysis was conducted so that corneal epithelial DCs could be examined in isolation of other
The results suggest that increased expression of CD86 (M600 = 10.57 ± 1.89% vs. M350 = 15.23 ± 1.38% vs. M450 = 15.73 ± 3.46%, P350 v 450 = 0.47, P350 v 600 = 0.36; Fig. 5c). However, the proportion of CD68 DCs was unchanged (M350 = 10.47 ± 0.46% vs. M600 = 15.23 ± 1.38% vs. M450 = 15.73 ± 3.46%, P350 v 450 = 0.47, P350 v 600 = 0.36; Fig. 5d).

**Discussion**

Corneal epithelial DCs are a key component of innate immunity at the ocular surface and are often considered in terms of their density and topographical distribution in the context of inflammation. However, the morphological and phenotypic responses of corneal epithelial DCs to acute topical hyperosmolar stress have not been previously examined despite the relevance of tear hyperosmolality to DED pathogenesis. In this study, we investigated the changes in murine corneal DCs after 2 hours of ocular surface stimulation with different concentrations of hyperosmotic saline. This experimental regimen intended to mimic tear hyperosmolar stresses considered to induce corneal inflammation in DED. Congruent with previous studies of DC maturation in patients with DED and murine models of DED, in our study acute hyperosmolar stress was associated with distinct morphological and phenotypic changes to DCs.

DCs are dynamic cells; the extension and retraction of their dendrites directly relate to antigen-sampling, cross-presentation, and migration functionalities. These properties are advantageous in the steady-state corneal epithelium as long dendritic projections allow efficient antigen capture over a large epithelial area despite the sparse presence of DCs. In our study, the steady-state cornea was mimicked by topical application of 350 mOsm/L saline, based on the reported tear osmolarity in young healthy mouse populations. DCs in mice treated with topical 350 mOsm/L saline were equivalent in density and morphology to previously reported healthy corneal DC populations, validating the use of 350 mOsm/L saline as an iso-osmotic control. DC immunophenotype was also evaluated using conventional and unconventional activation markers, CD86 and CD68, respectively. Although these molecules have been reported to be exclusively expressed by resident immune cells in the corneal stroma of mice during steady-state, we found low, but similar expression in naïve and 350 mOsm/L-treated corneal epithelial DCs.

Corneal DCs show a variety of stimulus-dependent morphological responses to inflammatory activation, including dynamic extension of existing and new dendrites, increased probing activity, and retraction of their dendrites to form a spherical morphology. In response to 600 mOsm/L corneal DCs showed elevations in CD86 (M350 = 28.34 ± 0.94 intensity units, p350 v 450 < 0.0001; Fig. 4) and CD68 staining (M350 = 3.39 ± 0.54 particles, p350 v 600 = 0.032). We next compared the activation status of all CD45^+CD11c^+ corneal DCs following hyperosmolar stress, using flow cytometry as a more sensitive measure of immunophenotype. Using the gating strategy (Fig. 5a), we found a significantly higher proportion of CD86^+CD14^+DC11c^+ DCs in the 450 mOsm/L (M350 = 18.38 ± 1.53% vs. M450 = 32.53 ± 1.72%, P350 v 450 = 0.006; Fig. 5c) and 600 mOsm/L groups relative to the 350 mOsm/L group (M600 = 40.38 ± 3.41%, P350 v 600 = 0.0003; Fig. 5c).

**Figure 4.** Immunofluorescence analysis of corneal epithelial dendritic cell (DC) phenotype. Increased expression of CD86 (A–F) and CD68 (G–L) in Iba1-labeled DCs exposed to 600 mOsm/L compared to 350 mOsm/L saline, which is also quantitatively presented as higher fluorescence intensity staining with CD86 (M) and a higher number of CD68 particles (N). Eyes treated with 450 mOsm/L saline showed a higher intensity of CD86 staining within Iba1-labeled DCs compared to the 350 mOsm/L saline condition. Data points represent individual DCs in both the central and peripheral corneal epithelium. N (CD86) = 82, 27, and 36 cells, and N (CD68) = 162, 34, and 41 cells in the 350, 450, and 600 mOsm/L groups for CD68 and CD68 analysis, respectively. Data are presented as mean ± SEM. Asterisks denote statistical significance between groups (*P < 0.05, **P < 0.01, ***P < 0.001). Scale bars for all images is 50 μm.

