Neuroprotectin D1 Synthesis and Corneal Nerve Regeneration after Experimental Surgery and Treatment with PEDF plus DHA

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Purpose. This study was conducted to define whether pigment epithelial–derived growth factor (PEDF), together with docosahexaenoic acid (DHA), enhances the synthesis of neuroprotectin D1 (NPD1) and the regeneration of corneal nerves damaged after surgery.

Methods. Corneal stromal dissection was performed in the left eyes of adult New Zealand rabbits treated with DHA+PEDF, PEDF, or DHA for 6 weeks. In vivo confocal images of the corneas were obtained at 2, 4, and 8 weeks, and nerve areas were quantified. At 8 weeks after treatment, corneas were stained with tubulin βIII antibody, and the epithelial nerve area and the sub-basal and stromal nerve plexus were quantified. At 1 week and 2 weeks after treatment, lipids were extracted from corneas, and the synthesis of NPD1 was analyzed by mass spectrometry. Epithelial cell density was quantified by confocal microscopy 8 weeks after surgery.

Results. In vivo confocal images at 2 and 4 weeks after surgery showed a 2.5-fold increase in corneal nerve area in PEDF+DHA-treated animals compared with control animals. Increased nerve surface areas in epithelia, subepithelia, and stroma were observed in rabbits treated for 8 weeks with PEDF+DHA. PEDF or DHA alone did not produce a significant increase. NPD1 synthesis peaked at 1 week and was four times higher in the PEDF+DHA-treated group than in the controls.

Conclusions. PEDF+DHA promotes the regeneration of corneal nerves. Neurotrophin-mediated NPD1 synthesis is suggested to precede nerve regeneration by demonstration of its accumulation upon addition of DHA and PEDF at earlier time points. Therefore, this signaling mechanism upregulates corneal nerve regeneration and may be targeted in neurotrophic keratitis, dry eye after refractive surgery, and other corneal diseases. (Invest Ophthalmol Vis Sci. 2010;51:804–810) DOI: 10.1167/iovs.09-3641

Trigeminal ganglion nerve fibers innervate both epithelial cells and stromal keratocytes, promoting corneal trophism and the overall maintenance of a healthy corneal surface. Alterations in corneal innervation cause neurotrophic keratitis and other dysfunctions.

It is estimated that 17 million people worldwide have had corneal surgery and that 1.5 million people undergo refractive surgery in the United States each year (Annual Report Vision-Watch, Vision Council of America, 2007). Complications arising from corneal nerve damage as a result of surgery include severe dry eye, neurotrophic keratitis, decreased blink reflex, and more susceptibility to injury and damage to the epithelium. Although the incidence of severe complications is relatively low, the rise in the number of surgeries increases complications. Up to 48% of patients report symptoms of ocular dryness, and between 15% to 20% report symptoms consistent with mild recurrent erosions after laser in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK) that affect their overall satisfaction. In fact, dry eye from sensory denervation of the cornea by refractive surgery can last up to 1 year. Corneal sub-basal nerve density does not recover to preoperative levels up to 5 years, and the number of stromal fiber bundles decreases by nearly 90% soon after LASIK and remains at less than 50% of that amount before surgery 1 year after LASIK.

Penetrating keratoplasty and the newer corneal transplant techniques, such as deep anterior lamellar keratoplasty, require transection of the host’s corneal nerves. Regeneration of nerve fibers in the graft is slow and incomplete, resulting in abnormal corneal sensation observable even 30 years after surgery and contributing to the high incidence of epithelial complications observed after this procedure.

In addition to corneal surgery, other frequent causes of neurotrophic keratitis are corneal transplantation, herpetic infection, injury to trigeminal nerves associated with cranial, orbital or retinal surgery, chemical burns, multiple sclerosis, and Sjögren syndrome. Recent studies show that dry eye associated with diabetes mellitus, contact lens use, and refractive surgery is also related to corneal innervation disturbances.

For years, patients with dry eye have used such treatments as artificial tears, ointments, and punctal plugs, which relieve only symptoms. Treatment with steroids, serum tears, and, more recently, low-concentration cyclosporin A aims to decrease the inflammatory response triggered by dry eye conditions. Although these treatments can be successful in alleviating dry eye symptoms and improving the ocular surface, they must be continuously administered, and the effects cease with discontinuation of the drug. No treatment can compensate for the underlying condition, which is innervation disturbance.

