Topical Combination of NGF and DHA Increases Rabbit Corneal Nerve Regeneration after Photorefractive Keratectomy

Salomon Esquesenazi,1,2 Haydee E. P. Bazan,1,2 Viet Bui,1 Jiucheng He,2 Dooho B. Kim,1 and Nicolas G. Bazan1,2

PURPOSE. To investigate the effect of nerve growth factor (NGF) in combination with docosahexaenoic acid (DHA) on corneal nerve regeneration in a rabbit model after PRK and correlate the findings with functional tear test results.

METHODS. Unilateral PRK was performed on 21 New Zealand albino rabbits. Three groups, each consisting of six rabbits, were randomized to receive twice-weekly treatments with DHA, NGF, or NGF plus DHA delivered by collagen shield. A fourth group, the control, received treatment with albumin. Rabbits were observed for 8 weeks, and tear secretion tests were conducted every 15 days. The eyes were prepared for immunostaining. Monoclonal antibodies for class II β-tubulin, calcitonin gene-related peptide (CGRP), substance P (SP), and Ki-67 were used. Cell nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI). Positive staining areas in relation to total area were calculated with image-analysis software.

RESULTS. There were no significant differences in the tear-secretion test results among the four groups. However, none of the eyes treated with NGF plus DHA showed rose bengal staining 30 days after PRK, compared with 50% in the control group and 33% in the DHA-treated group. A higher percentage of Ki-67-positive cells, a marker of cell proliferation, was observed in the DHA plus NGF–and NGF-treated groups compared with DHA alone or the control. Eight weeks after PRK, tubulin-positive and CGRP-positive epithelial and subbasal nerve bundle areas were significantly higher in the DHA plus NGF group than in the control and NGF or DHA alone. No differences were noted in the SP-positive nerve bundles between the different treatments and the control treatment.

CONCLUSIONS. NGF plus DHA treatment after PRK in rabbits is associated with increased corneal nerve surface area, increased epithelial proliferation, and decreased rose bengal staining compared with NGF, DHA, or vehicle control alone. The combination of NGF plus DHA yields faster nerve recovery after PRK and may have therapeutic usefulness in the treatment of post-PRK dry eye and other neurotrophic keratopathies. (Investigative Ophthalmology & Visual Science, September 2005, Vol. 46, No. 9 DOI:10.1167/iovs.05-0241)

The use of the excimer laser for the correction of refractive defects is widely accepted today. An annual survey that assesses the variety and volume of refractive surgeries showed that excimer ablative refractive procedures have been the predominant type performed since 1998.1 Photorefractive keratectomy (PRK) consists of the removal of the epithelium before applying the laser correction. In contrast, laser in situ keratomileusis (LASIK) requires the creation of a flap that includes epithelium and superficial stroma before the laser treatment. In both procedures, there is damage to the corneal nerve supply that may result in neurotrophic epitheliopathy and dry eye symptoms, characterized by punctuate epithelial erosions occurring days to weeks after the refractive procedure. In LASIK, hinge position and flap thickness seem to be important factors contributing to the rate of corneal sensation compromise.2 An impaired corneal sensitivity results in reduced afferent input and loss of the lacrimation reflex with a subsequent decrease in essential tear-derived trophic factors.3,4 Tears provide not only lubrication but also deliver growth factors and proteins to the compromised ocular surface that are essential for the maintenance of epithelial integrity after corneal refractive surgery. In addition, the local production of neuron-derived molecules from subbasal and epithelial nerve bundles may promote a healthy epithelium. There is evidence that the compromise of the corneal nerve bed disrupts the homeostasis of the cornea, resulting in impaired healing and persistent epithelial erosions.5,6 Therefore, by facilitating corneal reinnervation, physiologic functions of the cornea may be restored.

There is growing evidence that nerve growth factor (NGF), a neurotrophic and immunomodulatory mediator, is responsible for the growth, differentiation, and survival of sensory neurons and acceleration of wound healing.7–10 Keratocytes and epithelial and endothelial cells synthesize NGF, and epithelial cells express NGF receptors. After an injury, there is an upregulation of corneal NGF.11 Topical NGF promotes healing of refractory corneal neurotrophic ulcers, and a role of the growth factor in modulating epithelial–stromal communication, essential in the induction of stromal healing, has been postulated.12,13 In addition, corneal sensitivity after LASIK has been enhanced by the administration of topical NGF.14 If the effect of NGF on corneal wound healing could be potentiated, it would be invaluable for the restoration of ocular surface integrity and visual function.