similar cell-types like stromal DCs in situ—a feature that is not possible with flow cytometry. Eccentricity-based differences were also not examined; DCs in the central and peripheral corneal epithelium were concatenated to compensate for lower cell numbers in the 290 × 290 μm field of view. Throughout the naïve corneal epithelium, there was very low CD86 and CD68 expression within Iba1^+ DCs, although some CD86^high and CD68^high DCs were present in Iba1-labeled DCs exposed to 600 mOsm/L saline (Fig. 5c). However, the proportion of CD68^+ DCs was unchanged (M350 = 10.47 ± 0.46% vs. M600 = 15.23 ± 1.38% vs. M450 = 15.73 ± 3.46%, P350 v 450 = 0.47, P350 v 600 = 0.36; Fig. 5d).
our findings show that altered morphology is the earliest indicator of DC activation, occurring within 2 hours of hyperosmotic exposure and preceding changes in DC density.

In the present study, topical application of 450 mOsm/L saline was chosen to experimentally model the osmotic insult that occurs in DED. Although DED is a chronic condition characterized by a long-term elevation in tear osmolality,44 the TFOS DEWS II report indicates that the condition can be acutely modelled using a 450 mOsm/L stimulus; in vitro studies demonstrate this stressor induces DED-like inflammatory cytokine release, and epithelial cell death in the cornea8,9 and conjunctiva.53 This level of hypertonicity is also the lowest osmolarity at which nociceptors show electrical activity in mice,10 and corneal pain is perceived by humans.9 Hence, our finding of DC phenotypic and morphological activation in response to ocular surface stimulation with a 450 mOsm/L solution, being a conservative hyperosmotic stressor, confirms the corneal inflammatory potential of relatively modest elevations in tear osmolality.

The observed DC maturation response to ocular surface hyperosmolality also closely resembles the increase in DC size, field area, and dendritic complexity reported in DED patients.26,27 Morphological and phenotypic signs of DC maturation, as reported in our study in response to 450 mOsm/L stress, typically correspond with acute inflammation and upregulation of costimulatory molecules that are critical for antigen presentation.48 For example, DC maturation was correlated to tear concentrations of interleukin-1β, interleukin-6, and interleukin-7 in patients with bacterial keratitis.15 Thus, mature DCs likely play a critical role in DED pathogenesis, as supported by the increased presence and T cell stimulation by CD86+ DCs in the cervical lymph nodes in mice after 4 days of evaporative (hyperosmolar) stress.45

Hyperosmolar stress may also act as a DC primer during DED pathogenesis. Renal DCs and cultured bone marrow-derived DCs both upregulate CD86 and CD68 in response to hyperosmolar stress, although the capacity to stimulate T cells only occurred in the presence of a secondary bacterial stimulus (lipopolysaccharide [LPS]).55 Similarly, Vega-Ramos et al. reported that DCs lack the capacity to secrete cytokines when activated without an antigen, but remain primed until a secondary antigen is introduced, whereupon there is hypersecretion of inflammatory cytokines.55 As hyperosmolar stress does not constitute an antigen, by virtue of its inability to be cross-presented to T cells, it is likely that our findings provide evidence for DC priming without induction of the adaptive immune response. Consistent with this hypothesis, T cells derived from the submandibular lymph nodes of mice treated with 3000 mOsm/L saline for 5 days induce a heightened swelling response when injected into a mouse footpad with ovalbumin antigen, and accelerate the impediment to tear production when injected into mice undergoing evaporation stress. Concurrent to this T cell priming, conjunctival DCs augmented their morphology after 5-day treatment of 3000 mOsm/L saline, similar to our findings in the cornea.

