2013

Efficacy of osmoprotectants on prevention and treatment of murine dry eye

W Chen

X Zhang

J Li

Y Wang

Q Chen

See next page for additional authors

Follow this and additional works at: https://researchonline.nd.edu.au/med_article

Part of the [Medicine and Health Sciences Commons](#)

This article was originally published as:

Chen, W., Zhang, X., Li, J., Wang, Y., Chen, Q., Hou, C., & Garrett, Q. (2013). Efficacy of osmoprotectants on prevention and treatment of murine dry eye. *Investigative Ophthalmology and Visual Science*, 54 (9), 6287-6297.

Original article available here:

<http://iovs.arvojournals.org/article.aspx?articleid=2128072>

This article is posted on ResearchOnline@ND at
https://researchonline.nd.edu.au/med_article/781. For more
information, please contact researchonline@nd.edu.au.

Authors

W Chen, X Zhang, J Li, Y Wang, Q Chen, C Hou, and Q Garrett

This is an Open Access article distributed in accordance with the Creative Commons Attribution Non-Commercial No Derivatives 4.0 International license (CC BY-NC-ND 4.0), which permits others to copy and redistribute the material in any medium or form, provided the original work is properly cited.

See: <https://creativecommons.org/licenses/by-nc-nd/4.0/>

This article which was originally published in *Investigative Ophthalmology and Visual Science* available at:

<http://iovs.arvojournals.org/article.aspx?articleid=2128072>

Chen, W., Zhang, X., Li, J., Wang, Y., Hou, C., and Garrett, Q. (2013) Efficacy of osmoprotectants on prevention and treatment of murine dry eye. *Investigative Ophthalmology and Visual Science*, 54, 6287-6297. doi: 10.1167/iovs.13-12081

Efficacy of Osmoprotectants on Prevention and Treatment of Murine Dry Eye

Wei Chen,¹ Xin Zhang,¹ Jinyang Li,¹ Yu Wang,¹ Qi Chen,¹ Chao Hou,¹ and Qian Garrett^{2,3}

¹School of Ophthalmology and Optometry, Wenzhou Medical College, Wenzhou, China

²Brien Holden Vision Institute, Sydney, Australia

³School of Optometry and Vision Science, University of New South Wales, Sydney, Australia

Correspondence: Qian Garrett, Brien Holden Vision Institute, Level 4, Rupert Myers Building, The University of New South Wales, Sydney, NSW 2052, Australia; q.garrett@brienholdenvision.org.

WC and XZ contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Submitted: March 22, 2013

Accepted: August 14, 2013

Citation: Chen W, Zhang X, Li J, et al. Efficacy of osmoprotectants on prevention and treatment of murine dry eye. *Invest Ophthalmol Vis Sci*. 2013;54:6287–6297. DOI:10.1167/iov.12081

PURPOSE. To evaluate the efficacy of osmoprotectants on prevention and treatment of dry eye in a murine model.

METHODS. Dry eye was induced in mice by using an intelligently controlled environmental system (ICES). Osmoprotectants betaine, L-carnitine, erythritol, or vehicle (PBS) were topically administered to eyes four times daily following two schedules: schedule 1 (modeling prevention): dosing started at the beginning of housing in ICES and lasted for 21 or 35 days; schedule 2 (modeling treatment): dosing started after ICES-housed mice developed dry eye (day 21), continuing until day 35. Treatment efficacy was evaluated for corneal fluorescein staining; corneal epithelial apoptosis by TUNEL and caspase-3 assays; goblet cell numbers by PAS staining; and expression of inflammatory mediators, TNF- α , IL-17, IL-6, or IL-1 β by using RT-PCR on days 0, 14, 21, and/or 35.

RESULTS. Compared with vehicle, prophylactic administration of betaine, L-carnitine, or erythritol significantly decreased corneal staining and expression of TNF- α and IL-17 on day 21 (schedule 1). Treatment of mouse dry eye with osmoprotectants significantly reduced corneal staining on day 35 compared with day 21 (schedule 2). Relative to vehicle, L-carnitine treatment of mouse dry eye for 14 days (days 21 to 35) resulted in a significant reduction in corneal staining, number of TUNEL-positive cells, and expression of TNF- α , IL-17, IL-6, or IL-1 β , as well as significantly increased the number of goblet cells.

CONCLUSIONS. Topical application of betaine, L-carnitine, or erythritol systematically limited progression of environmentally induced dry eye. L-carnitine can also reduce the severity of such dry-eye conditions.

Keywords: L-carnitine, betaine, osmoprotectants, dry eye, animal model, apoptosis, inflammation, TNF- α , IL-17, IL-6, IL-1 β

Dry-eye disease is one of the most common ophthalmic pathologies and is associated with tear film hyperosmolarity and inflammation of the ocular surface.¹ Osmolarity has been reported to be highly correlated with severity of the disease across normal, mild/moderate, and severe categories² and can reach values of up to 360 mOsm^{3,4} compared with normal tear film, which ranges between 300 and 310 mOsm.⁵ It has been proposed that the tear film osmolarity over the ocular surface can reach much higher values than is measured in meniscus,⁶ and may reach values of up to 600 mOsm.⁷ Under a hyperosmolar environment, cells will lose water and/or gain salts, with concomitant changes in cell volume,^{8,9} leading to damage to DNA and proteins,¹⁰ and induction of ocular surface inflammatory responses.¹¹ Tear film hyperosmolarity induces hyperosmolarity of epithelial cells, unleashing a cascade of inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF- α ; and proteolytic enzymes, such as matrix metalloproteinase-9 (MMP-9).^{12,13} The production of inflammatory mediators on the ocular surface has been suggested as the primary cause of ocular discomfort, inflammation, and ocular surface cell apoptosis.^{9,13,14}

Hyperosmolarity-induced corneal epithelial apoptosis has been observed using both cultured human ocular surface

epithelial cells and animal dry-eye models.^{15–21} Generally, cells can adapt to a hyperosmotic environment by accumulating compatible organic osmolytes/osmoprotectants.²² Osmoprotectants, such as L-carnitine and betaine, are compatible solutes that act similarly to electrolytes to balance osmotic pressure yet do not interfere with cell metabolism and can aid survival of cells under extreme osmotic stress.⁹ L-carnitine and erythritol have been shown to protect human corneal epithelial cells in hyperosmotic conditions and to lower levels of mitogen-activated protein (MAP) kinases in response to hyperosmolar stress.²³ L-carnitine and betaine also stabilize corneal epithelial cell volume under hyperosmotic stress and ameliorate hyperosmotic stress-induced human corneal epithelial cell apoptosis (Garrett Q. *IOVS* 2012;53:ARVO E-Abstract 564; Willcox MD. *IOVS* 2012;53:ARVO E-Abstract 564).²⁴ Further, in dry-eye patients, reduced levels of tear L-carnitine compared with healthy subjects have been reported,²⁵ suggesting that carnitine might play a contributory role in the development of dry eye.