Docosahexaenoic acid (DHA) belongs to the ω-3 family of fatty acids and is concentrated in synapses and other cellular membranes of the brain and retina. Previous studies have shown that during postnatal development, accumulation of DHA in brain correlates to synaptogenesis, dendrite formation, and photoreceptor biogenesis. DHA is also involved in aging, memory formation, synaptic membrane function, and neuroprotection. In a previous study from our laboratory, we found that the application of nerve growth factor (NGF) and
DHA to rabbits after PRK produces faster nerve recovery.\textsuperscript{14} We proposed that the mechanisms could be facilitated through the action of the DHA-derived lipid mediator neuroprotectin D1 (NDP1).\textsuperscript{15-17} NDP1 is derived from DHA metabolism and has potent anti-inflammatory and neuroprotective actions. Its synthesis is stimulated by several growth factors; more recently, it has been found that pigment epithelial-derived factor (PEDF) is 10 times more potent than NGF in inducing NDP1 synthesis in retinal pigment epithelial (RPE) cells.\textsuperscript{18} PEDF is a broad-acting neurotrophic and neuroprotective factor that regulates processes associated with angiogenesis, neuronal cell survival, and cell differentiation.\textsuperscript{19,20} In this study, we used DHA in combination with PEDF in a rabbit model of lamellar keratectomy to enhance the regeneration of corneal nerves damaged after surgery and to assess the synthesis of NDP1.

**Materials and Methods**

**Animals**

Animals were treated according to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and of protocols approved by the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center.

New Zealand albino rabbits of either sex, each weighing 2 to 3 kg, were used. Each rabbit received intramuscular xylazine (10 mg/kg) and ketamine hydrochloride (50 mg/kg) anesthesia. Tetracaine eyedrops were used as topical anesthesia. Each rabbit underwent lamellar keratectomy in the left eye consisting of an 8-mm stromal dissection through a 3-mm incision. No sutures were used. Moxifloxacin ophthalmic eyedrops were instilled after surgery for infection prophylaxis. Animals were killed with an overdose of sodium pentobarbital through carotid vein injection at given time points.

PEDF was obtained from Chemicon International (Temecula, CA) with greater than 95% purity tested by SDS-PAGE. PEDF (400 ng) dissolved in PBS and 400 μg DHA (Cyamn Chemical, Ann Arbor, MI) complexed to 25% human albumin was used per shield. Collagen shields (24-hour; Oasis, Glendora, CA) were soaked in the drug or vehicle for 5 minutes and then placed on the rabbit cornea. Previous studies show that in using this method, DHA is absorbed with 25% efficiency into the collagen shield.\textsuperscript{14} Temporary tarsorrhaphy was performed and kept in place for the first 3 days. Collagen shields were changed twice a week for different times according to the experiments.

**Immunohistochemistry**

After rabbits were killed, whole corneas were excised and fixed in 2% fresh paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours at room temperature or overnight at 4°C. After three thorough washings in 0.1 M PBS for 5 minutes each, the whole cornea was incubated with mouse monoclonal anti-βIII-tubulin antibody (1:1000; Covance Antibody Services Inc., Berkeley, CA) in 1% goat normal serum plus 0.1% Triton X-100 in 0.1 M PBS for 24 hours at room temperature. After they were washed with 0.1 M PBS five times for 5 minutes each, the cornea was incubated with FITC-conjugated secondary antibody (1:1500; Molecular Probes, Eugene, OR) for 2 hours at room temperature. Finally, the injured area of the cornea was viewed and photographed with a fluorescence microscope (Eclipse TE200; Nikon, Tokyo, Japan) equipped with a digital camera (DXM1200; Nikon) using imaging software (MetaVue; MDS Analytical Technologies, Mississauga, ON, Canada). The βIII-tubulin–positive tissue nerve area was calculated and compared to the total area with an image analysis program (Image pro Plus 4.5; Media Cybernetics Inc., Silver Spring, MD). From the same area, epithelial, subepithelial, and stroma; nerves were visualized by adjusting the focus of the microscope in the different planes. Stromal nerves were from the anterior one-third thickness of the layer. Eight to 10 different areas were imaged and analyzed per cornea. In addition, 20-μm frozen sections of nerves were obtained, but cuts were made in a modified technique that orients the tissue obliquely, increasing the area and allowing for better visualization of corneal nerves. Sections were immunostained with anti-βIII-tubulin antibody, as described. For nuclear counterstaining, 4′,6′-diamino-2-phenyldino (DAPI; Sigma, St. Louis, MO) was used.