DCs did not show morphological changes in response to 600 mOsm/L stress, relative to 350 mOsm/L controls. Instead, there were signs of DC membrane blebbing and fragmentation in the central cornea, suggesting these cells were arrested and potentially undergoing cell death. DCs exposed to 600 mOsm/L saline also exhibited increased activation marker expression, consistent with evidence of starvation-induced apoptosis in cultured DCs.56 This was a somewhat unexpected result. Although in vitro exposure to a 600 mOsm/L stimulus can induce nociceptor injury54 and epithelial cell death,57 a topical drop of 600 mOsm/L saline
induces only mild pain perception in humans\(^8\) and is below the theorized 2000 mOsm/L osmotic level at the epicentre of tear film breakup spots.\(^9\) Many topical ophthalmic hyperosmolar preparations prescribed for treating corneal edema, such as Muro-2\% (≈700 mOsm/L\(^\text{10}\)) and Muro-5\% (≈1700 mOsm/L\(^\text{10}\)), are also considered safe, with a low reported incidence of ocular surface pain and corneal damage.\(^{60}\) To the best of the authors’ knowledge, the direct effect of acute hyperosmolar saline exposure on resident corneal DCs has not been reported. Hence, it is interesting that DC toxicity may have been observed in our study given the 600 mOsm/L stimulus was not within a known pathological range in an in vivo setting. The difference in exposure periods may also explain this finding. Mice in our study were exposed to 600 mOsm/L stress for 2 hours, whereas the typical residence time of topical hyperosmolar preparations in humans is considered in the order of a few minutes.\(^{61,62}\) Irrespective of this difference, our findings have implications for understanding how corneal DC physiology is altered after topical hyperosmolar treatment. Our findings may also be relevant to understanding cumulative alterations to DC physiology in populations with decreased tear stability, such as frequent blinkers,\(^{63}\) heavy computer users,\(^{64}\) and patients with DED.\(^5\)

This study was limited by the use of anesthetized mice. Not only did we detect retraction in the resting morphology of DCs in anesthetized mice, but general anesthesia is reported to disrupt corneal homeostasis by inducing corneal thinning,\(^{65}\) structural damage\(^{66}\) and altering tear film biochemistry.\(^{67}\) Hence, our findings after 2 hours of uninterrupted hyperosmolar stress in anesthetized mice are not completely generalizable to the natural in vivo conditions of acute hyperosmolar stress in humans. Further in vivo experiments in laboratory and clinical settings are required, with a focus on shorter exposures to hyperosmolar stress.

**Conclusion**

Corneal epithelial DCs undergo morphological and phenotypic changes when exposed to a hypertonic topical stimulus, with a larger and activated morphology in response to mild (450 mOsm/L) stress, and immunophenotypic activation and morphological alterations consistent with reported features of DC death in response to severe (600 mOsm/L) stress. Considering the role of hyperosmolar stress in the pathogenesis of DED, these data support a role for corneal DC activation following the loss of tear homeostasis.

**Acknowledgments**

The authors thank the Florey Advanced Microscopy Facility at the Florey Institute of Neuroscience and Mental Health Facility for provision of instrumentation, training, and general support.

**Grant Information:** Funding support (to H.R.C.) provided by the National Health and Medical Research Council (APP1126540).

All authors have no conflicts to disclose. L.E.D. has received funding from Alcon, Allergan, and Azura Ophthalmics for dry eye research, and consulting income from Seqlerus and Medmont, all unrelated to this work.

Author contributions: K.S., L.E.D., and H.R.C. designed and performed experiments and data analysis. H.J. performed experiments. K.S., L.E.D., and H.R.C. drafted the paper. All authors have read, reviewed, and approved the final paper as submitted.

**Disclosure:** K. Senthil, None; H. Jiao, None; L.E. Downie, None; H.R. Chinnery, None

**References**


Downloaded from iovs.arvojournals.org on 03/02/2021
Author/s: Senthil, K; Jiao, H; Downie, LE; Chinnery, HR

Title: Altered Corneal Epithelial Dendritic Cell Morphology and Phenotype Following Acute Exposure to Hyperosmolar Saline

Date: 2021-02-01


Persistent Link: http://hdl.handle.net/11343/265789

File Description: Published version

License: CC BY-NC-ND