Docosahexaenoic acid (DHA), a ω3 fatty acid that is concentrated in phospholipids of photoreceptor membranes, brain, and retinal synapses, as well as in the nerve membrane itself, has been used to slow the progression of X-linked retinitis pigmentosa.15,16 Based on its potent ability to inactivate proapoptotic and proinflammatory signaling, it has been postulated that DHA and its derivative, neuroprotectin D1 (NPD1)
have neuroprotective bioactivity in oxidative stress-challenged retinal pigment epithelial (RPE) cells. 17

Although there is increasing evidence to support the notion that NGF promotes corneal wound healing, there have been no studies that have investigated the combination of NGF with other factors to enhance corneal nerve regeneration after corneal lamellar refractive surgery. The goal of this study was to define the action of NGF plus DHA as an enhancer of nerve regeneration and as a potential therapeutic approach to control neurotrophic epitheliopathy after PRK and LASIK surgery. In this study, we used a PRK rabbit model as opposed to LASIK in the evaluation of nerve bundle areas, because during PRK, contrary to LASIK, all epithelial and subbasal nerve bundles are, with absolute certainty, newly regenerated nerves.

**Materials and Methods**

**Photorefractive Surgery**

Twenty-one New Zealand albino rabbits weighing 1.5 to 2.0 kg were used. The rabbits were treated in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Each rabbit received intramuscular xylazine (10 mg/kg) and ketamine hydrochloride (50 mg/kg) anesthesia. Tetracaine eye drops were used as topical anesthesia. Tear secretion tests consisting of tear breakup time, Schirmer’s test, and rose bengal staining were performed before surgery in animals under general anesthesia. Each rabbit received a unilateral PRK laser treatment. The corneal epithelium was removed with an epithelial scrubber (Katena, Inc., Denville, NJ). An excimer laser (Lasersight Technologies, Inc., Winter Park, FL) ablation was performed to correct −5 D of myopia with a 6.5-mm optical zone. The eye was washed with balanced salt solution. Lubricating eye drops and an ophthamlic solution of 0.5% ofloxacin (Allergan, Inc., Irvine, CA) eye drops were used after surgery.

**Preparation of NGF and DHA**

NGF (Sigma-Aldrich, St. Louis, MO) was prepared in a stock solution of 6.0 μg in 1.5 mL PBS and kept at 4°C. DHA (Sigma-Aldrich) was complexed to 25% human albumin (Baxter Healthcare, Deerfield, IL) in 0.1 M acetic acid, at a flow rate of 300 mL/min for 30 minutes. LC effluents were diverted to an electrospray-ionization probe (ESI) on a triple-quadrupole mass spectrometer (TSQ Quantum; Thermo Electron). DHA standards (Cyanam Chemical, Ann Arbor, MI) were used for tuning and optimization and to create calibration curves. The instrument was set on full-scan mode to detect parent ions and selected reaction mode for quantitative analysis to detect product ions simultaneously. The selected parent ion was 327.2 m/z, and the selected product ion was 283.5 m/z at a collision energy of 16 V, running on negative ion detection mode. Quantization was obtained by integration of peak areas of standards and samples.

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NGF (Sigma-Aldrich, St. Louis, MO) was prepared in a stock solution of 6.0 μg in 1.5 mL PBS and kept at 4°C. DHA (Sigma-Aldrich) was complexed to 25% human albumin (Baxter Healthcare, Deerfield, IL) in a proportion of 1 mL albumin to 1 mg of DHA and kept in the dark at 4°C in a sterile bottle.

**Animal Treatments**

Rabbits were divided into four groups. Groups 1 to 3 consisted of six rabbits each. Group 4 (control) had three rabbits. Each group was randomized to receive twice-weekly topical treatments with 0.1 μg of NGF (25 μL) plus 100 μL PBS, 100 μg (100 μL) DHA plus 25 μL PBS; 0.1 μg (25 μL) NGF plus 100 μg (100 μL) DHA; or 125 μL PBS through 7-hour collagen shields (Oasis, Glendora, CA) for 8 weeks. In all animals, a tarsorrhaphy was performed on the treated eyes, and the eyes were opened only twice a week to introduce the new collagen shield.

