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Authors
Butovich, Igor A
Yuksel, Seher
Leonard, Brian
et al.

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ORIGINAL ARTICLES

Novel Lipids of the Rabbit Harderian Gland Improve Tear Stability in an Animal Model of Dry Eye Disease

Igor A. Butovich,[1] Seher Yuksel,[1] Brian Leonard,[2] Tom Gadek,[3] Arthur S. Polans,[4] and Daniel M. Albert[5]

Abstract

Purpose: Instability of the tear film leads to evaporative dry eye disease (EDED), but the Harderian gland in some terrestrial vertebrates may produce novel lipids that stabilize the tear film and protect against dry eye. Here, the nonpolar lipids in the Harderian gland and tears of the rabbit but absent in human tears were identified and tested in preclinical studies to determine whether they could treat severe EDED.
Methods: Lipids were identified primarily by atmospheric pressure chemical ionization mass spectrometry (MS) and fragmentation MS/MS. An identified lipid was synthesized and formulated as an emulsion and as a cyclodextrin (CD) clathrate. Following doses with test agents and controls, tear film breakup time (TBUT), tear production, corneal fluorescein staining, macrophage infiltration, and goblet cell survival were measured using standard tests at 0, 2 and 4 weeks in an animal model of EDED.
Results: The lipid emulsion increased TBUT ($P < 0.01$) and tear production ($P < 0.05$), while it decreased corneal staining ($P < 0.01$) compared to controls. The lipid CD formulation increased TBUT ($P < 0.05$) and tear production ($P < 0.05$) but had no significant effect on the remaining test parameters. There were no differences in macrophage infiltration and conjunctival impression cytology scores between the formulations and their vehicle controls.
Conclusions: Lipids in the rabbit Harderian gland and tears differ from those identified in human meibum and tears. These unique rabbit lipids may confer a protective effect against EDED and, as supplements to human tears, fulfill a similar role.

Keywords: dry eye disease, Harderian gland, lipid analysis, tear film, preclinical model

Introduction

Tears cover the surface of the eye, providing lubrication and protection from environmental factors. The tear film is comprised of an external lipid layer produced by the meibomian glands, and it functions to retard tear evaporation and assist in spreading tears across the surface of the eye.[1] A middle aqueous layer is produced by the main and accessory lacrimal glands, while the inner mucin layer of the tear film is produced by the conjunctiva goblet cells and the ocular surface epithelium.

When the tear film is compromised, dry eye disease occurs, affecting $\sim$34 million Americans and over 300 million globally.[2] At least 50% of dry eye patients have evaporative dry eye disease (EDED), caused by instability of the tear film due, in part, to the insufficiency or composition of lipids, leading to more rapid aqueous evaporation. Therefore, a significant need exists to improve tear stability and avoid EDED.

In addition to the production of lipids by the meibomian gland, the Harderian gland is a source of tear film lipids.[3,4] The Harderian gland is found in all groups of terrestrial vertebrates. However, it is absent in some mammals, including humans. While early anatomical and histological studies of the Harderian gland were highly informative, little was determined about its secretory lipids due to limitations in the instrumentation available at the time. Overall, there have been few structural determinations of the lipids in the Harderian gland and their contribution to tears.

[1]Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, Texas, USA.
[2]Department of Surgical & Radiological Sciences, School of Veterinary Medicine, University of California, Davis, Davis, California, USA.
[3]MCAL Therapeutics, Inc., Park City, Utah, USA.
[4]Department of Ophthalmology and Visual Sciences, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin, USA.
[5]Casey Eye Institute, School of Medicine, Oregon Health & Science University, Portland, Oregon, USA.

Tear film breakup time (TBUT) and blinking rate correlate with the stability of the tear film. Since mouse, dog, and rabbit are often used for models of dry eye disease, they have been thoroughly investigated.[5,6] TBUT in the mouse is $\sim$7 s,[7] which is near the lower range of normal TBUT values measured in humans (10–45 s).[8] TBUT in canines is just under 20 s.[9] The blinking rate in rodents is $\sim$10/min[10] and in adult canines roughly 10–15/min,[11] which are close to the values obtained in humans (10–20 blinks/min).[12] In contrast, TBUT in rabbits can be extremely long (up to 30 min) and rabbits seldom blink (once every 10–20 min).[13] Yet, rabbits do not exhibit dry eye, despite their potentially harsh environmental conditions. Generally, therefore, rodents and dogs may serve as better models of human dry eye disease, but rabbits may be better suited to identify lipids that potentially can stabilize the tear film for longer periods, thus avoiding evaporative disease. The importance of the Harderian gland in providing these lipids is supported by the finding that the removal of the rabbit Harderian gland independent of the meibomian and lacrimal glands results in severe dry eye.[14]

In the studies reported here, the lipid composition of the rabbit Harderian gland and rabbit tears was investigated and revealed a small and unique family of lipids that resemble ether ester and ether-diester groups, which differ in the length of their carbon chains (C_{16}–C_{18}). These related lipids were not found in human tears in the current study, they were not observed in the meibomian secretions and tears of the mouse, dog, or humans[5] among major lipid species, nor were they identified in any databases during our mass spectrometry (MS) studies. We hypothesize that these unique nonpolar lipids secreted by the Harderian gland become an essential part of the protective tears, thereby making the ocular surface less susceptible to dry eye. We report that treatment with such lipids demonstrates statistically significant increases in TBUT and tear production and decreased fluorescein staining of the cornea in an animal model of dry eye. No toxicity was associated with lipid supplementation.

Methods

Reagents

Lipid standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Hydropropyl-β-CD was purchased from Ashland Specialty Chemical Company (Jacksonville, AR). HPMC 2910 is a product of Shin-Etsu (Tokyo, Japan). All other reagents were available from MilliporeSigma.

Collection of rabbit and human tears and preparation of extracts from the rabbit Harderian gland

Tears from 2 anesthetized male New Zealand white rabbits and 2 anesthetized female rabbits were collected using a capillary tube placed just outside the lower internal canthus or close to the nictitating membrane.[15] A ninefold volume of methanol was added, and samples were immediately stored at –80°C. The same procedures were used to collect tears from 3 male and 2 female rabbits without topical proparacaine. No significant differences were observed between male and female specimens, nor were differences observed between rabbits exposed to anesthesia and those that were not.

Initially, 4 Harderian glands were removed from 2 male and 2 female rabbits and stored at –80°C. Chloroform:methanol (2:1, v:v) was used to extract lipids from the tissue cut into small pieces to facilitate the lipid transfer. The samples were processed using methods described previously.[16] Additional glands were collected and processed for further analyses (Fig. 2) using the same procedures.

