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Topical Omega-3 and Omega-6 Fatty Acids for Treatment of Dry Eye

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Objective: To study the efficacy of topical application of alpha-linolenic acid (ALA) and linoleic acid (LA) for dry eye treatment.

Methods: Formulations containing ALA, LA, combined ALA and LA, or vehicle alone, were applied to dry eyes induced in mice. Corneal fluorescein staining and the number and maturation of corneal CD11b⁺ cells were determined by a masked observer in the different treatment groups. Real-time polymerase chain reaction was used to quantify expression of inflammatory cytokines in the cornea and conjunctiva.

Results: Dry eye induction significantly increased corneal fluorescein staining; CD11b⁺ cell number and major histocompatibility complex Class II expression; corneal IL-1 α and tumor necrosis factor α (TNF- α) expression; and conjunctival IL-1 α , TNF- α , interferon γ , IL-2, IL-6,

and IL-10 expression. Treatment with ALA significantly decreased corneal fluorescein staining compared with both vehicle and untreated controls. Additionally, ALA treatment was associated with a significant decrease in CD11b⁺ cell number, expression of corneal IL-1 α and TNF- α , and conjunctival TNF- α .

Conclusions: Topical ALA treatment led to a significant decrease in dry eye signs and inflammatory changes at both cellular and molecular levels.

Clinical Relevance: Topical application of ALA omega-3 fatty acid may be a novel therapy to treat the clinical signs and inflammatory changes accompanying dry eye syndrome.

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RY EYE SYNDROME (DES) IS a highly prevalent health problem that affects more than 10 million people, primarily women, in the United States alone.^{1,2} It is a frequent cause of office visits due to ocular discomfort and commonly leads to problems with sustained visual activities such as reading and driving.3 Inflammation has been recognized as an important component of DES.4 The recently introduced topical cyclosporin A (Restasis; Allergen, Irvine, California) has been shown to decrease ocular surface inflammation, stimulate tear production, and improve signs and symptoms of dry eye,⁵ further signifying the role of inflammation and anti-inflammatory agents for dry eye treatment.

Naturally occurring essential polyunsaturated fatty acids (PUFA) of omega-3 (n-3) and omega-6 (n-6) series are promising natural anti-inflammatory agents shown to have beneficial effects in many inflammatory conditions such as rheumatoid arthritis and ulcerative colitis.⁶ The n-3 FAs include alpha-linolenic acid (18: 3n-3; ALA) and its elongation and desaturation products, stearidonic acid (18:4n-3), eicosapentaenoic acid (20:5n-3; EPA), and docosahexaenoic acid (22:5n-3; DHA). The n-6 FAs include linoleic acid (18: 2n-6; LA) and its products, gammalinolenic acid (18:3n-6; GLA), dihomogammalinoleic acid (20:3n-6; DGLA), and arachidonic acid (20:4n-6; AA). Both ALA and LA are called "essential" FAs because they cannot be synthesized by mammals and must be supplied in diet.

Recent studies have shown beneficial effects of dietary supplementation of FAs in DES.⁷⁻⁹ In a cross-sectional study of 32 470 women, women with a higher n-3 FA intake (more than 5-6 tuna servings per week as opposed to less than 1) were found to have 68% lower prevalence of DES.⁹ In 2 randomized clinical trials, oral supplementation with LA and GLA ameliorated the signs and symptoms of dry eye.^{7.8} It is postulated that when the n-6 to n-3 ratio is approximately 4:1 or lower, the conversion of DGLA to AA undergoes competitive inhi-

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bition with enhanced metabolism of DGLA to prostaglandin E1 (PGE1) series,¹⁰ an eicosanoid with antiinflammatory properties. In aggregate, these data indicate that n-3 and n-6 FAs may play a role in the pathogenesis and treatment of DES. However, several important issues remain unresolved, in particular whether FAs can be provided topically, thereby bypassing excess caloric intake and gastrointestinal adverse effects associated with their oral supplementation. The purpose of this study was to evaluate the efficacy of topical n-3 and n-6 FAs using the controlled environmental chamber murine model of dry eye.¹¹