The intelligently controlled environmental system (ICES)-induced murine dry eye model has been used previously to assess therapeutic effects of trehalose eye drops.^{15,26} Continual exposure of mice to the low humidity and excessive air flow

TABLE. The Primer Sequences Used for qRT-PCR

Gene	Forward Primer, 5'-3'	Reverse Primer, 5'-3'
<i>IL-1β</i>	TGAGCTGAAAGCTCTCCACC	CTGATGTACCAGTTGGGGAA
<i>IL-6</i>	AGATAACAAGAAAGACAAAGCCAGAGTC	GCATTGGAAATTTGGGGTAGGAAG
<i>IL-17</i>	CTCAACCGTTCCACGTCACCCCT	CCAGCTTTCCCTCCGCATT
<i>TNF-α</i>	TCTACTGAACCTCGGGGTGATCG	ACGTGGGTACAGGCTTGTC
<i>GAPDH</i>	TGTCCGTCGTGGATCTGAC	CCTGCTTCACCACCTTCTTG

created in ICES for 14 or more days promotes tear evaporation that destabilizes the tear film and increases tear osmolarity, showing biological and morphologic characteristics of dry eye similar to those in humans, such as reduced aqueous tear production, presence of corneal epithelial defects and apoptosis of ocular surface epithelium, increased inflammatory responses and expression of proteolytic MMP-9, as well as a

decrease in the number of goblet cells.^{15,26,27} Using this animal model, the present study extended the previous in vitro studies of osmoprotectants (Garrett Q. *IOVS* 2012;53:ARVO E-Abstract 564; Willcox MD. *IOVS* 2012;53:ARVO E-Abstract 564)^{23,24} to determine the efficacy of osmoprotectants L-carnitine, betaine, and erythritol in protection and therapeutic treatment of ICES-induced murine dry eye.

FIGURE 1. Mean corneal staining score of mice in response to housing at ICES and topical treatment with betaine (0.23% in PBS), carnitine (0.25% in PBS), erythritol (0.25% in PBS), or vehicle PBS, and ICES with no treatment, on days 0, 14, 21, and/or 35. (A) Schedule 1: compounds were administered to mice at the beginning of their housing in ICES. *Statistically significant difference ($P < 0.05$) compared with the vehicle (PBS) treatment on the same day ($n = 22$ for days 14 and 21; $n = 10$ for day 35). (B) Schedule 2: compound administration following housing in ICES for 21 days while mice were housed in ICES for a total of 35 days. *Statistically significant difference ($P < 0.05$) between day 21 and day 35 for the same treatment group; #Statistically significant difference ($P < 0.05$) compared with the vehicle (PBS) treatment on day 35 ($n = 18$). Error bars present the SD. (C, D) Representative corneal staining images after the treatment ([a], betaine; [b], L-carnitine; [c], erythritol; [d], PBS, or [e], ICES with no treatment) for schedule 1, day 21 (C) or schedule 2, day 35 (D).

MATERIALS AND METHODS

Animals

All procedures were approved by the Animal Care and Ethics Committee of Wenzhou Medical College, Zhejiang, China, and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female C57BL/6 mice (age range, 4–6 weeks, supplied by the Animal Breeding Unit of Wenzhou Medical College) were used for this study. Only those healthy mice with no corneal infections, infiltrations, or leukoma, and with total scores of corneal fluorescein staining less than 10^{27} were selected for the study.

ICES-Induced Murine Dry Eye Model

A murine model of dry eye was induced by an ICES.^{15,26,27} The animals were maintained in relative humidity, 60% to 80%, no airflow, and at 21 to 23°C. Dry-eye desiccation was created through exposure to relative humidity of $13.1\% \pm 3.5\%$, airflow of 2.2 ± 0.2 m/s, and temperature of $22 \pm 2^\circ\text{C}$. After housing for 21 days, mice began developing dry-eye conditions similar to those observed in humans.²⁶ The relative humidity and temperature of ICES were monitored daily.

Compound Administration Schedules

Osmoprotectants for topical administration of betaine, L-carnitine, and erythritol were formulated in sterile PBS. Mice without any topical treatment were used as the dry-eye control group (ICES), whereas PBS-treated mice were the vehicle control (PBS). Two topical administration schedules were used for this study: schedule 1 was designed to model prevention in which dosing of a compound started at the beginning of housing in the ICES (on day 1) and lasted for 21 or 35 days; whereas schedule 2 was to model treatment where dosing started on day 22 after the housed mice developed dry eye (on day 21)²⁶ and lasted until day 35. During both schedules, mice remained housed in ICES. In both schedules, 10 μL per eye of each compound solution was topically administered to mice eyes bilaterally four times a day. Five treatment groups were classified as follows:

betaine (0.23% in PBS), L-carnitine (0.25% in PBS), erythritol (0.25% in PBS), PBS only, and no treatment (ICES), respectively. Five animals were used per treatment group. PBS was from Maixin Technology (Fujian, China) and betaine, L-carnitine, erythritol were all from Sigma-Aldrich (Shanghai, China). The concentrations of the test compounds were determined based on the previously published data where addition of L-carnitine (10–15 mM), erythritol (20–40 mM), or betaine (10 mM), to hyperosmolar media demonstrated osmoprotection of cultured human corneal epithelial cells.^{23,24} As well, the addition of betaine or L-carnitine at 10 mM, or erythritol at 40 mM, to the culture medium had no effect on viability of the cultured corneal epithelial cells.²³