**Tandem Mass Spectrometry Analysis**

Measurements of the absorption of the DHA-albumin solution by the collagen shields were performed through mass spectrometry. Corneal shields were soaked with the DHA-albumin solution overnight. The shields were washed in phosphate buffer (pH 7.4) and extracted in 1 mL methanol, followed by 1 mL methanol wash. Collected solvent extracts were dried under nitrogen and resuspended in 1 mL methanol. Samples were loaded on a liquid chromatograph-tandem mass spectrometer (LC-MSMS; LC-TSQ Quantum; Thermo Electron Corp., Waltham, MA) equipped with a column (Biobasic-AQ; Thermo-HyperSil-Keystone, Bellefonte, PA; 100% solution A: 40:60:0.01 methanol-water-acetic acid [pH 4.5]), to 100% solution B: 99.99:0.01 methanol-acetic acid), at a flow rate of 300 μL/min for 30 minutes. LC effluents were diverted to an electrospray-ionization probe (ESI) on a triple-quadrupole mass spectrometer (TSQ Quantum; Thermo Electron). DHA standards (Cyanam Chemical, Ann Arbor, MI) were used for tuning and optimization and to create calibration curves. The instrument was set on full-scan mode to detect parent ions and selected reaction mode for quantitative analysis to detect product ions simultaneously. The selected parent ion was 327.2 m/z, and the selected product ion was 283.5 m/z at a collision energy of 16 V, running on negative ion detection mode. Quantization was obtained by integration of peak areas of standards and samples.

**Tear Secretion Tests**

Tear secretion tests (tear breakup time, Schirmer’s test, and rose bengal staining) were performed every 15 days. All the tests were performed with animals under general anesthesia. Schirmer’s test was performed using the standard methods and test strips (Alcon Laboratories, Fort Worth, TX). The tear breakup time test was performed with fluorescein strips (Akorn, Inc., Lincolnshire, IL) that were moistened with nonpreserved saline solution. Rose bengal staining was performed with Barnes/Hind strips (Akorn, Inc.). Three or more punctate spots of staining on the cornea were required to consider the stain positive. All measurements were conducted in a masked fashion.

**Tissue Preparation**

Rabbits were humanely euthanatized at 8 weeks after surgery with an intravenous overdose of pentobarbital. The treated eyes were immediately enucleated, and the entire corneas were excised and fixed in neutral formalin (10%) for 24 hours. The corneas were removed, bisected, and embedded in optimal cutting temperature (OCT) compound (Miles, Inc., Pittsburgh, PA). Six-micrometer cryostat sections were prepared, air dried, and stored at −80°C until further use. They were evaluated with hematoxylin and eosin (H&E) stain and by immunohistochemical analysis.

**Immunostaining**

To identify epithelial and subbasal regenerating nerve bundle endings after PRK, monoclonal antibodies for class III β-tubulin, calcitonin gene-related peptide (CGRP), and substance P (SP) were used. Tissue sections were incubated with mouse anti-class III β-tubulin antibody at a concentration of 1:500 (Covance Research Products, Inc., Berkeley, CA) for 1 hour followed by the secondary antibody, fluorescein-conjugated horse anti-mouse (1:500; Vector Laboratories Inc., Burlingame, CA), applied for 45 minutes at room temperature. Chicken anti-CGRP monoclonal antibody (1:500; Chemicon International, Temecula, CA) was incubated at room temperature for 1 hour. The secondary antibody, fluorescein-conjugated goat anti-chicken (1:1000; Rockland, Gilbertsville, PA) was applied for 1 hour at room temperature. Guinea pig anti-SP monoclonal antibody (1:500; Chemicon International) was incubated at room temperature for 90 minutes followed by the secondary antibody, fluorescein-conjugated goat anti-guinea pig (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour at room temperature. Immunofluorescence with a monoclonal anti-chondroitin sulfate clone CS-56 (Sigma-Aldrich) was performed as previously described.18 To stain for rabbit corneal myofibroblasts (RCMs), tissue sections were incubated with (1:300) monoclonal mouse anti-α-smooth muscle (oSMA; Sigma-Aldrich) for 2 hours at room temperature, followed by incubation with the secondary antibody fluorescein-conjugated goat anti-mouse IgG (Vector Laboratories, Inc.) for 1 hour at room temperature.