METHODS

INDUCTION OF DRY EYE

All animals were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Schepens Eye Research Institute Animal Care and Use Committee. The details of the controlled environmental chamber and dry eye end points have been published previously.11 Dry eye was induced in 6- to 10-week-old C57BL/6 mice (Taconic Farms, Germantown, New York) for variable periods ranging from 2 to 10 days. Mice were placed in the controlled environmental chamber (relative humidity <30%, airflow 15 L/min, temperature 21-23°C),11 modified with subcutaneous scopolamine administration for maximal ocular dryness.11 Scopolamine (Sigma-Aldrich, St Louis, Missouri) was injected in dorsal skin of mice (0.5 mg per 0.2 mL at 9 AM, 12 PM, and 3 PM; 0.75 mg per 0.3 mL at 6 PM). Controls were age-matched mice (relative humidity >80%, no airflow, temperature 21-23°C, no scopolamine).

TOPICAL FA FORMULATIONS AND TREATMENT REGIMEN

Formulations tested included 0.2% ALA, 0.2% LA, and 0.1% ALA to 0.1% LA (1:1 ratio of n-3/n-6, total FA amount equal to the individual FA formulations). The FAs are water insoluble and hence require emulsification with compatible surfactants. The vehicle used consisted of the surfactants and emulsifiers Tween-80 (2.6%) and Glucam E-20 (2.6%), vitamin E as an antioxidant, mixed with a packing solution (water, boric acid, sodium borate, sodium chloride, and ethylenediamine-tetraacetic acid) and prepared in an emulsion (Johnson and Johnson Vision Care, Inc, Jacksonville, Florida).

Forty-eight hours after dry eye induction, each eye was randomized to receive one of the formulations or the vehicle. One microliter eye drop was applied topically to the eye of unanesthetized mouse once daily from 48 hours to day 4 (total 3 doses) or day 9 (total 8 doses) depending on the time point studied. The untreated group received no eye drops. Signs of dry eye were measured 24 hours after the last dose (day 5 or day 10). Mice were then killed for cellular and molecular studies.

MEASUREMENT OF CORNEAL FLUORESCEIN STAINING

Corneal fluorescein staining was performed at baseline (day 0), 48 hours (before administration of the first eye drop dose), day 5, and day 10. One microliter of 5% fluorescein was applied into the inferior conjunctival sac as previously described.¹² Eyes were flushed with phosphate-buffered saline (PBS) to remove excess fluorescein at 3 minutes and examined with slitlamp biomicroscope in cobalt blue light. Punctate staining was recorded in a masked fashion using a standardized National Eye Institute grading system of 0 to 3 for each of the 5 areas of the cornea.¹³ Kruskal-Wallis and Mann-Whitney tests (unpaired data set) and Wilcoxon test (paired data set) were used for statistical analysis.

IMMUNOHISTOCHEMICAL STAINING

The following primary antibodies (BD Pharmingen, San Diego, California) were used for immunohistochemical staining: FITCconjugated rat antimouse CD11b (monocyte/macrophage marker, catalog No. 557396; isotype FITC-conjugated rat antimouse IgG2bk, catalog No. 553988), purified hamster antimouse CD3e (T-cell marker, catalog No. 553057; isotype purified hamster IgG₁, catalog No. 553969), biotin-conjugated rat antimouse GR-1 (neutrophil marker, catalog No. 553124, isotype biotin-conjugated rat IgG2b, catalog No. 553987), biotin-conjugated rat antimouse Iab (C57BL/6 major histocompatibility complex [MHC] Class II marker, catalog No. 553546; isotype biotin-conjugated mouse IgG2bk, catalog No. 559531). The secondary antibodies (Jackson Laboratories, Bar Harbor, Maine) included Cy3conjugated goat anti-Armenian hamster (code No. 127165-160) and Cy3-conjugated Strepavidin antibodies (code No. 016-160-084). For whole-mount immunofluorescence corneal staining, freshly excised corneas were washed in PBS and acetone fixed for 15 minutes. Nonspecific staining was blocked with anti-FcR CD16/CD32 antibody (BD Pharmingen, catalog No. 553142), and Strepavidin and Biotin blocking solutions (Vector Laboratories, Burlingame, California). Next, the specimens were immunostained with primary or isotype antibodies for 2 hours, washed with PBS, incubated with secondary antibodies, and mounted using Vector Shield mounting medium (Vector Laboratories). Whole-mount corneal images were taken using confocal microscope (Leica TCS 4D; Lasertechnik, Heidelberg, Germany). Cells were counted in 8 to 10 areas each in the periphery (0.5-µm area from the limbus) and the center (central 2-µm area) of the cornea in a masked fashion using an epifluorescence microscope (model E800; Nikon, Melville, New York) at \times 40 magnification. The mean number of cells were obtained by averaging the cell number in the 8 to 10 areas studied. Cell number was compared using 1-way analysis of variance (ANOVA), followed by pairwise comparisons adjusted for multiple comparisons by the least significant difference method. P values less than .05 were deemed statistically significant.