Clinical Examination: Corneal Fluorescein Staining

Clinical examination using corneal fluorescein staining was performed on all eyes on days 0, 14, 21, and 35 by instilling via a micropipette 0.5 μL of 5% fluorescein PBS solution into the inferior conjunctival sac. The cornea was examined using a slit-lamp microscope (SLM-3; Kanghua Technology Co., Ltd., Chongqing, China) with cobalt blue light after fluorescein instillation. The stained area was assessed and graded by a masked observer using the 2007 Dry Eye WorkShop (DEWS)-recommended grading system.²⁸ Mouse corneas were rated from 0 to 4, with the cornea surface divided into five regions (0 dot, Grade 0; 1–5 dots, Grade 1; 6–15 dots, Grade 2; 16–30 dots, Grade 3; and 30 dots, Grade 4). The total score from the five regions was recorded.

Animal Euthanasia

For schedule 1, at the end time point of day 21 in ICES (schedule 1, day 21), mice from each group were killed with an overdose of a mixture of ketamine and xylazine for histological, immunohistochemical, and quantitative RT-PCR (qRT-PCR). For schedule 2, a subset of mice from ICES control was killed at day 21 and other mice were killed on day 35 (schedule 2, day 35). Additional healthy animals not housed in ICES and receiving no treatment were euthanized as untreated healthy controls for comparison.

FIGURE 2. Corneal epithelium of the normal healthy mice or the ICES-housed mice with or without the treatment with betaine (A), L-carnitine (B), erythritol (C), PBS (D), or with no treatment (E), for schedule 1, day 21 and schedule 2, day 35 (magnification: $\times 40$).

FIGURE 3. Bulbar conjunctival epithelium (*superior*, magnification: $\times 40$; scale bar: 50 μm) with expression of goblet cells (*pink*, positive to PAS staining) in response to (I) housing at ICES for 21 days with the schedule 1 treatment during which compounds were administered to mice at the beginning of their housing; or (II) housing in ICES for 35 days with schedule 2 treatment during which compound administration began following mouse housing in ICES for 21 days. The treatment groups are (A): betaine (0.23% in PBS), (B): L-carnitine (0.25% in PBS), (C): erythritol (0.25% in PBS), and (D): PBS. Mice housed in ICES with no treatment (E) and normal healthy mice without housing in ICES or receiving any treatment were used as comparisons. Graphs demonstrate the average number of goblet cells $\pm$ SD in both superior and inferior regions. *Statistically significant difference compared to the vehicle PBS ($P < 0.05$, $n = 6$). †Statistically significant difference of the ICES-housed mice between no treatment and the treatment with betaine, L-carnitine, erythritol, or PBS ($P < 0.05$, $n = 6$). #Statistically significant difference between the normal healthy mice and other groups ($P < 0.05$, $n = 6$). Error bars present SD.

PAS Staining of Goblet Cells

After animals were killed, eyes and ocular adnexa were surgically excised, fixed in 10% formalin, paraffin embedded, and cut into 8- μm sections. The bulbar conjunctival epithelium (superior and inferior regions) sections were stained with PAS (Sigma-Aldrich) reagent for measuring goblet cells and were examined and photographed with a microscope equipped with a digital camera (BX51; Olympus, Guangzhou, China). PAS-positive goblet cells in the conjunctiva were measured in five sections from each eye with image analysis software (available in the public domain at <http://rsb.info.nih.gov/ij/index.html>; ImageJ software; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Apoptosis

Immunohistochemical evaluation of caspase-3 expression and TUNEL assay were performed on corneal tissues collected on schedule 1, day 21, and schedule 2, day 35, to detect apoptosis of the corneal epithelial cells. The expression of caspase-3 was evaluated by laser scanning confocal microscopy (LSM 710; Zeiss with krypton-argon and He-Ne laser; Carl Zeiss Meditec,

Sartrouville, France) using frozen corneal tissue sections. Mice eyes (schedule 1, day 21, and schedule 2, day 35) from each treatment group were excised. Corneal section slides were fixed with methanol at 4°C for 10 minutes. After fixation, they were permeabilized with Triton X-100 (0.25% Triton X-100 in PBS) for 10 minutes and then blocked with 10% goat serum in PBS for 60 minutes. Caspase-3 antibody (1:100 dilution; Abcam, Cambridge, MA) was applied and incubated for 12 hours at 4°C followed by incubation with secondary antibody (Alexa-Fluor 594-conjugated goat anti-rabbit IgG, 1:300; Invitrogen-Molecular Probes, Eugene, OR) in a dark chamber for 1 hour at room temperature. Counterstaining with 4',6-diamidino-2-phenylindole (DAPI; 1:1000 dilution) was followed for 10 minutes. Sections were covered with Slowfade antifade mounting medium (Invitrogen, Eugene, OR) and sealed with a cover slip for microscopic observation.

DNA fragmentation detected by TUNEL assay was also evaluated using laser scanning confocal microscopy and frozen corneal tissue sections. Mice eyes (schedule 1, day 21, and schedule 2, day 35) from each treatment group were excised. Corneal section slides were fixed with 4% paraformaldehyde in PBS at room temperature for 10 minutes. After fixation, they were permeabilized with Triton X-100 (0.1% in PBS; Sigma, St.

FIGURE 4. Confocal microscopic observation of caspase-3 expression in mouse corneas (green, caspase-3-positive; blue, DAPI-positive cells) in response to (I) housing in ICES for 21 days with the schedule 1 treatment in which compounds were administered to mice at the beginning of housing in ICES; or (II) housing in ICES for 35 days with schedule 2 treatment in which compound administration began following housing in ICES for 21 days. The treatment groups are (A): betaine (0.23% in PBS); (B): carnitine (0.25% in PBS), (C): erythritol (0.25% in PBS), and (D): PBS. Mice housed in ICES with no treatment (E) and normal healthy mice without housing in ICES or receiving any treatment were used as comparisons (magnification: $\times 20$; scale bars: 50 μm). Error bars present SD.