Human tears were collected using Schirmer test strips (Alcon Laboratories, Ft. Worth, TX), and then extracted 3 times with chloroform:methanol (2:1, v:v), dried, and processed as described previously.[15] Samples from both eyes were combined from each volunteer and 3 volunteers were analyzed.

Procedures involving rabbits were approved by the Institutional Animal Care and Use Committees at the University of Nebraska Medical Center and UT Southwestern Medical Center and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The collection of tear samples from healthy human volunteers (Institutional Review Board Approvals, UT Southwestern STU 102010-195 and UW Madison M-2009-1065-CR006) was conducted with consent according to the Declaration of Helsinki.

Lipid identification

Routine high pressure liquid chromatography (HPLC)/MS experiments were conducted as previously described.[17,18] In some experiments, nonpolar lipids also were analyzed using normal phase and reversed phase HPLC and atmospheric pressure chemical ionization (APCI) MS. The structures of individual lipids were determined by fragmentation MS/MS and multistage mass MS^n spectrometry.

Exact molecular masses of the analytes were determined using a high-resolution Synapt G2-Si QToF mass spectrometer equipped with an IonSabre APCI ion source and a LockSpray unit (Waters Corp., Milford, MA).[19] The experiments were conducted in the MS^E mode. Chromatographic separation of lipids was conducted on an Acquity M-Class ultra-HPLC (Waters Corp.) in reversed-phase mode. The elemental compositions of lipids were computed using MassLynx EleComp v.4.0. (Waters Corp.).

Lipid formulation

The compositions of the lipid emulsion and the cyclodextrin (CD) formulation are provided in Tables 1 and 2, respectively.

Table 1. Lipid Emulsion Formulation

Purpose	*Name*	*/1 mL*	*/200 mL*
API	Compound 611	1.0 mg	0.2 g
Viscosity agent	Xanthan gum	0.5 mg	0.1 g
Solubilizing agent	Span65	3.0 mg	0.6 g
Solubilizing agent	Polysorbate80	10.0 mg	2.0 g
Emulsifying agent	Castor oil	4.0 mg	0.8 g
Stabilizing agent	PEG400	10.0 mg	2.0 g
Buffer	Boric acid	6.0 mg	1.2 g
Buffer	Borax	0.6 mg	0.12 g
Isotonic agent	Propylene glycol	9.0 mg	1.8 g
pH modifier	HCl or NaoH	q.s.	q.s.
Solvent	Purified water	q.s.	q.s.

TABLE 2. LIPID CYCLODEXTRIN FORMULATION

Purpose	*Name*	*/1 mL*	*/100 mL*
API	Compound 611	1.0 mg	0.1 g
Solubilizing agent	HPβCD	100.0 mg	10.0 g
Solubilizing agent	HPMC2910	3.0 mg	0.3 g
Buffer	Anhydrous dibasic sodium phosphate	5.0 mg	0.5 g
Isotonic agent	Glycerin	6.0 mg	0.6 g
pH modifier	HCl or NaoH	q.s.	q.s.
Solvent	Purified water	q.s.	q.s.

Preclinical studies of dry eye

Testing was conducted by KNOTUS Ltd. (Republic of Korea). Thirty-five male New Zealand white rabbits, 2.0–2.5 kg (Hallym Laboratory, Republic of Korea) were acclimated for 7 days. Animals were provided water and food (Cargill Agri Purina, Inc., Minneapolis, MN) *ad libitum*. This study was performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, the Animal Experimentation Policy of KNOTUS Ltd. in compliance with the Korea Food and Drug Administration Notification No. 2014-67 and tenets of the Organization for Economic Co-operation and Development Principles of Good Laboratory Practice.

Animals were divided into 7 groups:

Group	*Sex*	*No. of animal*	*Induction of dry eye*	*Treatment*	*Dose volume ([a]drop/eye)*	*Frequency (times/day)*
G1	M	5	N	—	—	—
G2	M	5	Y	0.9% NaCl	1	2
G3	M	5	Y	Lipid/Emulsion	1	2
G4	M	5	Y	Emulsion Vehicle	1	2
G5	M	5	Y	Lipid/CD	1	2
G6	M	5	Y	CD Vehicle	1	2
G7	M	5	Y	Restasis®	1	2

([a]1 drop is about 50 μL.)

The induction of dry eye was initiated by the topical administration of 0.5% benzalkonium chloride solution once daily for 2 weeks to both eyes of each rabbit. Test articles, vehicle controls, and saline were administered from 2 weeks after the initiation of dry eye induction, 1 drop/eye/time, and 2 times/day (8-h interval) for 4 weeks. Restasis was chosen for comparison since it is the most frequently used of the few prescription drugs available for the treatment of dry eye.

Tear production was measured with Schirmer paper strips (COLOR BARTM; EagleVision, Tucson, AZ) at week 0, and 2 and 4 weeks after the initiation of treatment. Animals were anesthetized with Zoletil 50 (VIRBAC, France) and xylazine (Rompun; Bayer AG, Germany)[20] and the Schirmer paper strip was inserted into the lower lid of each right eye. The wetted length (mm) of the paper strip was read after 5 min. TBUT was measured in the right eye by staining with fluorescein strips (HAAG-STREIT AG, Switzerland) at weeks 0, 2, and 4. Corneal epithelial damage was graded with a cobalt blue light at weeks 0, 2, and 4 using the National Eye Institute grading system.

Conjunctival impression cytology[21] was assessed at weeks 0, 2, and 4, using circular disks of nitrocellulose filter paper (Pall, New York) placed in the left eye of each animal. The filter paper was pressed for 10 s and fixed with 10% neutral buffered formalin. Periodic acid-Schiff reagents were used to stain the specimens. A conjunctival impression cytology grading system was used.[22]

To detect inflammatory cells,[23] eyes were fixed with 10% neutral buffered formalin after the last evaluation. The tissues were embedded in paraffin and sectioned. Immunohistochemical stained slides (leukocyte-specific protein 1, F4/80) were prepared and examined by light microscopy. Images were taken at 200× magnification and analyzed using Image Pro Plus 7.0 (Media Cybernetics, Rockville, MD).

Statistical analyses

The results from preclinical studies were assumed to be normally distributed and analyzed by parametric multiple comparison or nonparametric multiple comparison procedures. For parametric multiple comparisons, 1-way ANOVA testing was applied. When the result of ANOVA testing was significant, Dunnett's multiple comparison test was used. For nonparametric multiple comparison procedures, a Kruskal–Wallis *H*-test was applied. When the result of testing was significant, a Dunn's multiple comparison test was used. The results of day 0 were analyzed between all groups. For the results of day 14 and 28, they were analyzed relative to the saline control (G2) with exception of the normal control G1. All statistical analyses were performed with Prism 5.03 (GraphPad Software, Inc., San Diego, CA), and the significance level was judged at $P < 0.05$.