RNA ISOLATION, REVERSE TRANSCRIPTASE– POLYMERASE CHAIN REACTION, AND REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was isolated from the cornea and conjunctiva (2 pooled corneas per group and 6 pooled conjunctivae per group) using Trizol (Invitrogen, Carlsbad, California, catalog No. 15596-026) for tissue homogenization and 70% ethanol for RNA precipitation, followed by extraction and purification using RNeasy Microkit (Qiagen, Valencia, California, catalog No. 74004). RNA was stored at -80° C until further use.

The first strand complementary DNA (cDNA) was synthesized from 300 ng of total RNA using SuperScript III Reverse Transcriptase (Invitrogen, catalog No. 18080) per manufacturer's protocol. Real-time polymerase chain reaction (PCR) was performed with FAM-MGB dye-labeled predesigned primers (Applied Biosystems, Foster City, California) for IL-1 α (catalog No. 4329586), tumor necrosis factor α (TNF- α) (Assay ID Mm99999068_m1), GAPDH (Mm99999915_g1), IL-2 (Mm00801778_m1), IL-4 (Mm00445259_m1), IL-6 (Mm00446190_m1), IL-10 (Mm00439616_m1), and interferon γ (IFN- γ) (Mm00801778_m1) per manufacturer's pro-

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Figure 1. Real-time polymerase chain reaction results showing increased relative expression of IL-1 α and tumor necrosis factor α (TNF- α) transcripts in dry eye corneas compared with normal corneas. Asterisk indicates P<.001. Data are presented as mean and standard error (error bars); n=6 for day 0, n=4 for day 2, n=8 for day 5, and n=4 for day 10.

tocol. One microliter of cDNA was loaded in each well and assays were performed in duplicates. A nontemplate control was included to evaluate DNA contamination of isolated RNA and reagents. The results of quantitative real-time PCR were analyzed by the comparative threshold cycle (C_T) method and normalized by GAPDH as an internal control. The relative expression of cytokines at different time points was compared using 1-way ANOVA, post hoc test least significant difference with P < .05 deemed significant.

RESULTS

CLINICAL AND MOLECULAR SIGNS OF DRY EYE

Compared with day 0 (mean [SD] score, 1.3 [1.5]), corneal fluorescein staining scores were significantly higher at day 2 (5.4 [2.13]), day 5 (8.4 [1.2]), and day 10 (7.9 [3.6]) (Wilcoxon test, P=.01, n=8). No significant difference was found between groups at day 2, day 5, or day 10. Thus, dry eye induction led to a significant increase in staining that remained steadily elevated through day 10.

The normal cornea has a resident population of bone marrow–derived immature (MHC Class II–CD80–CD86–) CD11b⁺ antigen presenting cells (APCs) that acquire MHC Class II in response to inflammation.^{14,15} Induction of dry eye for 10 days increased the CD11b⁺ cell number in the periphery by 44% (mean [SEM], 240 [23.2] vs 345.8 [15], P=.02, n=3) and the center by 45% (183.4 [20.2] vs 265.5 [27.8], P=.09, n=3). MHC Class II expression by CD11b⁺ cells, an important marker for the cells' maturation and T-cell stimulatory capacity, was increased by 104% in the periphery (75.1 [8.2] vs 152.9 [33.7], P=.07, n=3) and 146% in the center (30.4 [6.5] vs 74.8 [13.6], P=.04, n=3) of the dry eye cornea.