Louis, MO) for 10 minutes and then 50 μL (5 μL enzyme solution in 45 μL label solution) TUNEL reaction mixture (In Site Cell Death Detection Kit; Roche, Mannheim, Germany) was applied and incubated for 1 hour at 37°C in a humidified atmosphere. Counterstaining with DAPI (1:1000 dilution) was followed for 30 minutes. Sections were covered with antifade mounting medium and sealed with a cover slip for microscopic observation.

Inflammatory Responses

qRT-PCR was used for detection of the expression of IL-1 β , IL-6, IL-17, TNF- α , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Total RNA from conjunctivas was extracted and pooled from two eyes of the same experimental group and same compound administration schedule using the RNA isolation kit according to the manufacturer's instructions (PicoPure RNA isolation kit; Applied Biosystem, Foster City, CA). cDNA was synthesized from 1 μg total RNA using random primers and Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase. The primer sequences for qRT-PCR detection of the inflammatory mediators of interest are listed in the Table.

The RNA concentration was measured at 260 nm and stored at -80°C before use. qRT-PCR analysis was employed by using the SYBR Green PCR Core Reagents System (Applied Biosystems, Paisley, UK) and Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Assays were performed in duplicate and repeated three times. The qRT-PCR results were analyzed using the comparative threshold cycle method and normalized with GAPDH as an endogenous reference.

Statistical Analysis

The 2(-Delta Delta C[T]) method²⁹ was used to analyze the relative changes in gene expression from real-time RT-PCR experiments. Statistical comparisons of four treatment groups and the group without treatment (ICES) for real-time PCR were performed with ANOVA. Post hoc multiple comparisons were adjusted using Tukey correction. The Mann-Whitney *U* test was used to compare the controls and the treatment groups for mouse corneal fluorescein staining scores. *P* less than 0.05 was considered statistically significant. Analyses were performed using SPSS 13.0 software (IBM SPSS Statistics, IBM Corporation, Chicago, IL).

FIGURE 5. Apoptosis of the mouse ocular surfaces (green, positive to TUNEL staining) in response to (I) housing in ICES for 21 days using the schedule 1 treatment in which compounds were administered to mice at the beginning of their housing in ICES; or (II) housing in ICES for 35 days using schedule 2 treatment in which compound administration began following housing in ICES for 21 days. The treatment groups are betaine (A), 0.23% in PBS), carnitine (B), 0.25% in PBS), erythritol (C), 0.25% in PBS), and PBS vehicle (D). Mice housed in ICES with no treatment (E) and normal healthy mice without housing in ICES or receiving any treatment were used as comparisons (magnification: $\times 20$; scale bars: 50 μm). Graphs demonstrate the mean $\pm$ SD of apoptotic cell density (number of cells/100 μm). *Statistically significant difference compared with vehicle PBS ($P < 0.05$, $n = 6$). †Statistically significant difference of the ICES-housed mice between no treatment and the treatment with betaine, L-carnitine, erythritol, or PBS ($P < 0.05$, $n = 6$). #Statistically significant difference between the normal healthy mice and other groups ($P < 0.05$, $n = 6$). Error bars present SD.

RESULTS

Corneal Fluorescein Staining

Fluorescein staining was used to assess changes in corneal epithelial integrity. For schedule 1, all three osmoprotectants (betaine, L-carnitine, or erythritol) reduced corneal staining on day 21 compared with the vehicle PBS and the ICES control receiving no treatment ($P < 0.05$) (Figs. 1A, 1C). Reduced corneal staining was also evident on day 14 for betaine- or L-

carnitine-treated mice; on day 35, L-carnitine treated mice corneas still exhibited significantly lower staining compared with the PBS control and ICES (Figs. 1A, 1C).

For schedule 2, Figures 1B and 1D show mouse corneas treated with betaine, L-carnitine, or erythritol for 14 days, following 21 days of desiccating conditions (by which time mice displayed typical signs of dry eye). All compounds demonstrated therapeutic action with significant reductions in mean corneal staining on day 35 compared with day 21 ($P <$

FIGURE 6. Real-time PCR analysis of TNF- α (A), IL-17 (B), IL-6 (C), and IL-1 β (D) expression after mice were housed in ICES for 21 days using the schedule 1 treatment in which compounds (0.23% betaine in PBS, 0.25% carnitine in PBS, 0.25% erythritol in PBS, or PBS vehicle) were administered to mice at the beginning of their housing in ICES. Mice housed in ICES with no treatment and normal healthy mice without housing in ICES or receiving any treatment were used as comparisons. *Statistically significant difference compared with the vehicle PBS ($P < 0.05$, $n = 3$). †Statistically significant difference compared with the ICES control ($P < 0.05$, $n = 3$). #Statistically significant difference between the normal healthy mice and other groups ($P < 0.05$, $n = 3$). Error bars present SD.

0.05). Corneas treated with L-carnitine or erythritol showed significant reduction in mean corneal staining relative to vehicle PBS on day 35. Betaine showed some effect in reducing corneal staining, but the effect was not significantly different compared with PBS ($P = 0.252$) on day 35. No statistically significant difference was found between PBS and ICES on day 21 or day 35.

Morphology of the Corneal Epithelium

Corneal epithelium thinning in the mice eyes after housing in ICES for 21 or 35 days was observed (Fig. 2E) compared with the normal healthy mice eyes (Fig. 2 normal). Although slight thinning was observed in all the treatment groups (Figs. 2A–D) for both schedule 1, day 21, and schedule 2, day 35, the ICES-housed, untreated mice showed greater thinning compared with the treatment or normal mice: from $32.7 \pm 1.1 \mu\text{m}$ to $24.3 \pm 2.1 \mu\text{m}$ and $23.5 \pm 1.2 \mu\text{m}$ (normal to ICES housed) for schedule 1, day 21, and schedule 2, day 35, respectively.