At the next level of comparison, the treatment effect was determined as the difference between the mean change from baseline for the formulated lipids and their paired vehicle controls. Means and standard deviations were calculated from all individual data measurements for each endpoint (eg, TBUT, Fluorescein Corneal Staining and Schirmer Tear Test) at Baseline (week 0), week 2 and 4 as reported in Tables 4–6. The 95% confidence interval (CI) was calculated from the standard deviations for the mean changes from baseline for lipid and vehicle. If the 95% CIs did not touch or cross the vertical null effect line at 0, then the result was statistically significant at the $P < 0.05$ level.

Results

Identification of novel lipids in the Harderian gland and tears of the rabbit by MS

The objective of this study was to identify lipids, primarily nonpolar lipids, contained within the Harderian gland and tears of rabbits but absent in human tears. Such lipids might contribute to the remarkable stability of rabbit tears and protect the corneal surface from EDED. To accomplish this objective, APCI MS was first utilized to identify potentially novel lipids in rabbit specimens of the Harderian gland and tears. As illustrated in Fig. 1A, the mass spectrum of an extract from the rabbit Harderian gland revealed several prominent peaks in the positive ion mode used to detect nonpolar lipids. Attention became focused on peaks referred to as 1–4 at *m/z* values of 593.6, 791.5, 819.5, and 847.5, correspondingly. An additional prominent peak in the spectrum from the Harderian gland extract at the *m/z* value of 621.6 either was absent from or varied between different rabbit tear specimens (Fig. 1B), while peaks 1–4 were reproducibly detected in the tear samples.

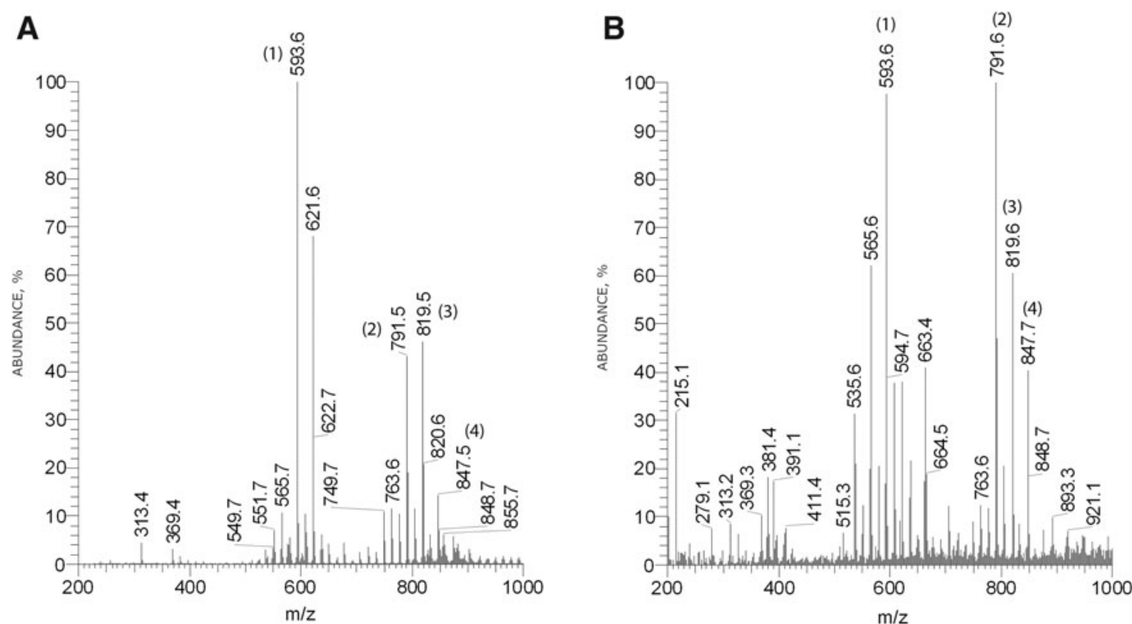

FIG. 1. Representative mass spectra of a rabbit Harderian gland extract **(A)** and a rabbit tear specimen **(B)** obtained by positive ion mode atmospheric pressure chemical ionization MS. Peaks 1–4 are denoted in *parentheses*. MS, mass spectrometry.

Additional peaks also were present in the tear spectrum, for example at *m/z* values of 535.6 and 663.4, but these peaks were absent in the lipid profile of the Harderian gland and may represent lipid contributions to the tear film originating from the rabbit meibomian gland.

The main MS peaks observed in the rabbit Harderian lipid samples were characterized further using high-resolution MS^E (Fig. 2 and Table 3). Their elemental composition indicated that compound 1 was an alkyl-acylglycerol with a formula of $C_{39}H_{77}O_3$, while compounds 2–4 were various alkyl-diacylglycerols. These lipids were present as a rather diverse mixture of various isobaric compounds as evidenced by their major fragments that differed by 1 or 2 methylene groups. Only a few selected major compounds were evaluated and listed in Table 3.