**Confocal Imaging**

Confocal microscopy was performed in the center of the cornea. For this examination, rabbits were anesthetized and the heads fixed in a special mounting system. During the examination, a drop of gel (preservative-free GenTeal Tears; Novartis, Basel, Switzerland) was placed on the objective lens. A retina tomograph (Heidelberg Retina Tomograph [HRT-II]; Heidelberg Engineering GmbH, Heidelberg, Germany) equipped with a confocal scanning microscope (Rostock Cornea Module [RCM]; Heidelberg Engineering GmbH) was used. Images consisted of 584 × 584 pixels covering an area of 400 × 400 μm/pixel and an acquisition time of 0.024 seconds. Images were acquired using an automatic-gain mode. The depth of the optical section was controlled manually. During the examination, the microscope was focused from the tear film to the anterior chamber, and images were taken from each layer. For the nerve area analysis, images were obtained from above the plane of dissection, evidenced by activated keratocytes, up to the epithelium.

For each eye, 20 images were obtained. An image was considered to be above the plane of dissection when it involved the stroma lying between the epithelium and the activated keratocytes in mid-stroma. The nerves in each image were traced using a caliper tool. Then the percentage nerve area for each image was quantified using an image analysis program (Image Pro Plus 4.5; Media Cybernetics, Silver Spring, MD). The corneal epithelium superficial cellular density was evaluated using the program associated with the HRT-II/RCM. The cells present in one image (74,340 μm²) were marked using the interactive computer display. Eight images per rabbit were counted and averaged. Results are expressed in cells per square millimeter.

**Lipid Extraction and NDP1 Analysis**

A longitudinal cornea strip from the surgical area was homogenized with chloroform/methanol 2:1 by volume using a mechanical homogenizer (Ultra-Turrax T8; Fisher Scientific, Pittsburgh, PA) set at high speed to disrupt the tissue. PGD2-d4, as an internal standard, was added at this point. Protein precipitates were separated by centrifugation at 10000g for 30 minutes and washed again with chloroform/methanol 2:1, and the lipid extract was combined. The protein extract was measured as reference.

Lipid samples were kept at this point under nitrogen at −80°C. Before use, 0.2 μl 0.05% CaCl₂ (pH 3.5) was added to wash the extract, and the upper layer was discarded. Samples were evaporated under a nitrogen stream evaporator and resuspended in 120 μL methanol. Ten microliters of each sample were loaded onto a liquid chromatography photodiode array electrospray ionization-tandem mass spectrometer (LC-TSQ Quantum, Thermo-Finnigan; Thermo Fisher Scientific, Waltham, MA) equipped with a guard column (Javelin; Thermo Scientific, Bellefonte, PA) attached to another column (Hypersil GOLD; Thermo Scientific) and eluted in linear gradient (100% solution A [40:60:0.01 methanol/water/acetic acid, pH 3.5] to 100% solution B [99:0.01 methanol/acetic acid]) at a flow rate of 300 μL/min for 30 minutes. Liquid chromatography effluents were diverted to the electron-spray-ionization probe of the quadrupole mass spectrometer.\textsuperscript{18} The selected parent/product ions (m/z) and collision energy obtained by running on negative ion detection mode were 359.2/15.3 for NDP1, and 555.2/275.2/20 for PGD2-d4. Quantification was conducted by integrating the peak areas of samples and standards.