Conjunctival Goblet Cells

For both schedule 1, day 21 (Fig. 3 [I]) and schedule 2, day 35 (Fig. 3 [II]), there was a significant reduction in the number of goblet cells for all the ICES-housed mice (with or without treatments) compared with normal healthy mice that had not been housed in ICES or received any treatment (all $P < 0.05$). The ICES-housed mice presented with increased numbers of goblet cells after receiving the treatment with betaine, L-carnitine, erythritol, or vehicle PBS relative to those with no treatment (Fig. 3, all $P < 0.05$). Although an increasing trend in

goblet cell number was observed with betaine or erythritol, only L-carnitine treatment showed significantly increased goblet cell numbers compared with the vehicle PBS control (Fig. 3, $P = 0.0001$ [superior], 0.0002 [inferior] for schedule 1, day 21, and $P = 0.0001$ [superior and inferior] for schedule 2, day 35, respectively).

Apoptosis: Caspase-3 Expression

Immunohistochemistry for caspase-3 expression (green staining) in the corneal epithelium revealed much reduced immunoreactivity (similar to the level in normal healthy mice) under schedule 1 following daily treatment with each osmoprotectant, betaine (A), L-carnitine (B), or erythritol (C), compared with the treatment vehicle PBS (D), or with no treatment (ICES, E) (Fig. 4 [I]). For schedule 2, on day 35, the L-carnitine-treated mice (Fig. 4 [IIB]) presented similar immune reactivity of caspase-3 compared with the normal healthy mice, whereas for betaine (A), erythritol (C), or ICES (E), immune reactivity of caspase-3 was not reduced relative to PBS control.

Apoptosis: TUNEL Staining

Figure 5 shows that all the ICES-housed mice, with or without treatment, had a significantly higher number of apoptotic cells (positive to TUNEL staining; green) compared with the unhoused normal healthy mice on day 21, schedule 1 and day 35, schedule 2 (all $P < 0.05$). Furthermore, a significant reduction in the number of ICES-induced apoptotic cells was observed by day 21, schedule 1, in corneas that had received daily administration of the osmoprotectants or PBS vehicle (Fig.

FIGURE 7. Real-time PCR analysis of TNF- α (A), IL-17 (B), IL-6 (C), and IL-1 β (D) expression after mice housing in ICES for 35 days using schedule 2 treatment in which compound administration began following housing in ICES for 21 days. The treatment groups are betaine ([A], 0.23% in PBS); carnitine (0.25% in PBS), erythritol (0.25% in PBS), or PBS vehicle. Mice housing in ICES with no treatment and normal healthy mice without housing in ICES or receiving any treatment were used as comparisons. *Statistically significant difference compared with the vehicle PBS ($P < 0.05$, $n = 3$). †Statistically significant difference compared with the ICES control ($P < 0.05$, $n = 3$). #Statistically significant difference between the normal healthy mice and other groups ($P < 0.05$, $n = 3$). Error bars present SD.

5 [I], $P < 0.001$). There was a further reduction, relative to PBS, in apoptotic cell density with all the osmoprotectant treatments on day 21, schedule 1, and on day 35, schedule 2; however, this reduction reached statistical significance only for the L-carnitine treatment group ($P = 0.032$ and 0.0005 , Fig. 5 [I] and [II], respectively).

Inflammatory Responses

qRT-PCR was used to evaluate TNF- α , IL-17, IL-6, and IL-1 β mRNA expression levels (Fig. 6 for schedule 1, day 21; Fig. 7 for schedule 2, day 35). For both schedules, mRNA expression for each inflammatory mediator was significantly higher in the conjunctiva of all the ICES-housed mice than those in the unhoused normal healthy mice ($P < 0.01$, Figs. 6, 7). L-carnitine treatment in both schedules showed a statistically significant reduction in expression level of each mediator, relative to the vehicle PBS samples, as well as to the other treatment groups, betaine and erythritol (all $P < 0.05$, Figs. 6, 7). Furthermore, compared with the ICES control with no treatment, systematic administration of osmoprotectants (betaine, L-carnitine, or erythritol), starting at the beginning of housing in the ICES, significantly reduced the expression level of TNF- α , IL-17, IL-6, and IL-1 β (Fig. 6, all $P < 0.05$). Compared with the vehicle PBS, expression of TNF- α and IL-17 was significantly reduced by the treatment with each osmoprotectant (Fig. 5, all $P < 0.05$). For schedule 2, day 35, treatment with each of the osmoprotectants significantly reduced expression of TNF- α , IL-17, and IL-6 compared with the ICES control with no treatment (Fig. 7, all $P < 0.05$). Betaine, L-

carnitine, or erythritol treatment also significantly reduced TNF- α expression relative to vehicle PBS (Fig. 7, $P < 0.05$), but not the expression of IL-17, IL-6, and IL-1 β .

DISCUSSION

In the present study, we used a murine dry eye model to evaluate the efficacy of prophylactic and therapeutic treatment of L-carnitine, betaine, and erythritol in maintaining and restoring ocular surface health in a desiccating environment created by ICES. We showed that systematic administration with these compounds during the establishment of murine dry eye (by housing in ICES) limited desiccation-induced clinical signs of dry eye, ocular surface inflammatory responses, and apoptosis. We also showed that on onset of mouse dry-eye conditions, systematic treatment with L-carnitine, or to a lesser extent, with betaine or erythritol, attenuated the deleterious effects by reduction of corneal staining, ocular epithelial cell apoptosis, and expression of TNF- α , IL-17, IL-6, and IL-1 β , as well as maintaining the number of PAS-positive conjunctival goblet cells, thus demonstrating the potential of L-carnitine not only in the prevention but also in the therapeutic treatment of dry eye.

Epitheliopathy is one of the most easily recognizable clinical features of dry-eye disease. Environmental desiccation stress-induced dry-eye conditions decrease epithelial cell size and increase epithelial cell turnover.³⁰ Staining the ocular surface with diagnostic dyes, such as fluorescein, provides a practical method for evaluating ocular surface integrity. Treatment with betaine, L-carnitine, or erythritol during the development of

dry eye reduced corneal staining once dry-eye conditions were established (day 21). These observations suggest that osmoprotectants might help maintain and protect murine ocular surface integrity from ICES desiccation-induced damage. A similar effect was also observed with the L-carnitine or erythritol treatment of the mice whose condition of dry eye was already developed (housing in ICES for 21 days) before systematic treatment (for 14 days), suggesting that these compounds could also help to restore mouse ocular surface health.