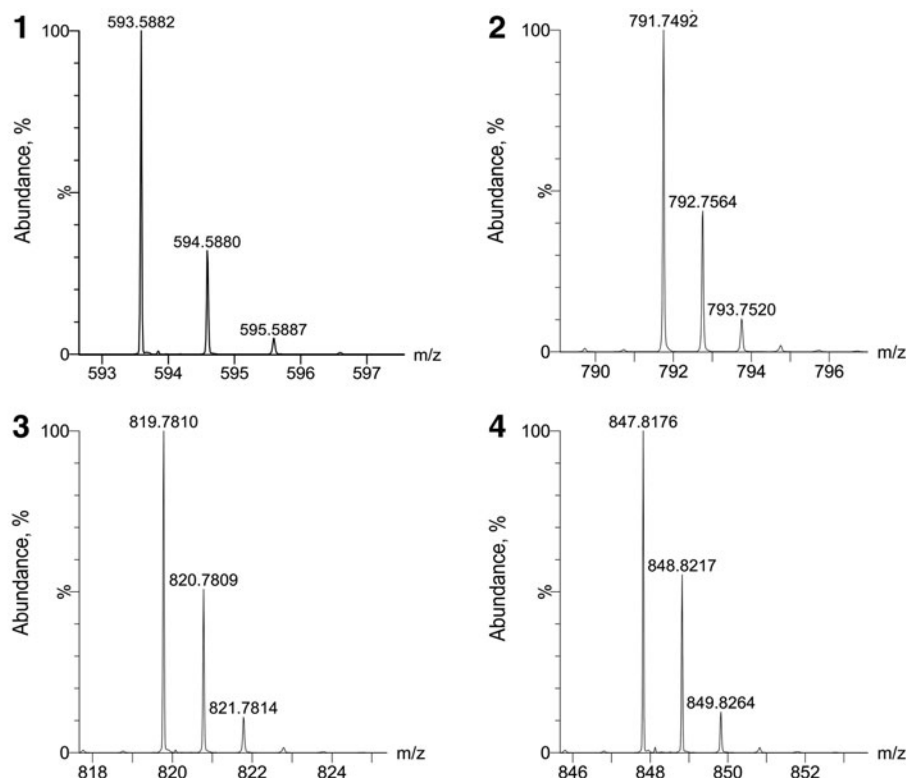

FIG. 2. Exact molecular masses of peaks **(1–4)** were determined using a high-resolution time-of-flight mass spectrometer in the MS^E mode that allowed for determination of accurate *m/z* values and major fragments of the analytes.

Table 3. Major Mass Spectrometry Signals Detected in the Rabbit Harderian Gland Extract

No.	*Experimental m/z*	*Elemental composition*	*Theoretical m/z*	*ΔM, mDa*	*Major fragment, experimental m/z*	*Elemental composition*	*Major fragment, theoretical, m/z*	*ΔM, mDa*
1	593.5882	$C_{39}H_{77}O_3$	593.5873	0.9				
2	791.7615	$C_{51}H_{99}O_5$	791.7493	12.2	535.5209	$C_{35}H_{67}O_3$	535.5090	11.9
3	819.7935	$C_{53}H_{103}O_5$	819.7806	12.9	563.5490	$C_{37}H_{71}O_3$	563.5403	8.7
					535.5109	$C_{35}H_{67}O_3$	535.5090	1.9
4	847.8176	$C_{55}H_{107}O_5$	847.8119	5.7	591.5698	$C_{39}H_{75}O_3$	591.5716	−1.8
					563.5386	$C_{37}H_{71}O_3$	563.5403	−1.7

Peaks 1–4 were consistently observed in rabbit tears, as confirmed in Fig. 3A. This spectrum was obtained from a tear sample from a different rabbit than the one used for the spectrum in Fig. 1B. Note the reproducibility of the 2 rabbit tear samples and particularly the presence of peaks 1–4. For comparison, the APCI MS spectrum of a human tear sample is shown in Fig. 3B. Peaks 1–4 were absent in the human specimen. The reproducibility of the human tear analysis can be seen by comparing the spectrum from Fig. 3B with a different human tear specimen shown in Fig. 3C. While lipid profiles from a variety of animal tears are like those associated with human tears, lipids from the rabbit were significantly different. Lipids in peaks 1–4 differed from human tear and meibum lipids, for example, in that they did not match any of the major human tear film lipids—wax esters, cholesteryl esters, triacylglycerols, etc.—and did not form anions which would be visible in the negative ion mode in HPLC/MS experiments as, for example, $(M-H)^-$ species.

To further characterize peaks 1–4, the structures of individual lipids were determined by HPLC-coupled fragmentation MS/MS. Retention times for each of the 4 peaks are

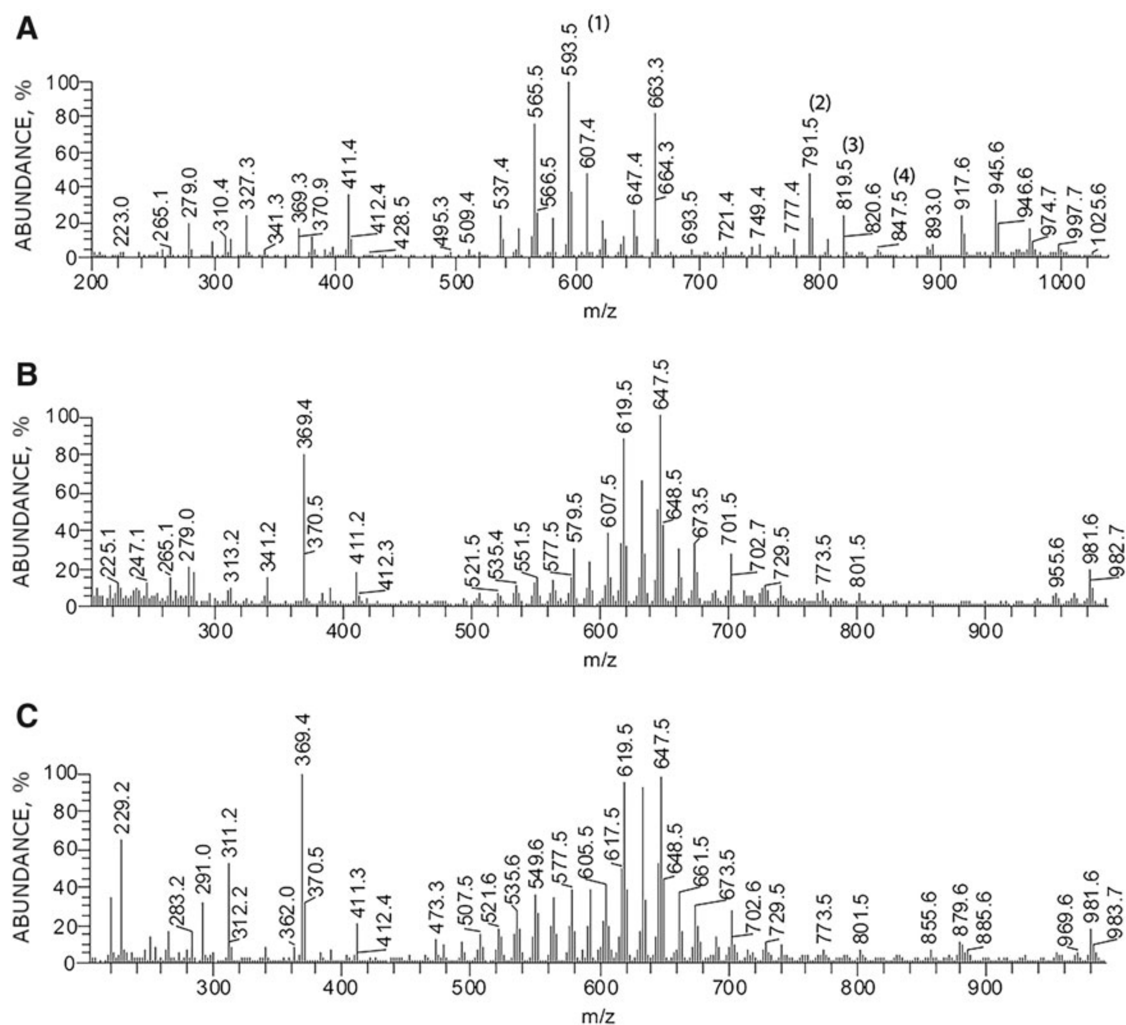

FIG. 3. Representative mass spectra of a rabbit tear specimen **(A)** and 2 human tear specimens **(B, C)** obtained by positive ion mode atmospheric pressure chemical ionization MS. Note the presence of peaks 1–4 in the rabbit specimen but not the 2 human samples.