Corneal and conjunctival expression of proinflammatory cytokines IL-1 α and TNF- α , was increased in dry eye relative to the normal eye (**Figure 1** and **Figure 2**). However, expression of T_H1 (IL-2 and IFN- γ) and T_H2 cytokines (IL-4, IL-6, and IL-10) was not detected in the cornea. On the contrary, the conjunctiva showed increased expression of IL-6 (14.5-fold), IL-2 (4.9-fold),



Figure 2. Real-time polymerase chain reaction results showing increased relative expression of various cytokine transcripts in dry eye conjunctiva (day 10) compared with normal conjunctiva. Data are presented as mean and standard error (error bars); n=18 for IL-1 α and tumor necrosis factor α (TNF- α), and n=6 for remaining cytokines. IFN- γ indicates interferon γ .



Figure 3. Topical alpha-linolenic acid (ALA) treatment produces a sustained decrease in corneal fluorescein staining compared with the vehicle-treated and untreated controls at days 5 and 10. Asterisk indicates $P_{=}.001$ vs untreated and $P_{=}.04$ vs vehicle; dagger, $P_{=}.007$ vs untreated and $P_{=}.02$ vs vehicle. Data are presented as mean and standard error (error bars); n=8 for the untreated, vehicle, ALA, and linoleic acid (LA) groups and n=6 for the combined ALA and LA treatment group.

IFN- γ (16.3-fold), and IL-10 (97.6-fold) (Figure 2). IL-4 expression was not increased.

CORNEAL FLUORESCEIN STAINING IN DRY EYE TREATED WITH FA FORMULATIONS

Two days after dry eye induction, eyes were randomized to receive 1 µL of ALA, LA, combined ALA and LA, or vehicle or no eye drops. Corneal fluorescein staining scores were measured at days 5 and 10. Only ALAtreated eyes showed a sustained and significant decrease in staining compared with the untreated and vehicle control groups at both days 5 and 10 (**Figure 3**). At day 5, all the treatment groups showed a significant decrease in staining compared with the untreated group. However, only ALA-treated eyes showed a significant decrease compared with the vehicle (45%, P=.04); no significant difference was noted between the eyes treated with LA, combined ALA and LA, and vehicle. At day 10, only the ALA-treated eyes showed a significant decrease in the corneal fluorescein staining compared with the vehicle (62%, P=.02) and untreated controls (71%, P=.007). No difference was seen between the eyes treated with vehicle, LA, and combined ALA and LA and untreated eyes at day 10 (Figure 3).



Figure 4. Alpha-linolenic acid (ALA) treatment decreases the number of CD11b⁺ cells in the periphery and center of dry eye corneas. Asterisk indicates P=.03 vs untreated; dagger, P=.001 vs untreated and P=.07 vs vehicle; and double dagger, P=.01 vs untreated and P=.03 vs vehicle. Data are presented as mean and standard error (error bars) and n=3. LA indicates linoleic acid.

ENUMERATION OF CD11b⁺ MONOCYTES IN EYES TREATED WITH FA FORMULATIONS

The number of CD11b⁺ cells was found to be significantly decreased (P=.03) in ALA-treated eyes in the center of the cornea as compared with the untreated group and the vehicle, LA, and combined ALA and LA groups (**Figure 4** and **Figure 5**). In the periphery, there was no significant difference between vehicle and ALA groups, although ALA treatment showed a significant decrease compared with untreated eyes (P=.001) (Figure 4). Treatment with ALA decreased the cell number by 37% (periphery) and 42% (center) compared with the untreated group and 21% (periphery) and 37% (center) compared with the vehicle.

CORNEAL AND CONJUNCTIVAL EXPRESSION OF IL-1α AND TNF-α IN EYES TREATED WITH FA FORMULATIONS

Among-group comparisons showed that only ALA treatment persistently decreased corneal and conjunctival expression of IL-1 α and TNF- α at days 5 and 10 compared with untreated eyes and eyes treated with vehicle, LA, and combined ALA and LA (**Figure 6** and **Figure 7**). The conjunctival expression was only studied at day 10



Figure 5. Representative confocal images of center of whole-mount corneas showing CD11b⁺ cells (green). Images show normal eyes (A); untreated eyes (B); and eyes treated with vehicle (C), alpha-linolenic acid (ALA) (D), linoleic acid (LA) (E), and combined ALA and LA (F). The number of CD11b⁺ cells is comparable with the normal (nondry) cornea only in the ALA-treated group.

