The Influence of a Fish Oil Dietary Supplement on Immunogenic Keratitis

Nicolos L. J. Verbey* and Nicolas J. van Haeringen†

Fish lipids contain large amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid. These fatty acids are known to have an influence on prostaglandin (PG) and leukotriene (LT) synthesis. We studied the effect of a fish oil dietary supplement on an immune-complex-induced keratitis of the rabbit eye and compared it with the effect of a sunflower seed oil dietary supplement, rich in linoleic acid. Immune complex keratitis induced by intrastromal injection of human serum albumin (HSA) was characterized by leukocyte infiltrate, neovascularization, and corneal edema. Animals given a fish oil diet showed significantly less leukocyte infiltrate, neovascularization, and corneal edema, compared to those given a sunflower seed oil diet. Invest Ophthalmol Vis Sci 31:1526–1532, 1990

Prostaglandins (PGs) and leukotrienes (LTs) play an important role in ocular inflammation.1–8 PGs contribute to the formation of edema and erythema.9,10 LTB₄ is a potent chemotactic agent that plays a role in the migration of leukocytes into sites of inflammation.11–13 These inflammatory mediators are derived from the fatty acid arachidonic acid (AA) by metabolic conversion catalyzed by the enzymes cyclooxygenase and lipoxygenase (Fig. 1). AA is released from the phospholipids of the cell membrane in response to injury or inflammation by phospholipase A₂.14 In addition to AA, some other fatty acids, such as eicosapentaenoic acid (EPA) and dihomo-γ-linolenic acid (DHGL), may also give rise to PG and LT formation. If PGs and LTs are synthesized from a fatty acid, each fatty acid has its own PG and LT counterparts with different chemical structures and biologic behaviors.15

The fatty acid composition of the diet determines the ratio in which fatty acids are incorporated into the phospholipids of the cell membrane.16 There is considerable evidence that human populations whose dietary protein is mainly derived from fish are at low risk for cardiovascular disease.17–22 A starch- and casein-derived diet containing 6.7% fish oil has shown a beneficial influence on edema formation, systemic lupus erythematosus, immunologically induced arthritis, and glomerulonephritis in experimental animals.23–26 Fish lipid contains large amounts of EPA and docosahexaenoic acid (DCHA), which are only very minor components of food derived from terrestrial plants and animals. EPA and DCHA as dietary supplements lead to diminished synthesis of PGE₂ and LTB₄ as a result of competitive inhibition of metabolism of AA in the cyclooxygenase and lipoxygenase pathways by EPA and the formation of PGE₃ and LTB₅ from EPA (Fig. 1).15–28 Rabbit, monkey, and human uvea and conjunctiva are able to metabolize EPA to PGE₃ and LTB₅ in vitro.34,35

A type III immune complex reaction of the rabbit cornea can be used as a model for testing anti-inflammatory properties of drugs. In this animal model, after intrastromal injection of antigen, the eye first remains quiet for 10–14 days. During this time, specific antibody production is generated in the local draining lymph nodes and spleen. An abrupt onset of inflammation occurs when sufficient specific antibody complexes with local antigen. These antibody–antigen complexes activate the complement cascade with the production of chemotactic factors that are responsible for polymorphonuclear cell infiltration.36 These polymorphonuclear leukocytes (PMLs) produce, among others, AA products. PGs and LTs are major factors in neovascularization and edema formation of the cornea as well as in the positive feedback mechanism of PML infiltrate formation.37

We compared the influence of a diet containing 6.7% fish oil and 2.7% sunflower seed oil with a diet containing 9.4% sunflower seed oil, rich in linoleic acid (LA), on the clinical appearance of human serum albumin (HSA)-induced immunogenic keratitis in rabbits.
Fig. 1. PGE and LTB formation from their precursor fatty acids.
DCHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; DHGL, dihomo-γ-linolenic acid. *, Competitive inhibition. HPDCHA, hydroperoxydocosahexaenoic acid.