shown in Figs. 4A–D, while the fragmentation patterns for each of the major peaks are presented in 4E–H. Where possible, the animal lipids were compared with lipid standards. As a result of these experiments, the structures of peaks 1–4 were elucidated (Fig. 5). As can be seen, compounds 2–4, that were tentatively identified as 1-(O)-alkyl-2,3-diacylglycerols (or 1-ether-2,3-diesters), are closely related and differ only by minor changes in the length of their fatty acid chains. However, a more hydrophilic peak 1 with the shortest retention time of the 4 analytes was composed of a compound with an *m/z* value of 611.59 Da (tentatively, a proton adduct of an alkyl-acylglycerol), which was unstable under the conditions of the MS experiment and underwent spontaneous in-source loss of H_2O to produce an $(M-H_2O + H)^+$ adduct with an *m/z* value of 593.59. To confirm this assignment, the fragmentation spectra of a commercially synthesized standard—1-(O)-eicosanyl-2-palmitoylglycerol (MW 611.59) and compound 1 from a rabbit specimen are shown in Fig. 6, upper and lower panels, respectively. The fragmentation spectrum of the standard matched that of the rabbit lipid, confirming the structural assignments.

Formulation of a novel lipid from the Harderian gland and tears of rabbit

To determine whether lipids corresponding to peaks 1–4 could enhance tear stability and thereby provide a protective role against dry eye, the lipid identified in peak 1 as 1-(O)-eicosanyl-2-palmitoylglycerol was synthesized and formulated for preclinical trials. As expected by its structure, lipid 1 was insoluble in aqueous solutions, forming 100–200 μm diamond-shaped particles at room temperature, as observed by light microscopy. Two initial strategies therefore were adopted to formulate lipid 1 for drug delivery; the first involved the formulation of an emulsion, while the second strategy focused on the development of a CD clathrate. The final formulation of lipid 1 as an emulsion is given in Table 1. The white, opaque emulsion, with an osmolality of 267 mOsmol/kg and pH = 7.0, remained stable at room temperature for at least 8 weeks. The final CD formulation is provided in Table 2. The final white, opaque formulation, with an osmolality of 290 mOsmol/kg and pH = 7.2, also remained stable at room temperature for at least 8 weeks.

Preclinical studies of dry eye using 2 formulations of a novel lipid associated with the Harderian gland and tears of rabbits

The different control and experimental groups comprising the preclinical study were provided in Methods section. Five measurements were made to assess the degree of dry eye for each of the treatment and control conditions: TBUT, tear production, corneal staining, macrophage score, and conjunctival impression cytology. During the studies, there were no unscheduled deaths or notable clinical signs related with treatment.

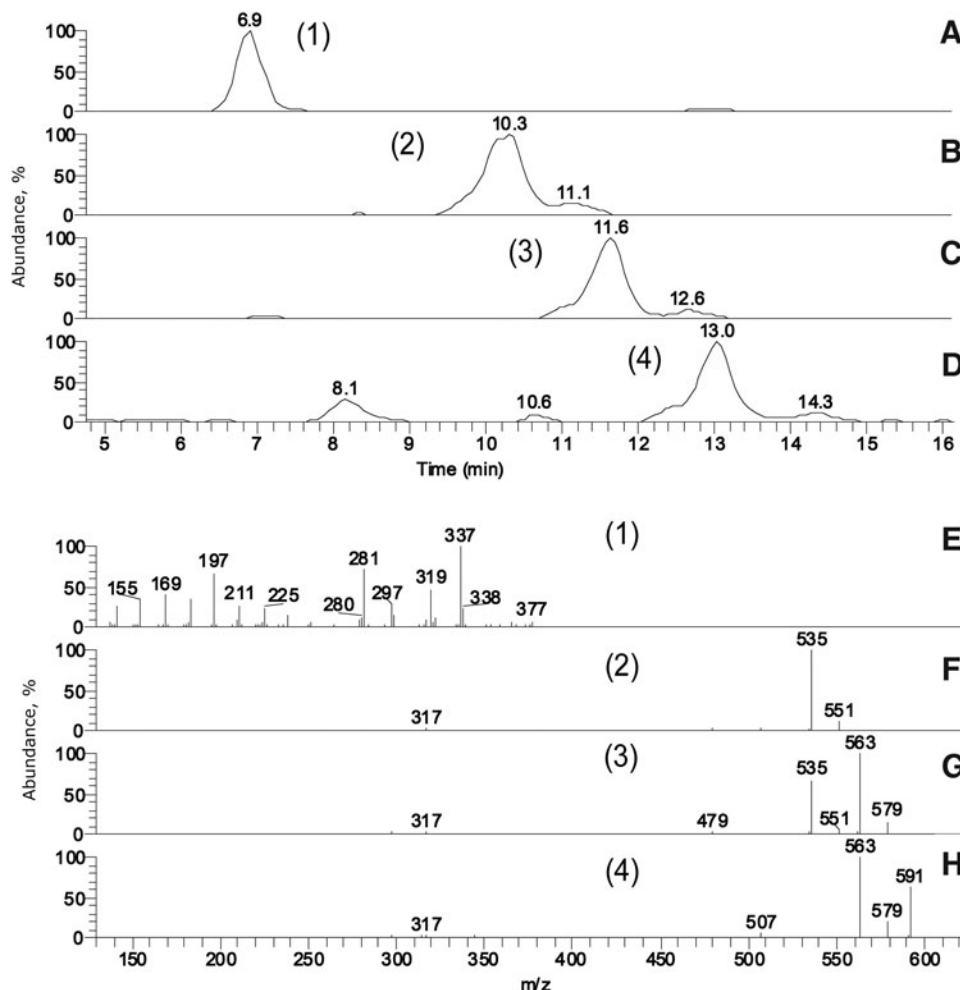

FIG. 4. Structures of individual lipids corresponding to peaks 1–4 were determined utilizing HPLC-coupled fragmentation MS/MS. Extracted ion chromatograms of analytical ions with *m/z* values of 593.5, 791.5, 819.5, and 847.5 are shown in panels **(A–D)**, respectively. Retention times for each of the 4 peaks are shown in the *top portion* of the figure, while the fragmentation patterns for each of the major peaks are presented in the *bottom panels* **(E–H)**. HPLC, high pressure liquid chromatography.