**Immunofluorescence with a monoclonal anti-chondroitin sulfate clone CS-56 (Sigma-Aldrich) was performed as previously described.18**
TABLE 1. Tear Secretion Tests After PRK in Rabbit Corneas

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Schirmer's Test (mm)</th>
<th>Tear Breakup Time (s)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>15 Days</td>
<td>30 Days</td>
</tr>
<tr>
<td>Control</td>
<td>10.5 ± 3.8</td>
<td>11.0 ± 4.2</td>
</tr>
<tr>
<td>NGF</td>
<td>11.5 ± 3.5</td>
<td>12.0 ± 2.8</td>
</tr>
<tr>
<td>DHA</td>
<td>10.0 ± 3.7</td>
<td>11.5 ± 3.4</td>
</tr>
<tr>
<td>NGF + DHA</td>
<td>12.0 ± 3.4</td>
<td>12.5 ± 3.7</td>
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</tbody>
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Data are the average ± SE of six samples in each condition and three in the control condition.
Increased staining with CGRP antibody of subbasal and epithelial nerve bundle areas was seen in the presence of NGF and DHA (Fig. 3B, 3D). Eight weeks after PRK, the average epithelial and subbasal CGRP-positive nerve areas in the control were 0.68 and 0.62 mm² (nerve and corneal tissue), respectively (Figs. 3A, 3C). A statistically significant increase in the CGRP-positive subbasal and epithelial nerve bundle areas was noted in the NGF group compared with the controls (P < 0.05). No statistically significant difference was observed between the DHA-treated and control groups (Figs. 3A, 3B). Treatment with both NGF and DHA gave highly significant differences compared with NGF (P < 0.001).

Nerve staining with SP was much lower than with tubulin or CGRP. Two months after PRK, the nerve bundle area without treatment was 0.38. No significant differences were observed between the four groups (Fig. 4).

Collagen III expression and chondroitin sulfate staining was observed in the anterior stroma 8 weeks after PRK. No α-SMA staining was observed, nor were any differences found between the four groups analyzed.

**DISCUSSION**

The cornea expresses NGF receptors (TrKA) that are upregulated after trauma. After an injury, there is a transient increase in corneal NGF levels that may induce sprouting of neurites and restore the function of damaged neurons. Our study showed a decrease in rose bengal staining in the eyes treated with NGF and no staining when the combination of NGF plus DHA was used. These results suggest that acceleration of nerve regeneration is associated with improved epithelial cell integrity. However, we found no difference between the treatment and control groups with regard to Schirmer testing and tear breakup time. This is in agreement with a recent study with patients who underwent LASIK surgery in which no correlation between decrease in tear production using Schirmer’s test and changes in corneal sensitivity was found. These findings suggest that the punctate epithelial erosions and rose bengal staining that develop after PRK are not attributable to diminished tear production. They may be the result of a PRK-induced neurotrophic epitheliopathy caused by diminished neurotrophic factors released from the injured and partially regenerated nerve endings. The combination of DHA and NGF completely inhibited epithelial defects. In fact, there was an increase in epithelial proliferation with this treatment. One possibility is that, under these conditions, DHA could be converted to NPD1 in the cells. Human RPE cells synthesize NPD1; and, more recently, it has been shown that mouse corneas generate endogenous NPD1 which, in turn, upregulates antiapoptotic proteins protecting the cells. Also, DHA is needed for nerve synthesis as a major component of their phospholipid membranes.

At this time, the mechanisms by which NGF and DHA exert their effects on the cornea are not well understood. Neurons and epithelial cells interact with each other. The neurons release neurotransmitters and neuropeptides that stimulate epithelial proliferation, and the epithelial cells can also release growth factors, such as NGF and other mediators that induce neurite survival. NGF may stimulate the synthesis of NPD1 through its precursor DHA. In fact, our laboratory has recently shown that several growth factors, including NGF, stimulate the synthesis of NPD1 in RPE cells.

The development of significant rose bengal staining may be associated with regression of the PRK correction. In a previous clinical study with a follow-up of 5 years after hyperopic LASIK, we demonstrated enhanced, long-term stability of the refractive results in eyes that did not have dry eye symptoms in the early postoperative period compared with eyes that had chronic dryness. These findings suggest that the epithelial surface changes noted in eyes with rose bengal staining may be associated with an upregulation of proapoptotic cytokines, activation of keratoctyes, and modification of the extracellular matrix associated with unpredictable corneal healing after refractive surgery. Besides NGF, it is postulated that many other substances, such as SP and CGRP, drive corneal wound healing. Matsu moto et al. demonstrated that the topical application of autologous serum, which harbors various neurotrophic factors, promotes healing in neurotrophic keratopathy. It is believed that serum contains factors that potentiate local trophic factors and facilitate epithelization by inducing cellular migration and adhesion.