**Statistical Analysis**

All data are expressed as mean ± SEM. The number of rabbits analyzed in each experiment is described in the figure legends. Statistical comparison between PEDF+DHA and vehicle groups was performed by
Rabbits were treated for a total of 6 weeks and were killed at 8 weeks after surgery, and the corneas were processed for immunohistochemistry. We reasoned that treatment for an additional 2 weeks would produce a greater effect and that by 8 weeks we would be able to observe whether the treatment did, in fact, induce differences in the innervations. The animals tolerated the treatment well, and no adverse reactions were noted. Quantification of the epithelial, subepithelial, and stromal nerve plexus areas was conducted in the injured area of corneal wholemounts by immunofluorescence with anti–βIII tubulin antibody, as described in Materials and Methods. We found that the use of corneal wholemounts with immunofluorescence staining was better for evaluating the nerve area because it allowed for visualization of corneal nerves from the stroma to the superficial terminal branches by focusing the microscope on different planes. Large areas could be studied without difficulty. It also allowed for comparison of the surgical area to the adjacent intact cornea (see Fig. 5). Analysis of nerve density in the injured area showed that there was an increase in the subepithelial nerve area from 10% in the untreated group to 29% in the PEDF+DHA-treated group (Figs. 2A, 2B). This represents a threefold increase in corneal innervation in the treated group. Stromal nerves showed an increase in nerve area in the treated group (18.8%) compared with the untreated group (6.9%). The epithelial nerve area was also increased in the treated group (6%) compared with the untreated group (3.7%). Increased epithelial nerve density was also observed in corneal sections obtained from three different rabbits and stained with βIII tubulin (Figs. 2C, 2E).

An experiment was then performed using only PEDF or DHA and compared to the combination treatment of PEDF+DHA (Fig. 3). Analysis of subepithelial nerve density showed that DHA and PEDF, when administered individually, did not significantly increase corneal nerve regeneration, as opposed to their combined administration. This suggested that both PEDF and DHA are needed to induce a synergistic effect. In addition, DHA is found in the cornea in very low quantities21; therefore, supplementation of this fatty acid may be needed to achieve neurotrophic effects.

NPD1 Synthesis after PEDF+DHA Treatment

In retinal pigment epithelial cells, PEDF is a potent stimulus for the synthesis of NPD1.18 We investigated whether this mediator was synthesized in the cornea after 1 and 2 weeks of treatment with PEDF+DHA and with PEDF and DHA alone. These times were chosen because we postulated that synthesis of NPD1 would occur before the increase of nerve regeneration observed at 8 weeks. To identify NPD1, a liquid chromatography coupled to a low collision-energy tandem mass spectrometer (LC-MS/MS) was used. Because NPD1 is present in very low amounts, this kind of instrument is the most appropriate for its quantification.22

The instrument was set on full-scan mode to detect parent and product ions simultaneously. A pure standard of NPD1 was run as a reference. Its retention time of 15.99 coincided with the peak of a cornea lipid extract from a PEDF+DHA-treated sample (Fig. 4A).

We found that NPD1 increases after surgery when corneas were treated with DHA for 1 week (Fig. 4B); however, a higher increase was seen when PEDF+DHA was administered. High levels of NPD1 were observed 1 week after surgery, but these levels declined by 2 weeks. The results indicated that the synthesis of NPD1 seemed to occur early.

In light of these results, nerve density was also studied by immunohistochemistry in rabbits treated with PEDF+DHA for only 1 and 2 weeks. In these experiments, the surgical area and the area surrounding the injury were imaged. One week after

RESULTS

Effect of Topical PEDF+DHA on Corneal Nerve Surface Area after Lamellar Keratectomy

With the use of lamellar dissection, we transected all stromal nerves and then followed corneal nerve regeneration. After surgery, rabbits were treated by topical application of PEDF+DHA to the cornea by means of a 72-hour collagen shield, as described in Materials and Methods, and the animals were examined under anesthesia with the confocal microscope at 2 and 4 weeks after treatment. There was an increase in the regeneration of sensory nerves in the PEDF+DHA-treated group compared with vehicle in the area above the plane of dissection (Fig. 1A). Imaging analysis showed a significant increase in nerve density in the stroma at 2 weeks that was further increased at 4 weeks in vehicle-treated corneas (Fig. 1B).