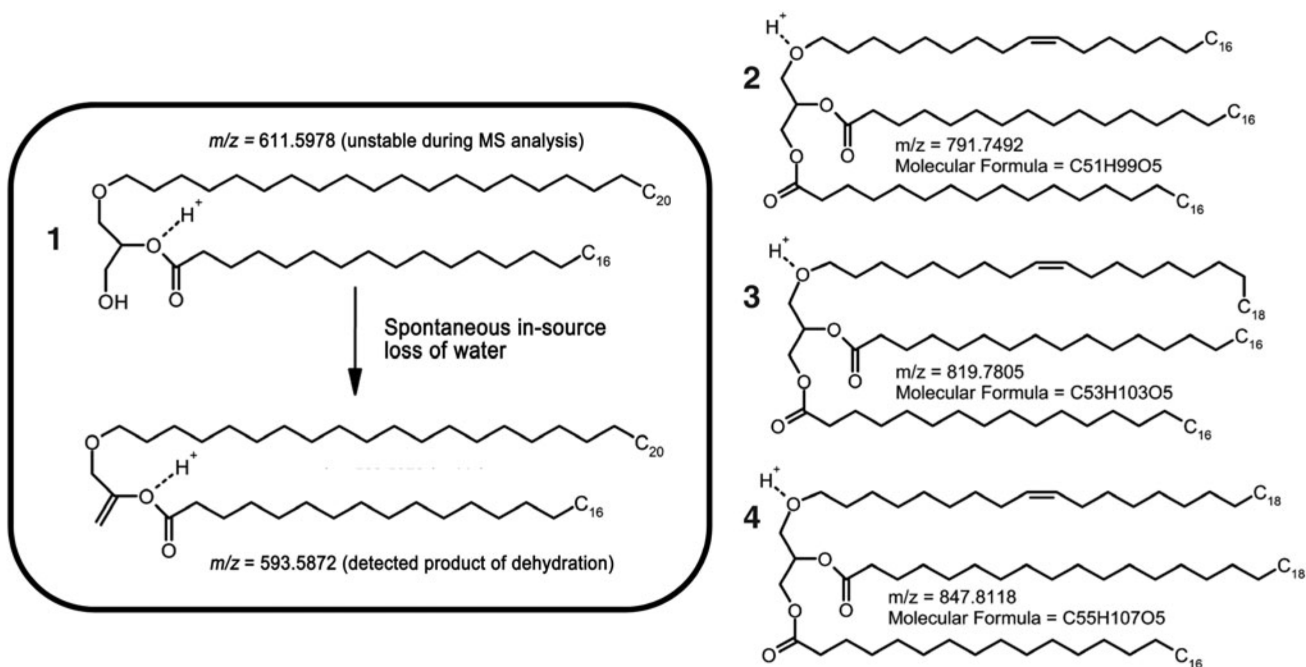

FIG. 5. Lipid structures associated with peaks **(1–4)**. Compound 1, which is stable in normal conditions, in MS experiments undergoes spontaneous insource loss of water to form a stable analytical ion $(M-H_2O + H)^+$ with *m/z* 593.58.

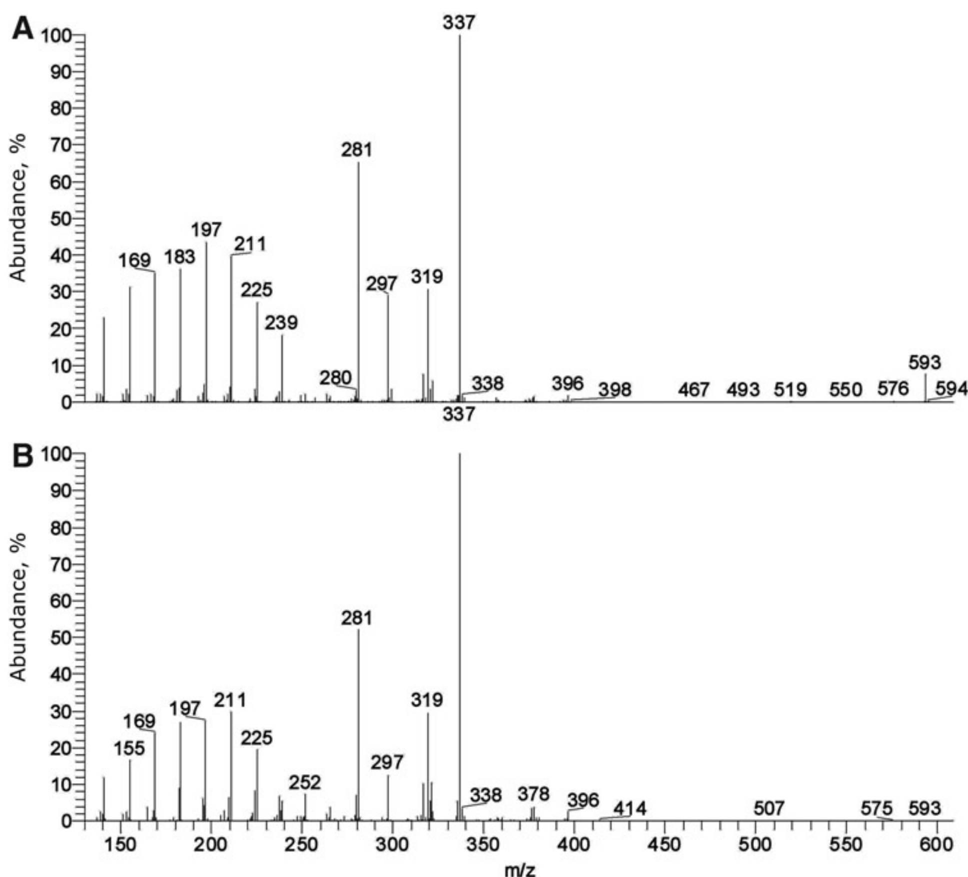

FIG. 6. The structural assignments of lipids were confirmed by comparing the fragmentation spectra of a commercially synthesized MW 611 standard **(A)**, for example, and the lipid corresponding to peak 1 from a rabbit Harderian gland extract **(B)**. Both compounds had identical *m/z* values and fragmented in similar manner.