Mucins released into the tear film are crucial for maintaining a healthy ocular surface.^{31,32} Hyperosmolarity in dry eye can induce cornification of conjunctival epithelial cells, entrapping the goblet cells, blocking mucus secretion, and subsequently degrading tear quality and stability.³³ Goblet cell populations are suggested to be sensitive indicators of ocular surface disease.³⁴ In our study, L-carnitine restored (although not completely) the loss of PAS-positive goblet cells caused by ICES desiccation (schedule 1) and dry eye conditions (schedule 2). Kunert et al.³⁴ similarly reported an increase (relative to untreated controls) in goblet cell density on treatment of dry eye syndrome with topical application of cyclosporin, suggesting that the anti-inflammatory properties of cyclosporin reduced ocular surface inflammation and concomitantly restored goblet cell density. That we observed similar increased goblet cell density with L-carnitine therapy suggests an additional influence of L-carnitine on reducing ocular surface inflammation.

It is recognized that inflammation plays a prominent role in the development and magnitude of signs and symptoms of dry eye. Tear hyperosmolarity and intracellular signaling pathway activation induced by desiccating stress initiate the production of proinflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF- α , and proteolytic enzymes, such as MMP-9.^{18,35,36} Using the same ICES murine dry-eye model, we previously reported dry-eye-associated increased ocular surface inflammation (a hallmark of dry-eye disease) as indicated by increased expression of IL-1 β , TNF- α , IL-6, and IL-17.²⁶ Application of trehalose eye drop restored ocular integrity in this model, reducing expression of IL-17, which in turn reduced the IL-17-mediated expression of IL-1, TNF- α , and IL-6.²⁶ That TNF- α , IL-17, IL-6, and IL-1 β were similarly upregulated in ICES controls in the present study further supports the use of the ICES model to mimic physiological stress experienced in dry eye. Activated MAP kinases can initiate expression of transcription factors, leading to expression of inflammatory cytokines, chemokines, and MMPs.³⁷ Epithelial and inflammatory cell production of MMP-9, stimulated by IL-1 and TNF- α , is suggested to impede reepithelialization of the cornea after injury.^{38,39} Previous studies have shown the osmoprotectants L-carnitine and erythritol, alone or in combination, can reduce the activation/phosphorylation of MAP kinases in cultured human corneal epithelial cells in response to hyperosmolar stress.²³ Reduced expression of MAP kinases would reduce production of MMP-9, suppressing the innate immune responses associated with expression of IL-1, TNF- α , and IL-17. In the present study, continual treatment of mice with betaine, L-carnitine, or erythritol suppressed expression of proinflammatory cytokines during the development of dry eye, as well as in dry-eye mice whose dry-eye conditions were developed before the treatment. This indicates that these compounds were able to at least partially ameliorate the inflammatory responses, consistent with previous reports of the anti-inflammatory properties of L-carnitine and betaine.^{40,41} It should be noted that among all the compounds, L-carnitine presented the only statistically significant, and the most beneficial effect, on lowering inflammatory responses of all the mediators investigated and demonstrated in both schedules.

L-carnitine is essential in maintaining the health of cells⁴² and is best known for its important role in the mitochondrial oxidation of long-chain fatty acids.⁴²⁻⁴⁴ Reports further indicate that L-carnitine also possesses anti-inflammatory and immunosuppressive activities.^{40,41} An in vivo study using a murine model of Crohn's disease revealed that systemic administration/treatment with L-carnitine during the establishment of colonic inflammation successfully reduced cytokine production and intestinal inflammation, and this protection was associated with a suppressive effect of L-carnitine on the colonic mRNA expression and serum of IL-1 β and IL-6.⁴³ In humans, an immunosuppressive role of L-carnitine is also observed with reduction of TNF- α levels in surgical and AIDS patients after receiving L-carnitine supplementation.^{45,46} Like L-carnitine, betaine has also been reported to possess anti-inflammatory properties.³⁸ Although the molecular events associated with the anti-inflammatory effects of L-carnitine or betaine in dry eye are not yet fully understood, we have demonstrated these activities also play a role in prevention and treatment of murine dry eye.

Long-term exposure of eyes to a desiccating environment increases tear evaporation, leading to hyperosmolarity. In an experimental mouse model of dry-eye disease, desiccating stress increased mouse tear osmolarity nearly 2-fold.⁴⁷ Hyperosmolarity is known to induce ocular surface epithelial apoptosis both in vitro (Garrett Q. *IOVS* 2011;52:ARVO E-Abstract 290-D754; Garrett Q. *IOVS* 2012;53:ARVO E-Abstract 564; Willcox MD. *IOVS* 2012;53:ARVO E-Abstract 564)^{16,24,19,48-54} and in vivo.^{13,26} In the present study, we also observed corneal epithelial apoptosis in ICES-induced murine dry eyes. However, treatment with osmoprotectants, in particular L-carnitine, appears to limit the ICES housing-induced apoptosis and contributes to stabilization of the ocular cell in the presence of desiccating conditions (although not completely). Although regulatory volume increase, the initial protective response to hypertonic induced cell shrinkage, restores cell volume within minutes through rapid uptake of inorganic ions,⁵⁵ the concentration of intracellular ions and, consequently, the intracellular ionic strength, remain abnormally high, which perturbs intracellular macromolecules.⁵⁶ However, if compatible organic osmolytes are available, they can enter and accumulate in cells and lower the ionic strength toward the isotonic state while maintaining cell volume without destabilizing proteins.⁵⁷ Our previous in vitro studies using cultured human corneal epithelial cells demonstrated that exogenous osmoprotectants L-carnitine (Garrett Q. *IOVS* 2012;53:ARVO E-Abstract 564; Willcox MD. *IOVS* 2012;53:ARVO E-Abstract 564) and betaine²⁴ could help stabilize corneal epithelial cell volume under hyperosmotic stress and limit hyperosmotic stress-induced apoptosis. Among the compounds tested, L-carnitine demonstrated the greatest ability. It should be kept in mind though that the ICES murine model in the present study represents only one form of dry-eye condition that is induced environmentally. Whether the effects of the osmoprotectants observed in the present study are applicable to other forms of dry eye needs to be investigated.