Diet and Feeding Procedures

The rabbits were maintained on standard rabbit diet (Hope Farms, Woerden, The Netherlands) prior to commencing the experimental diet. Because sufficient control of the exact fatty acid composition of a commercial rabbit diet is not possible, we chose a semi-synthetic experimental diet in which the composition of nutrients can be controlled exactly. Both diets contained 2.7% sunflower seed oil, and the total fat was increased to 9.4% by adding either 6.7% more sunflower seed oil or 6.7% fish oil. These two diets will be referred to as “the sunflower seed oil diet” and “the fish oil diet”. The composition of the experimental diets is given in Tables 1 and 2.

Diet and water were given ad libitum to both groups. Body weight was measured twice a week.

The diet for the two groups of rabbits contained 30.1 g fat per 1.000 kcal (energy = 28%, weight = 9.4%). The fish oil was extracted from mackerel and contained 13.3% EPA (20:5) and 7.1% DCHA (22:6).

The animals were gradually acclimated to their experimental dietary supplements in five steps of 2 weeks each. At each step a higher percentage of the standard diet was replaced by the experimental diets. The animals were kept for at least 6 months on a 100% semi-synthetic diet prior to the experiments. (Materials for the diets were provided by Unilever, Vlaardingen, The Netherlands.) Weights were similar in rabbits fed with fish-oil-enriched or sunflower seed oil enriched food at the completion of this feeding period.

Parameters of Ocular Inflammation

The effect of the diet on the clinical appearance of this HSA-induced immunogenic keratitis of the rabbit eye was evaluated by measuring corneal edema.
Table 1. Composition of semi-synthetic rabbit diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Sunflower seed oil (g/1000 kcal)</th>
<th>Weight (%)</th>
<th>Energy (%)</th>
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</thead>
<tbody>
<tr>
<td>Corn starch*</td>
<td>140</td>
<td>43.7</td>
<td>49</td>
</tr>
<tr>
<td>Casein†</td>
<td>62</td>
<td>19.3</td>
<td>23</td>
</tr>
<tr>
<td>Crude fiber§</td>
<td>60</td>
<td>18.7</td>
<td>—</td>
</tr>
<tr>
<td>Salts mixture§</td>
<td>25</td>
<td>7.8</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin mixture”</td>
<td>2</td>
<td>0.6</td>
<td>—</td>
</tr>
<tr>
<td>Grass meal‖</td>
<td>1.5</td>
<td>0.5</td>
<td>—</td>
</tr>
<tr>
<td>Sulfathiazide</td>
<td>0.04</td>
<td>0.0</td>
<td>—</td>
</tr>
<tr>
<td>Fish oil</td>
<td>21.5</td>
<td>6.7</td>
<td>20</td>
</tr>
<tr>
<td>Sunflower seed oil</td>
<td>8.6</td>
<td>2.7</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>320.6</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* 4.1 kcal/g (N.V. Honig's Artikelen. Koog a/d Zaan. The Netherlands).
† 4.1 kcal/g (D.M.V., Veghel. The Netherlands).
‡ Sawdust (sterilized) (Broekman Instituut, Someren. The Netherlands).
§ Composition in milligrams: CaCO₃ 7250; CoSO₄ 0.3; Cu citrate 12; Fe(III) citrate 290; KCl 5970; KH₂PO₄ 1930; KIO₃ 0.3; MgHPO₄·3H₂O 6320; MnSO₄·4H₂O 72; Na acetate 3100; Zn citrate 35.

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1 Composition in milligrams: vitamin A acetate 12; p-amino-benzoic acid 147; biotin 0.5; cholecalciferol 5; choline 587; all-rac-o-tocopherol-acetate 60; folic acid 0.3; myo-inositol 294; vitamin K 5; nicotinamide 45; pantotenic acid (Ca salt) 17; pyridoxine 7; riboflavin 7; thiamin (mononitrate) 18.
* Union N.V., Antwerp, Belgium.
† Unimills B.V., Zwijndrecht. The Netherlands.

formation, neovascularization, and the occurrence of an annular leukocyte infiltrate in the cornea (Wessely's ring).39 These three parameters of corneal inflammation can be well observed in vivo. The clinical observation was organized in a masked fashion.

**Corneal aspect:** The number of days during which opaque rings or a diffuse completely opaque cornea were visible were recorded from color slides taken daily (flash intensity 50 mW, distance 5 cm). For each animal the values of both eyes were averaged.