Table 4. Tear Break-up Time

	TBUT			Unit: s
	Week			
Group	Week 0	Week 2	Week 4	
G1	29.2 ± 4.3	28.6 ± 3.6	29.2 ± 3.0	
G2	8.8 ± 0.8***	7.8 ± 0.4***	7.6 ± 0.5***	
G3	8.8 ± 1.6***	9.4 ± 2.1***	10.4 ± 1.5***,##	
G4	9.0 ± 1.2***	8.6 ± 0.9***	8.8 ± 0.4***	
G5	8.6 ± 0.9***	9.0 ± 0.7***	9.4 ± 0.9***,#	
G6	8.6 ± 1.1***	8.6 ± 0.5***	8.2 ± 1.1***	
G7	9.0 ± 1.6***	9.6 ± 1.5***	10.8 ± 1.3***,###	

Data are expressed as mean ± SD.
***A significant difference at $P < 0.001$ level compared to G1.
###/##/#A significant difference at $P < 0.001/P < 0.01/P < 0.05$ level compared to G2.
G1, normal control; G2, saline control; G3, lipid emulsion; G4, lipid emulsion vehicle; G5, lipid cyclodextrin (CD) formulation; G6, lipid CD formulation vehicle; G7, Restasis®; TBUT, tear film breakup time.

Tear film breakup time

The results of the TBUT analysis are given in Table 4. All test and control conditions showed a statistically significant decrease ($P < 0.001$) in TBUT compared to the normal control without dry eye beginning at day 0 of the study and continuing through day 28.

Next, treatment with both the lipid emulsion and the lipid CD formulation showed a significant increase in TBUT ($P < 0.01$ and $P < 0.05$, respectively) relative to the saline control by day 28. The lipid emulsion and Restasis showed comparable results. In contrast, TBUT values did not increase with treatment with the vehicle controls for the lipid emulsion or the lipid CD formulation compared to the saline control.

A further analysis was then performed to compare the lipid emulsion and the lipid CD formulation with their paired vehicle controls. The increase in TBUT, observed with the lipid emulsion was significantly different when compared with its vehicle control (Fig. 7A). The lipid emulsion reached statistical significance by day 28 ($P < 0.05$). The lipid CD formulation also reached statistical significance compared with its vehicle control by day 28 ($P < 0.05$) as shown in Fig. 7B.

Tear production

In this study, the tear production associated with all test and control conditions was significantly lower than the normal control group without dry eye at day 0 and persisted through day 28 ($P < 0.001$), Table 5.

The tear production of the lipid emulsion group and Restasis was significantly higher than the saline control after 14 days from the day of test article administration ($P < 0.05$ and $P < 0.001$, respectively) and persisted through day 28 ($P < 0.05$ and $P < 0.01$, respectively).

The increase in tear production observed with the lipid emulsion also was significantly different in comparison with its vehicle control (Fig. 7A). The lipid emulsion reached statistical significance by day 28 ($P < 0.05$). The lipid CD formulation also reached statistical significance compared with its vehicle control by day 28 ($P < 0.05$) as shown in Fig. 7B.

Fluorescein staining

Fluorescein staining scores associated with the lipid emulsion and Restasis were significantly lower than the saline control group after 28 days of treatment ($P < 0.01$ for both; Table 6).

The decrease in corneal staining observed with the lipid emulsion also was significantly different in comparison with its vehicle control (Fig. 7A). The lipid emulsion reached statistical significance from its vehicle control by day 28 ($P < 0.05$). However, the lipid CD formulation group was not statistically different from the saline control (Table 6) or its own vehicle control group within the timespan of this study (Fig. 7B).

Conjunctival impression cytology

The conjunctival impression cytology score for Restasis was significantly lower than that of the saline control ($P < 0.05$), Table 7. However, there were no statistically

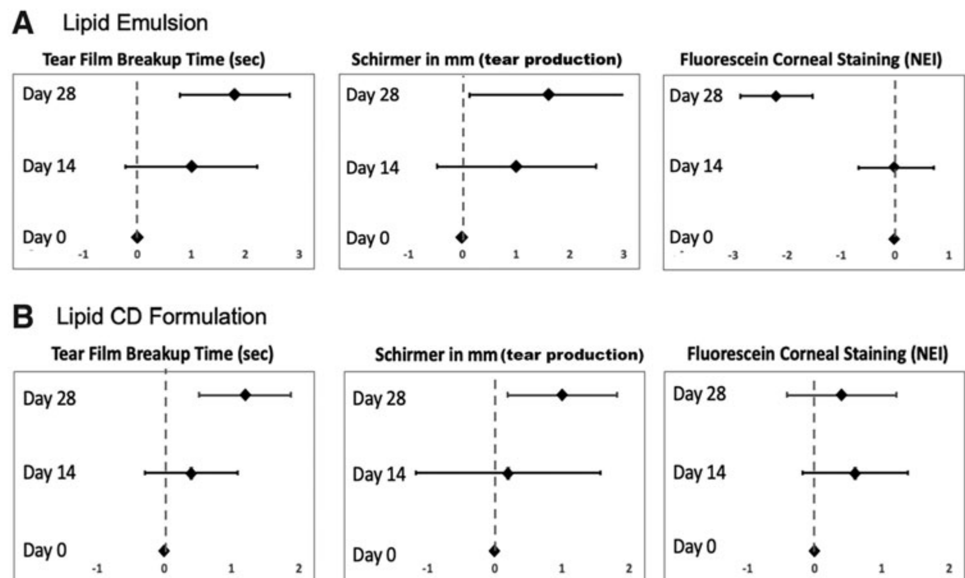

FIG. 7. Forest plots of Therapeutic Effect (*black diamond*) for lipid emulsion **(A)** and lipid cyclodextrin formulation **(B)** with 95% confidence intervals (*horizontal lines* on either side of *black diamond*). Therapeutic effect is the difference in mean change from baseline for lipid treatment minus vehicle control (Placebo). *Dashed vertical line* at 0 is the null effect and results are statistically significant at $P < 0.05$ level when the confidence intervals do not touch or cross the null effect.

Table 5. Tear Production

	Tear production		*Unit: mm*
		Week	
Group	*Week 0*	*Week 2*	*Week 4*
G1	29.4 ± 5.0	28.8 ± 5.3	29.8 ± 3.0
G2	12.6 ± 2.1***	11.4 ± 1.3***	11.6 ± 1.1***
G3	12.8 ± 2.6***	14.2 ± 1.3***,#	15.2 ± 1.9***,#
G4	12.4 ± 0.9***	12.8 ± 2.2***	13.2 ± 1.5***
G5	12.8 ± 1.5***	13.4 ± 1.5***	14.2 ± 1.8***
G6	12.2 ± 2.3***	12.6 ± 1.3***	12.6 ± 2.4***
G7	12.2 ± 0.8***	15.6 ± 0.9***,###	16.2 ± 1.5***,##

Data are expressed as mean ± SD.
***A significant difference at $P < 0.001$ level compared to G1.
###/##/#A significant difference at $P < 0.001/P < 0.01/P < 0.05$ level compared to G2.