Taken together, we conclude that systematic administration of osmoprotectants L-carnitine, betaine, or erythritol can limit progression and reduce the severity of environmentally induced dry eye.

Acknowledgments

The authors thank Judith Flanagan (Brien Holden Vision Institute) and Haixia Liu (Allergan) for their review of the manuscript.

Supported by Allergan, Inc., and research grants from National Natural Science Foundation of China (81170820 [WC, QG]) and

Zhejiang Provincial Natural Science Foundation of China (Y2090821 [WC]; LQ12H12002 [QC]).

Disclosure: **W. Chen**, None; **X. Zhang**, None; **J. Li**, None; **Y. Wang**, None; **Q. Chen**, None; **C. Hou**, None; **Q. Garrett**, Allergan Inc. (F), P

References

1. The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007). *Ocul Surf.* 2007;5:75-92.
2. Sullivan BD, Whitmer D, Nichols KK, et al. An objective approach to dry eye disease severity. *Invest Ophthalmol Vis Sci.* 2010;51:6125-6130.
3. Gilbard JP, Farris RL. Tear osmolarity and ocular surface disease in keratoconjunctivitis sicca. *Arch Ophthalmol.* 1979;97:1642-1646.
4. Lemp MA, Bron AJ, Baudouin C, et al. Tear osmolarity in the diagnosis and management of dry eye disease. *Am J Ophthalmol.* 2011;151:792-798.e1.
5. Tomlinson A, Khanal S, Ramaesh K, Diaper C, McFadyen A. Tear film osmolarity: determination of a referent for dry eye diagnosis. *Invest Ophthalmol Vis Sci.* 2006;47:4309-4315.
6. Bron AJ, Tiffany JM, Yokoi N, Gouveia SM. Using osmolarity to diagnose dry eye: a compartmental hypothesis and review of our assumptions. *Adv Exp Med Biol.* 2002;506:1087-1095.
7. Liu H, Begley C, Chen M, et al. A link between tear instability and hyperosmolarity in dry eye. *Invest Ophthalmol Vis Sci.* 2009;50:3671-3679.
8. Burg MB, Ferraris JD, Dmitrieva NI. Cellular response to hyperosmotic stresses. *Physiol Rev.* 2007;87:1441-1474.
9. Lang F. Mechanisms and significance of cell volume regulation. *J Am Coll Nutr.* 2007;26:613S-623S.
10. Garner MM, Burg MB. Macromolecular crowding and confinement in cells exposed to hypertonicity. *Am J Physiol.* 1994;266:C877-C892.
11. Baudouin C. A new approach for better comprehension of diseases of the ocular surface. *J Fr Ophtalmol.* 2007;30:239-246.
12. De Paiva CS, Corrales RM, Villarreal AL, et al. Corticosteroid and doxycycline suppress MMP-9 and inflammatory cytokine expression, MAPK activation in the corneal epithelium in experimental dry eye. *Exp Eye Res.* 2006;83:526-535.
13. Baudouin C. The pathology of dry eye. *Surv Ophthalmol.* 2001;45:S211-S220.
14. Enriquez-de-Salamanca A, Castellanos E, Stern ME, et al. Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. *Mol Vis.* 2010;16:862-873.
15. Chen W, Zhang X, Liu M, et al. Trehalose protects against ocular surface disorders in experimental murine dry eye through suppression of apoptosis. *Exp Eye Res.* 2009;89:311-318.
16. Clouzeau C, Godefroy D, Riancho L, Rostene W, Baudouin C, Brignole-Baudouin F. Hyperosmolarity potentiates toxic effects of benzalkonium chloride on conjunctival epithelial cells in vitro. *Mol Vis.* 2012;18:851-863.
17. Gao J, Schwalb TA, Addeo JV, Ghosn CR, Stern ME. The role of apoptosis in the pathogenesis of canine keratoconjunctivitis sicca: the effect of topical Cyclosporin A therapy. *Cornea.* 1998;17:654-663.
18. Luo L, Li DQ, Corrales RM, Pflugfelder SC. Hyperosmolar saline is a proinflammatory stress on the mouse ocular surface. *Eye Contact Lens.* 2005;31:186-193.
19. Png E, Samivelu GK, Yeo SH, Chew J, Chaurasia SS, Tong L. Hyperosmolarity-mediated mitochondrial dysfunction requires Transglutaminase-2 in human corneal epithelial cells. *J Cell Physiol.* 2011;226:693-699.
20. Strong B, Farley W, Stern ME, Pflugfelder SC. Topical cyclosporine inhibits conjunctival epithelial apoptosis in experimental murine keratoconjunctivitis sicca. *Cornea.* 2005;24:80-85.
21. Yeh S, Song XJ, Farley W, Li DQ, Stern ME, Pflugfelder SC. Apoptosis of ocular surface cells in experimentally induced dry eye. *Invest Ophthalmol Vis Sci.* 2003;44:124-129.
22. Wehner F, Olsen H, Tinel H, Kinne-Saffran E, Kinne RKH. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. *Rev Physiol Biochem Pharmacol.* 2003;148:1-80.
23. Corrales RM, Luo L, Chang EY, Pflugfelder SC. Effects of osmoprotectants on hyperosmolar stress in cultured human corneal epithelial cells. *Cornea.* 2008;27:574-579.
24. Garrett Q, Khandekar N, Flanagan JL, Simmons PA, Vehige J, Willcox MD. Betaine stabilizes cell volume and protects against apoptosis in human corneal epithelial cells under hyperosmotic stress. *Exp Eye Res.* 2013;108:33-41.
25. Pescosolido N, Imperatrice B, Koverech A, Messano M. L-carnitine and short chain ester in tears from patients with dry eye. *Optom Vis Sci.* 2009;86:E132-E138.
26. Li J, Roubeix C, Wang Y, et al. Therapeutic efficacy of trehalose eye drops for treatment of murine dry eye induced by an intelligently controlled environmental system. *Mol Vis.* 2012;18:317-329.
27. Chen W, Zhang X, Zhang J, et al. A murine model of dry eye induced by an intelligently controlled environmental system. *Invest Ophthalmol Vis Sci.* 2008;49:1386-1391.
28. Methodologies to diagnose and monitor dry eye disease: report of the Diagnostic Methodology Subcommittee of the International Dry Eye WorkShop (2007). *Ocul Surf.* 2007;5:108-152.
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402-408.
30. Beardsley RM, De Paiva CS, Power DE, Pflugfelder SC. Desiccating stress decreases apical corneal epithelial cell size—modulation by the metalloproteinase inhibitor doxycycline. *Cornea.* 2008;27:935-940.
31. Gipson IK, Argueso P. Role of mucins in the function of the corneal and conjunctival epithelia. *Int Rev Cytol.* 2003;231:1-49.
32. Argueso P, Baram M, Spurr-Michaud S, Keutmann HT, Dana MR, Gipson IK. Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjogren syndrome. *Invest Ophthalmol Vis Sci.* 2002;43:1004-1011.
33. Corrales RM, de Paiva CS, Li DQ, et al. Entrapment of conjunctival goblet cells by desiccation-induced cornification. *Invest Ophthalmol Vis Sci.* 2011;52:3492-3499.
34. Kunert KS, Tisdale AS, Gipson IK. Goblet cell numbers and epithelial proliferation in the conjunctiva of patients with dry eye syndrome treated with cyclosporine. *Arch Ophthalmol.* 2002;120:330-337.
35. Li DQ, Chen Z, Song XJ, Luo L, Pflugfelder SC. Stimulation of matrix metalloproteinases by hyperosmolarity via a JNK pathway in human corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2004;45:4302-4311.
36. Li DQ, Luo L, Chen Z, Kim HS, Song XJ, Pflugfelder SC. JNK and ERK MAP kinases mediate induction of IL-1beta, TNF-alpha and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp Eye Res.* 2006;82:588-596.
37. Barchowsky A, Frleta D, Vincenti MP. Integration of the NF-kappaB and mitogen-activated protein kinase/AP-1 pathways at the collagenase-1 promoter: divergence of IL-1 and TNF-