since higher level of these cytokines was seen in the cornea at day 10. Only ALA-treated eyes showed significant decrease in IL-1 α expression compared with untreated corneas at day 10 (*P*=.04), significant decrease in TNF- α expression compared with untreated (*P*=.02) and vehicle-treated corneas (*P*=.04) at day 5, and a decreased trend at day 10 (*P*=.07). Expression of TNF- α in ALA-treated conjunctivae was significantly decreased at day 10 compared with untreated eyes (*P*=.004) and a downward trend was also seen in IL-1 α expression. No significant difference was seen between the untreated eyes and eyes treated with vehicle, LA, or combined ALA and LA in either IL-1 α or TNF- α expression.

COMMENT

The preponderance of evidence suggests that inflammation, whether a cause or effect or both, frequently accompanies DES in rodents¹⁶ and humans.¹⁷ Artificial tears, the most common therapy for DES, often provide temporary symptomatic relief but do not address the underlying pathogenic mechanisms that lead to DES. The current study demonstrates for the first time a beneficial effect of topical application of the n-3 FA ALA in treating the ocular signs and reversing the inflammatory changes of dry eye at both molecular and cellular levels.

The ALA-treated eyes showed a significant reversal in corneal epithelial damage, manifested by decreased fluorescein staining as compared with the untreated eyes and eyes treated with vehicle, LA, or combined ALA and LA. The exact mechanism of corneal epithelial repair in the ALA-treated eyes is unknown but could theoretically be mediated directly by ALA or its metabolites, EPA and DHA. In healthy individuals, nearly 5% to 10% of dietary ALA is converted sequentially to EPA and DHA^{18,19} by delta-5 and delta-6 desaturase enzymes. Human corneal epithelial cells express the enzyme 15-lipoxygenase (ALOX15),^{20,21} and the endogenous formation of neuroprotectin D1 (NPD1), a novel DHA-derived ALOX15 product, has been reported in the murine cornea.²² Topical NPD1 application increases the reepithelization rate in a mouse corneal wound model.²² Thus, endogenous production of NPD1 from topically administered ALA may be one of the mechanisms for reversing corneal epitheliopathy in DES.

At the molecular level, dry eye induction leads to a persistent increase in corneal expression of IL-1 α and TNF- α . These cytokines are important mediators of inflammation implicated in the pathogenesis of corneal ulceration, uveitis, and corneal transplant rejection.²³⁻²⁶ Produced constitutively in the corneal epithelium and on release by injury or death,^{27,28} IL-1 α can up-regulate TNF- α release and its own autocrine production.²⁹ Tumor necrosis factor α has been implicated as an important mediator of pathogenesis in DES.³⁰ Elevated gene expression of IL-1, IL-6, IL-8, and TNF- α in the conjunctival epithelium³¹ and a higher tear concentration of IL-1 has been reported in patients with DES.³²

Of the formulations tested, only ALA treatment was effective in decreasing the corneal and conjunctival expression of IL-1 α and TNF- α . Because these cytokines



Figure 6. Real-time polymerase chain reaction results showing that corneas treated with alpha-linolenic acid (ALA) have significantly decreased relative expression of (A) IL-1 α (asterisk indicates *P*=.04 vs untreated) and (B) tumor necrosis factor α (TNF- α) transcripts (asterisk indicates *P*=.02 vs untreated and *P*=.04 vs vehicle). Data are presented as mean and standard error (error bars); n=6 for day 5 and n=4 for day 10. LA indicates linoleic acid; mRNA, messenger RNA.



Figure 7. Real-time polymerase chain reaction results showing that conjunctiva treated with alpha-linolenic acid (ALA) has decreased relative expression of IL-1 α and tumor necrosis factor α (TNF- α) transcripts. Asterisk indicates *P*=.004 vs untreated. Data are presented as mean and standard error (error bars) and n=18. LA indicates linoleic acid; mRNA, messenger RNA.

are released early in response to epithelial cell damage and are also released by activated macrophages, the epithelial repair and decreased macrophages infiltration in the ALA-treated cornea may account for the decreased cytokine expression. Dietary ALA has been shown to decrease endotoxin-induced macrophage production of TNF- α .³³ Healthy humans, when fed an ALA-rich diet, have shown to suppress IL-1 β and TNF- α production by 30%.^{34,35} However, the precise mechanism of this suppression is not yet understood.