Table 7. Conjunctival Impression Cytology

	Conjunctival impression cytology		*Unit: score*
		Week	
Group	*Week 0*	*Week 2*	*Week 4*
G1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
G2	3.2 ± 1.3	3.8 ± 0.8**	4.0 ± 0.7***
G3	2.8 ± 1.3	2.4 ± 1.7	2.2 ± 1.1
G4	3.0 ± 1.4	3.4 ± 0.9*	3.4 ± 1.1*
G5	3.0 ± 1.2	2.6 ± 0.9	2.6 ± 1.1
G6	3.2 ± 1.5	3.0 ± 0.7	3.2 ± 0.8*
G7	3.2 ± 1.3	2.2 ± 1.1	1.8 ± 0.8#

Data are expressed as mean ± SD.
***/**/*A significant difference at $P < 0.001/P < 0.01/P < 0.05$ level compared to G1.
#A significant difference at $P < 0.05$ level compared to G2.

significant differences between the saline control and the 2 lipid formulations or between the 2 formulations and their vehicle controls.

Immunohistochemical staining

In this study, the number of macrophages was determined by immunohistochemical staining (Table 8). The number of macrophage positive cells in the lipid emulsion and Restasis groups was significantly lower than the saline control ($P < 0.05$ and $P < 0.01$, respectively). However, least squares mean differences (95% CI) from baseline between the lipid emulsion and its vehicle control did not reach statistical significance.

In summary, the lipid 1 emulsion increased TBUT and tear production, while it decreased fluorescein staining compared to its vehicle control in an animal model of dry eye. The lipid 1 CD clathrate also increased TBUT and tear production compared to its vehicle control, but had no statistically significant effect on fluorescein staining within the timespan of the study. Overall, the results from the current study demonstrate that novel lipids from the rabbit Harderian gland have the potential to improve tear stability in humans and protect against EDED.

Discussion

Dry eye disease can arise either from a decrease in the production of tears or the loss of water from the tear film by evaporation. Decreased tear production is caused most often by inflammation and damage to the lacrimal gland. Fortunately, prescription eye drops such as cyclosporine (Restasis), lifitegrast (Xiidra), or loteprednol (Eysuvis) are available for the treatment of the inflammatory component of dry eye disease.[24] However, EDED accounts for 50%–80% of all dry eye cases and is caused most often by a deficiency in the meibomian glands leading to aberrations in the tear film lipids. Unfortunately, for most patients, there is no prescription drug to deal effectively with EDED. Rather, such patients often rely on short-acting over-the-counter lubricants to help ameliorate their discomfort.

In this study, we demonstrate that novel lipids are produced by the Harderian gland of the rabbit and found in rabbit but not human tears. They may be responsible for the exceptional tear stability observed in rabbits compared to humans and other animal species. We determined that treatment with one of these lipids increased TBUT and tear production, while decreasing corneal staining compared to

Table 6. Fluorescein Staining Score

	Fluorescein staining score		*Unit: score*
		Week	
Group	*Week 0*	*Week 2*	*Week 4*
G1	0.2 ± 0.4	0.4 ± 0.9	0.0 ± 0.0
G2	7.8 ± 0.8*	7.6 ± 1.7**	7.4 ± 0.5***
G3	7.4 ± 1.1	5.8 ± 0.8	4.0 ± 0.7##
G4	7.6 ± 0.5	6.0 ± 1.0	6.4 ± 0.9*
G5	7.0 ± 0.7	6.4 ± 0.9	5.8 ± 0.8
G6	7.8 ± 1.5*	6.6 ± 0.5*	6.2 ± 0.8*
G7	7.6 ± 0.5	5.6 ± 0.5	3.8 ± 0.8##

Data are expressed as mean ± SD.
***/**/*A significant difference at $P < 0.001/P < 0.01/P < 0.05$ level compared to G1.
##A significant difference at $P < 0.01$ level compared to G2.

Table 8. Immunohistochemistry

Group	*Immunohistochemistry Tests Cell count*
G1	7.0 ± 2.6
G2	259.2 ± 118.7***
G3	124.4 ± 36.9#
G4	202.0 ± 83.4***
G5	137.0 ± 54.6*
G6	170.4 ± 43.7**
G7	59.2 ± 28.7##

Data are expressed as mean ± SD.
***/**/*A significant difference at $P < 0.001/P < 0.01/P < 0.05$ level compared to G1.
##/#A significant difference at $P < 0.01/P < 0.05$ level compared to G2.

vehicle controls in an animal model of dry eye. The supposition is that these lipids can act as supplements to mitigate dry eye symptoms in humans, but this determination awaits clinical trials.

While the data presented here are compelling, further investigations are warranted. It is reasonable to assume that the novel lipids reported here acted by stabilizing the tear film. However, determining the precise mechanism of action of these lipids could be furthered by *in vitro* studies, for example, measuring real-time surface-tension, interfacial dilatational visco-elastic properties, as well as other parameters[25–28] of lipid layers in the presence and absence of the novel rabbit lipids. Additional *in vivo* measurements of tear film thickness and tear film dynamics using optical coherence tomography also could help establish the mechanism of action of the rabbit lipids in animal models of EDED.[29]

In this report, we did not determine whether the lipids identified in rabbits are present in other mammalian species with Harderian glands. Unfortunately, the number of such species is vast, making the evaluation difficult. Until now, most research has been limited to those animals most often used as models of dry eye, including rodents, rabbits, and dogs.

Finally, it should be noted that while the lipids utilized in the studies reported here were synthesized, they correspond to natural products. Long-term toxicity therefore seems unlikely, but that also will require further investigation along with determining the proper dosing regimen and durability of the lipids in a final formulation.

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Author Disclosure Statement

A.S.P. and D.M.A. are inventors of U.S. patents US9289494B2 and US9994511B2, whose value may be affected by this publication. T.G. is the Executive Chairman of MCAL Therapeutics, whose value may be affected by this publication. Remaining authors have no competing financial interests.

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Address correspondence to:
Dr. Daniel M. Albert
Casey Eye Institute
School of Medicine
Oregon Health and Science University
515 SW Campus Drive
Portland, OR 97239
USA

E-mail: albedan@ohsu.edu