**Neovascularization:** The extent of vessel growth into the cornea was determined every other day from the color slides. Diapositives were projected and enlarged 12 times. By planimetry the area with new vessels was determined as a percentage of the total corneal surface. The percentage of vessel growth was plotted against time in days, and from these graphs the area under the curve was measured by planimetry. For each animal the values of both eyes were averaged.

**Pachymetry:** A Haag-Streit slit lamp with a pachymeter fitted with central fixation lights according to Mishima and Hedbys40 was used for these measurements. The use of the fixation lights is essential in order to obtain consistent measurements of central thickness. In inflamed edematous corneas, pachymetry measurements were subject to greater inaccuracy, which, however, did not exceed a relative measurement error of 5%. From each eye the mean of three measurements was taken. For each animal the values of both eyes were averaged.

Central corneal thickness was measured before intracorneal injection with HSA and every other day from day 7 until day 29. For each animal the difference between pachymetry measurement before intra-
ocular injection with HSA and the measurement at any time thereafter was recorded as the change in corneal thickness. For each animal the corneal thickness was plotted in time, and from this graph the area under the curve was measured.

**Determination of Anti-HSA Immunoglobulins by Enzyme-Linked Immunosorbent Assay (ELISA)**

Serum was obtained from the rabbits of the two dietary regimens on day 29 after the first intracorneal injection with HSA. Microcuvettes (Gilford, Cleveland, OH) were coated with coating buffer containing 10 μg/ml of the HSA, used as antigen for the intracorneal injections on day 0. After a coating period of 1 hr at room temperature the cuvettes were washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 80. Subsequently 250-μl serum samples, diluted 1/10,000 in 0.1% Tween in PBS were added to the cuvettes. After an incubation period of 1 hr at room temperature the cuvettes were washed three times with 0.1% Tween in PBS. Subsequently the cuvettes were incubated with a 1/500 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin for 1 hr at room temperature (Nordic, Tilburg, The Netherlands). After being washed three times with 0.1% Tween in PBS, bound peroxidase-labeled antibody was developed at room temperature by adding 350 μl ABTS solution (0.16 mM 2,2-azino-di-3-ethyl-benzthiazoline-6-sulphonate [Boehringer, Mannheim, West Germany] + 0.15% H₂O₂ in 0.05 M citric acid, pH 4.0). The green reaction product was measured after 20 min in a spectrophotometer (EIA; Gilford) at 405 mm.

As a standard, a sample of purified rabbit anti-HSA immunoglobulin was used. This standard contained 10,000 ELISA units per 0.37 μg rabbit anti-HSA immunoglobulin.

**Statistical Analysis**

Data were analyzed by nonparametric methods to avoid assumptions about the distribution of the variables involved.

Wilcoxon's signed-rank test was applied for pachymetry data obtained in the treated and untreated groups during the period of inflammation. The Mann-Whitney U-test was applied for the difference in the area under the curve of neovascularization and for the difference in the duration of corneal opacification between dietary groups. Significance of difference was obtained from a table for two-tailed observations, and *P* values < 0.05 were regarded as significant. All values are given in mean ± SEM.

**Results**

**Clinical Observations**

The appearance of keratitis in rabbits fed a sunflower seed oil diet was as follows. One week to 10 days after intracorneal injection of HSA, clouding of the cornea started at the limbus, and on approximately days 14–17 a white ring of opacification, also called Wessely’s ring, formed. This ring was present for 1–8 days. Two to 4 days after Wessely’s ring was first noted, neovascularization of the cornea started from the limbus. The neovascularization progressed until approximately days 22–25 and then regressed quickly. In all cases we observed a clear cornea 30 days after the injection of the HSA.

All animals injected with HSA responded with white ring formation and neovascularization. Corneal edema formation, recorded with pachymetry, started around day 7 and lasted until day 30 (Fig. 2, Table 3).