In vivo confocal images of rabbit corneas at 2 and 4 weeks after surgery. (A) Samples of images obtained above the plane of dissection (white arrows) show stromal nerves at 2 and 4 weeks after surgery and treatment with PEDF+DHA. (B) For each rabbit an average of 20 images was taken. Quantification of the stromal nerve area is expressed as the average of three (vehicle, 4 weeks) or four rabbits ± SEM. *Significant differences with respect to vehicle (Student’s t-test).

Student’s t-test. Comparison between several groups was performed by one-way ANOVA. P < 0.05 was considered significant.
surgery, no nerves were observed in the epithelial and subepithelial surgical areas, whereas dense innervation was seen in the area adjacent to the surgical area. However, in the surgical area in the stroma, there was already growth of nerve fibers from the sectioned branches in PEDF/H11001 DHA–treated corneas (Fig. 5A, arrows).

Two weeks after surgery, there were epithelial nerves in the surgical areas of rabbit corneas treated with PEDF/H11001 DHA, but few nerves were found in the vehicle-treated corneas (Fig. 5B). Images obtained at a deeper plane showed more stromal nerves growing in the surgical area of the PEDF/H11001 DHA–treated group compared with the vehicle-treated group.

**FIGURE 2.** Effect of DHA and PEDF on nerve density 8 weeks after surgery. (A) Immunohistochemistry of epithelia, subepithelia, and stroma of rabbit corneal wholemounts stained with anti–βIII tubulin antibody 8 weeks after surgery. (B) Quantification of βIII tubulin stains of PEDF+DHA–treated group compared with animals receiving only vehicle. Eight to 10 images were analyzed per rabbit, and the values correspond to the average ± SEM of six (vehicle) to nine (PEDF+DHA) rabbits. In some of the groups, the SEM is not shown with the scale used. (C) Corneal sections of samples from three rabbits treated or untreated with PEDF+DHA for 6 weeks. Sections were obtained at 8 weeks after surgery and stained with anti–tubulin βIII antibody (red). DAPI was used to stain the nuclei (blue). (D) Negative controls omitting the first antibody show no nerve staining. (E) Quantification of tubulin staining in corneal sections showed significantly more nerves in the treated versus the untreated groups. *Statistical significance (Student’s t-test).

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**FIGURE 3.** Nerve density after PEDF, DHA, and PEDF+DHA treatment. Nerve density was measured by βIII tubulin staining 8 weeks after lamellar keratectomy and treatment for 6 weeks with PEDF, DHA, and a combination of PEDF+DHA. *ANOVA test showed that only PEDF+DHA was statistically significant compared with vehicle treatment. n, number of rabbits.

Two weeks after surgery, there were epithelial nerves in the surgical areas of rabbit corneas treated with PEDF+DHA, but few nerves were found in the vehicle-treated corneas (Fig. 5B). Images obtained at a deeper plane showed more stromal nerves growing in the surgical area of the PEDF+DHA–treated group compared with the vehicle-treated group.

**Increase in Epithelial Corneal Cells after PEDF and DHA Treatment**

Dry eye reduces the number of superficial epithelial cells. To assess the effect that DHA and PEDF have on the epithelium, rabbits were treated for 6 weeks with PEDF+DHA after surgery, and epithelial cell counts were performed at 8 weeks by confocal microscopy. There was a significant increase in cells per square millimeter in the group treated with PEDF+DHA compared with the vehicle-treated group (Fig. 6). This suggests that the increased innervation seen in the treated group leads to a healthier corneal epithelium.

**DISCUSSION**

Our experiments show convincing evidence that the combination of PEDF and DHA increases the regeneration of damaged corneal nerves after lamellar keratectomy compared with results in untreated corneas. The mechanisms require a synergism between these two compounds because neither DHA nor PEDF alone significantly increases nerve regeneration.

Injury to the cornea disrupts the homeostasis of the tissue and triggers a rapid repair mechanism. Several growth factors, such as epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor, are secreted by epithelial and
stromal cells and the lacrimal gland and are important in promoting epithelial wound healing. NGF has also been shown to increase in tears after LASIK and PRK. PEDF is a potent and broad-acting neurotrophic and neuroprotective factor that regulates processes associated with angiogenesis, neuronal cell survival, and cell differentiation. PEDF is expressed in several eye tissues. In the cornea it is expressed most strongly by epithelial cells. PEDF prevents photoreceptors from degenerating in models of retinal degeneration, such as exposure to light. It also protects retinal neurons from damage caused by increased intraocular pressure. In chick spinal cord cultures, PEDF exerts a strong neurotrophic effect, increasing cell survival and neurite length.