**Table 2. Schirmer’s Test Results in Rabbits with Positive or Negative Rose Bengal Staining**

<table>
<thead>
<tr>
<th>Days after PRK</th>
<th>Rose Bengal Staining</th>
<th>No Rose Bengal Staining</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>15 Days</td>
<td>12.7 ± 4.2 mm</td>
<td>13.2 ± 5.2 mm</td>
<td>0.79</td>
</tr>
<tr>
<td>30 Days</td>
<td>13.4 ± 5.1 mm</td>
<td>13.7 ± 5.4 mm</td>
<td>0.82</td>
</tr>
<tr>
<td>45 Days</td>
<td>13.7 ± 4.8 mm</td>
<td>13.8 ± 4.6 mm</td>
<td>0.91</td>
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</tbody>
</table>

Samples were grouped according to Rose Bengal staining without considering the treatment given.

![Figure 1](https://iovs.arvojournals.org/pdfaccess.ashx?url=data/journals/iovs/933712/ on 11/22/2018)
DHA is most concentrated in photoreceptors, brain, and retinal synapses. In cornea, DHA is a minor component of membrane phospholipids. In our study, DHA alone caused no increase in nerve density in the subbasal and epithelial areas, compared with the control. However, when DHA was combined with NGF, there was a twofold increase in positive nerve tissue stained with tubulin and CGRP compared with the NGF group. The number of identifiable SP-positive neurons was very low and difficult to detect. It has been shown that approximately 58% of corneal neurons are CGRP positive, whereas only 20% are SP positive.

Future studies with esthesiometry will evaluate the functionality of the newly regenerated nerve fibers.

The molecular mechanisms underlying DHA potentiation of NGF are not clear. We propose two mechanisms: (1) DHA is the precursor of NPD1, a mediator that inhibits oxidative stress-induced apoptosis by upregulating antiapoptotic proteins, such as Bcl-2 and Bcl-xL, and downregulating proapoptotic proteins, such as Bax and Bad, and by inhibiting caspase-3 activation; and, (2) DHA is incorporated as an acyl chain of membrane phospholipids. DHA being a polyunsaturated fatty acyl chain could be a target of lipid peroxidation activated during corneal injury. Thus, the addition of DHA may contribute to the repair of the damaged nerves and render them more receptive to the action of NGF. We hypothesize that after PRK, there is a shortage of neurotrophic factors, of DHA acyl groups in phospholipids, and of neuroprotective mediators such as NPD1.

In conclusion, the results of this study suggest that topical DHA potentiates the effects of NGF in reinnervating the cornea. This enhancement in corneal nerve regrowth may yield a faster anatomic and functional recovery after PRK or LASIK. In a recent study in which reinnervation of the cornea was measured in patients 3 years after LASIK, it was found that the subbasal corneal nerves did not return to preoperative densities. Injury to the cornea by PRK reduces nerve bundle density by 60% up to 1 year after surgery. Hence, topical NGF plus DHA may accelerate the reinnervation after PRK and LASIK and avoid dry eye or other neurotrophic keratopathies. Further studies are warranted to determine the molecular mechanisms by which NGF aids in restoring the ocular surface integrity and how DHA enhances the effect of NGF on corneal wound healing.

**Acknowledgments**

The authors thank Hilary Thompson, PhD, for assistance with the statistical analysis of the data and Victor Marcheselli for assistance with tandem mass spectrometry.
Figure 3. Effect of NGF and DHA in CGRP-positive epithelial and subbasal nerve bundles 8 weeks after PRK. (B, D) CGRP immunofluorescence nerves (green). Nuclei of epithelial and stromal cells were counterstained with DAPI. (A, C) Nerve areas with compared with total areas. NGF, DHA, and NGF plus DHA represent the mean ± SE of six samples. Control data are the average ± SE of three samples. *P < 0.05 compared with the control; **P < 0.001 compared with NGF.

Figure 4. (A) Substance-P-positive nerve bundles 8 weeks after PRK in the control group. (B) Similar nerve areas were found in all treatment groups.
References