Our study showed a nearly 100-fold increased expression of IL-10 in dry eye conjunctiva. Interleukin 10 is produced by activated macrophages and some lymphocytes. The 2 major activities of IL-10 are to inhibit IL-1 and TNF production by macrophages and to inhibit the accessory function of macrophages in T-cell activation through reduced expression of MHC Class II.³⁶ Consequently, IL-10 inhibits both innate and T-cell-mediated immunity. The enhanced IL-10 expression may represent a regulatory mechanism in the ocular surface to promote quiescence and maintain normal homeostasis.

Our study showed the novel finding of corneal infiltration by mature MHC Class II-expressing APCs in dry eye. The normal central cornea has a resident population of CD45⁺ bone marrow-derived cells that are uniformly of a highly immature (MHC Class II-CD80-CD86-) phenotype14,37 and acquire high expression of maturation markers in response to inflammation, thereby enhancing their capacity to stimulate T-cell-mediated responses.14,15 The vast majority of these resident cells are CD11b⁺ and of a monocyte/macrophage lineage.38 Up-regulation of HLA-DR in the conjunctiva of patients with DES has been reported,³⁹ but to our knowledge, this is the first study to report corneal leukocytic infiltration and activation in dry eye. Interestingly, topical ALA application showed a reduction in the corneal leukocytic infiltration. This may be partly accounted for by the decreased cytokine expression, especially TNF- α . Both IL-1 α and TNF- α can induce corneal APC mobilization and IL-1 induction of these cells' migration is largely mediated by TNF receptor function.⁴⁰ These changes are important in disease pathogenesis and severity since the corneal APC mobilization is known to have a significant effect on the degree and rapidity of corneal immune reactions, including graft rejection⁴¹ and herpes keratitis.^{42,43}

Previous studies have shown variable effects of LA and GLA oral intake in dry eye.^{7,8,44} In 2 clinical trials, oral LA and GLA intake was shown to ameliorate dry eye associated ocular discomfort,^{7,8} corneal epitheliopathy,⁷ and lissamine green staining of the conjunctiva⁸ and increase the tear PGE1 content.⁷ However, another randomized trial comparing GLA and placebo in 90 patients with Sjögren syndrome found no significant difference in dry eye signs and symptoms between the treatment and placebo groups.⁴⁴ Another crosssectional study showed no independent relationship between dietary LA intake and dry eye.⁹

In our study, we found no beneficial effect of combined LA and ALA formulation. The lack of beneficial effect in this combination may in part be dosedependent. For example, it may be possible that a beneficial effect would be seen at higher doses of LA and ALA or with a different n-3 to n-6 ratio. However, the consistent failure of LA to demonstrate any positive effect clinically and cellularly, and its reversal of ALA therapeutic efficacy when a combination is used, suggests that at least for topical therapy, ALA (n-3 FA) is preferred. Recent data have emerged showing polymorphic variations in the desaturase enzymes, delta-5 and delta-6 desaturase, that can affect the FA composition in phospholipids.45 These insights suggest that common genetic variations in these and other genes might influence LA metabolism toward greater production of the more inflammatory eicosanoid products of AA rather than the relatively less inflammatory products of DGLA.45 Therefore, the combination of an n-3 FA (eg, ALA) with one of n-6 FA conversion products, such as DGLA, may very well demonstrate efficacy levels beyond what our current data suggest. Our present study does not compare the changes seen with topical ALA treatment with other available topical anti-inflammatory agents, such as steroids; such direct comparisons may be worthwhile future studies.

In summary, our study shows the beneficial effect of ALA omega-3 FA topical application in reversing the signs and the underlying inflammatory changes seen in dry eye. The use of these fatty acids in topical formulations to treat dry eye and potentially other inflammatory ocular surface conditions, would allow more flexibility in dosing without the accompanying systemic, in particular gastrointestinal, adverse effects that can be seen with oral intake of these fatty acids. Further studies are clearly indicated to optimize dosing and formulations that are maximally effective.

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