One possible mechanism by which DHA is important to increase corneal nerves after injury could be that DHA is the precursor of the recently discovered NPD1, a lipid messenger with potent anti-inflammatory and neuroprotective actions. This mediator has been found to protect against oxidative stress in retinal cells and in the human brain by upregulating anti-apoptotic proteins such as Bcl-2 and Bcl-XL and downregulating proapoptotic proteins such as Bax and Bad. Corneas contain very low amounts of DHA, but recent studies show that mice fed with DHA-enriched diets have corneas that generate NPD1 and that NPD1 also promotes corneal wound healing.

Several growth factors stimulate the synthesis of NPD1 in retinal pigment epithelial cells. A previous study shows that a combination of NGF and DHA increases rabbit corneal nerve regeneration after photorefractive keratectomy. However, subsequent studies show that PEDF is 10 times more potent than any other growth factor tested, including NGF, in stimulating the synthesis of NPD1 from DHA. A recently identified lipase-linked cell membrane receptor for PEDF implicates a relation between the growth factor and lipid metabolism. An early increase in NPD1 was observed in the presence of PEDF and DHA, which correlates with an increase in stromal nerves after 2 weeks and a significant increase in nerve regeneration 8 weeks after surgery. However, the stimulation of NPD1 by DHA treatment was not correlated with a significant increase in stromal nerves and the lacrimal gland and are important in promoting epithelial wound healing.

![Figure 4](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932966/)

**Figure 4.** Synthesis of NPD1 in injured corneas treated with PEDF, DHA, and PEDF + DHA. Lipids from treated corneas were extracted and analyzed using LC-MS/MS. (A) The molecular structure of NPD1 is shown. The line with the arrow indicates a typical product ion breakpoint at the MS collision cell. A chromatogram of NPD1 standard and a PEDF + DHA-treated sample (monitoring for the parent ion 359 and the product ion 153) are also shown. (B) Quantification of NPD1 after 1 week and 2 weeks of lamellar keratectomy in rabbit corneas. Authentic standards of NPD1 were used to confirm the identification. The samples were run in duplicate, and the values correspond to an average ± SEM of two or three rabbits per condition. *Significant differences with respect to vehicle. **Significant differences between DHA and DHA + PEDF (ANOVA).

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932966/)

**Figure 5.** Cornea epithelial and stromal nerves stained with βIII tubulin after 1 and 2 weeks of surgery and PEDF + DHA treatment. (A) Representative images of four different rabbit corneas 1 week after PEDF + DHA treatment. Arrows: branches in the stroma. Surgical and adjunct areas are shown in the epithelium. (B) Immunocytochemistry of rabbit epithelia and stroma 2 weeks after surgery. Images are representative of corneas from four different rabbits. Only the surgical area is shown.
some preliminary studies which indicate that NPD1 could act through a receptor.\textsuperscript{17} Identifying that receptor could also be important in elucidating the molecular mechanism of NPD1 action in corneal nerve regeneration. Future studies will also have to address the kind of sensory neurons regenerated and the functionality of these nerves.

Our results also show that the health of the corneal epithelium after surgery is improved with P PEDF+DHA treatment, as evidenced by the decreased corneal epithelium shedding that probably results from restoration of the trophic influence provided by corneal nerves. Dry eye after refractive surgery is a complication resulting from damage to the nerves, and this damage produces loss of corneal sensitivity that delays the blink reflex and decreases tear secretion by the lacrimal gland.\textsuperscript{56} In more severe cases such as neurotrophic keratitis, healing of the ocular surface can be difficult and can lead to scarring and loss of vision. A combination of a substance P-derived neuropeptide and insulin growth factor-1 has been used with some success for nonhealing epithelial defects caused by neurotrophic keratitis.\textsuperscript{37,38} Our approach is unique in that it aims at reverting the underlying physiopathologic process of these conditions. Based on our observations, a combination of PEDF and DHA could result in an effective method of treatment.

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References