In the rabbits fed a fish oil diet, the period of corneal opacification was significantly shorter compared to that of animals fed a sunflower seed oil diet (Fig. 3, Table 3). Moreover, the white ring frequently appeared incomplete, and corneal opacification was less intense. Vessel growth measured as area under the

![Fig. 2. Comparison of changes in mean corneal thickness during immunogenic keratitis treated with two different dietary regimens (fish oil diet, *n* = 8 or sunflower seed oil diet, *n* = 10). Δ corneal thickness was calculated from the difference between the pachymetry measurement before intracorneal injection with HSA and at any given time thereafter.](image-url)
Table 3. Immunogenic keratitis in rabbits on a supplemented diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>Duration of corneal opacity* (days)</th>
<th>Vessel growth AUC* (%)</th>
<th>Corneal thickness AUC† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower seed oil (n = 10)</td>
<td>4.8 ± 0.5</td>
<td>100 ± 15</td>
<td>100 ± 15</td>
</tr>
<tr>
<td>Fish oil (n = 8)</td>
<td>2.6 ± 0.6‡</td>
<td>16 ± 8§</td>
<td>27 ± 6§</td>
</tr>
</tbody>
</table>

All values are mean ± SEM.

* Significance of difference for duration of corneal opacity and vessel growth was calculated with the Mann-Whitney U-test.
† Mean corneal thickness values of the animals on the two dietary regimens at various time points during the inflammation were tested for significance with Wilcoxon’s signed rank test.
‡ P < 0.01.
§ P < 0.001.
† P < 0.005.

A significant inhibition of corneal edema was noted in rabbits given a fish oil diet as compared to those given a sunflower seed oil diet (Fig. 2, Table 3).

Systemic Antibody Response to Intracorneal Injected HSA

There was no significant difference in systemic response to HSA in the two different dietary regimens. Animals on a sunflower seed oil diet showed a serum level of 8.12 ± 2.17 μg/ml (mean ± SEM) of anti-HSA IgG immunoglobulins, and those on a fish oil diet 8.38 ± 3.26 μg/ml serum (mean ± SEM).

Discussion

The three parameters of corneal inflammation we used, Wessely’s ring, swelling, and neovascularization, can be observed well in the model of immunogenic keratitis and are known to be at least partially mediated by the formation of PGs and LTs. Rabbits on a diet containing 9.4% sunflower seed oil were the controls in our study. For health reasons, in order to avoid deficiency of essential fatty acids the experimental group received 2.7% sunflower seed oil made up to 9.4% with fish oil. The values found for the parameters during immunogenic keratitis in the control group were not significantly different from those in rabbits maintained on standard rabbit diet. The suppressive effect of the fish oil dietary supplement was significant on all three clinical parameters. Apart from a shorter period of infiltration with leukocytes, the observed amelioration of the immunogenic keratitis by the diet may be due also to altered metabolic activity of the invaded leukocytes. Most authors consider the presence of PML in corneal inflammation responsible for the later development of neovascularization and the concomitant edema formation. Others believe that the corneal tissues, especially the epithelium, can produce chemotactic and neovascularogenic substances without the infiltration of the PML. In this case leukocytes are not a prerequisite of neovascularization, but rather by their presence merely potentiate the neovascular response.

Fish oil dietary supplementation may markedly alter the eicosanoid formation in endotoxin-induced anterior uveal inflammation: in contrast to our findings in immunogenic keratitis, however, it has no effect on cellular infiltration. This difference in inflammatory response is due possibly to differences in tissue synthesis of LTB₄, a potent chemoattractant for leukocytes. The iris ciliary body of the rabbit has low levels of lipooxygenase activity, unlike the cornea, in
which lipoxygenase activity is more prominent.\textsuperscript{7} In our study the effect of fish oil supplement on corneal inflammation may be ascribed to a diminished synthesis of PGs in the cornea by EPA- and DGLA-induced inhibition of cyclooxygenase and to the formation of the less active LTB\textsubscript{5} and PGE\textsubscript{3} from EPA (Fig. 1).\textsuperscript{27-33}

In immunogenic keratitis the reaction in the cornea starts with the formation of antibody-antigen complexes.\textsuperscript{46} Although an effect of the EPA-enriched diet on antibody formation has been observed,\textsuperscript{32} in our experiments a diet containing EPA compared to one containing LA does affect the systemic antibody response to HSA.

Key words: fish oil, keratitis, eye, prostaglandin, leukotriene

Acknowledgments

The authors thank Mr. W. Heerooms for assistance with animal experiments: Unilever, Vlaardingcn, for providing the material for the animal diets; and Mrs. M. Wissing and Mrs. C. H. M. Muijlwijk-Planting for secretarial assistance.

References

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