- dependent signal transduction in rabbit primary synovial fibroblasts. *Cytokine*. 2000;12:1469–1479.
38. Li DQ, Lokeshwar BL, Solomon A, Monroy D, Ji Z, Pflugfelder SC. Regulation of MMP-9 production by human corneal epithelial cells. *Exp Eye Res*. 2001;73:449–459.
 39. Woessner JF. Matrix metalloproteinases and their inhibitors in connective-tissue remodeling. *FASEB J*. 1991;5:2145–2154.
 40. Detopoulou P, Panagiotakos DB, Antonopoulou S, Pitsavos C, Stefanadis C. Dietary choline and betaine intakes in relation to concentrations of inflammatory markers in healthy adults: the ATTICA study. *Am J Clin Nutr*. 2008;87:424–430.
 41. Miguel-Carrasco JL, Mate A, Monserrat MT, Arias JL, Aramburu O, Vazquez CM. The role of inflammatory markers in the cardioprotective effect of L-carnitine in L-NAME-induced hypertension. *Am J Hypertens*. 2008;21:1231–1237.
 42. Flanagan JL, Simmons PA, Vehige J, Willcox MD, Garrett Q. Role of carnitine in disease. *Nutr Metab (Lond)*. 2010;7:30.
 43. Fortin G, Yurchenko K, Collette C, et al. L-carnitine, a diet component and organic cation transporter OCTN ligand, displays immunosuppressive properties and abrogates intestinal inflammation. *Clin Exp Immunol*. 2009;156:161–171.
 44. Evans AM, Fornasini G. Pharmacokinetics of L-carnitine. *Clin Pharmacokinet*. 2003;42:941–967.
 45. De Simone C, Tzantzoglou S, Famularo G, et al. High dose L-carnitine improves immunologic and metabolic parameters in AIDS patients. *Immunopharmacol Immunotoxicol*. 1993;15:1–12.
 46. Delogu G, De Simone C, Famularo G, Fegiz A, Paoletti F, Jirillo E. Anaesthetics modulate tumour necrosis factor alpha: effects of L-carnitine supplementation in surgical patients. Preliminary results. *Mediators Inflamm*. 1993;2:S33–36.
 47. Stewart P, Chen Z, Farley W, Olmos L, Pflugfelder SC. Effect of experimental dry eye on tear sodium concentration in the mouse. *Eye Contact Lens*. 2005;31:175–178.
 48. Smith J. The epidemiology of dry eye disease: report of the Epidemiology Subcommittee of the International Dry Eye WorkShop (2007). *Ocul Surf*. 2007;5:93–107.
 49. Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y. Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci U S A*. 2000;97:9487–9492.
 50. Lang KL, Fillon SE, Schneider DS, Rammensee HGR, Lang FL. Stimulation of TNF α expression by hyperosmotic stress. *Pflügers Arch*. 2002;443:798–803.
 51. Friis MB, Friberg CR, Schneider L, et al. Cell shrinkage as a signal to apoptosis in NIH 3T3 fibroblasts. *J Physiol*. 2005;567:427–443.
 52. Luo L, Li DQ, Pflugfelder SC. Hyperosmolarity-induced apoptosis in human corneal epithelial cells is mediated by cytochrome c and MAPK pathways. *Cornea*. 2007;26:452–460.
 53. Clouzeau C, Godefroy D, Riancho L, Rostene W, Baudouin C, Brignole-Baudouin F. Hyperosmolarity potentiates toxic effects of benzalkonium chloride on conjunctival epithelial cells in vitro. *Mol Vis*. 2012;18:851–863.
 54. Png E, Samivelu GK, Yeo SH, Chew J, Chaurasia SS, Tong L. Hyperosmolarity-mediated mitochondrial dysfunction requires transglutaminase-2 in human corneal epithelial cells. *J Cell Physiol*. 2011;226:693–699.
 55. Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y. Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci U S A*. 2000;97:9487–9492.
 56. Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN. Living with water stress: evolution of osmolyte systems. *Science*. 1982;217:1214–1222.
 57. Street TO, Bolen DW, Rose GD. A molecular mechanism for osmolyte-induced protein stability. *Proc Natl Acad Sci U S A*. 2006;103:13997–14002.